EPIcentro

Modulo 6

Iper-reattività bronchiale in asma severo: l'altra faccia della medaglia

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Iper-reattività bronchiale in asma severo: l'altra faccia della medaglia

SOMMARIO

1. Calzetta L, Aiello M, Frizzelli A, Bertorelli G, Ritondo BL, Rogliani P, Chetta A. The Impact of Monoclonal Antibodies on Airway Smooth Muscle Contractility in Asthma: A Systematic Review. Biomedicines. 2021;9(9):1281.
2. Koefoed HJL, Zwitserloot AM, Vonk JM, Koppelman GH. Asthma, bronchial hyperresponsiveness, allergy and lung function development until early adulthood: A systematic literature review. Pediatr Allergy Immunol. 2021;32(6):1238-1254.
3. Porsbjerg CM, Sverrild A, Lloyd CM, Menzies-Gow AN, Bel EH. Anti-alarmins in asthma: targeting the airway epithelium with next-generation biologics. Eur Respir J. 2020;56(5):2000260.
4. Peters MC, Wenzel SE. Intersection of biology and therapeutics: type 2 targeted therapeutics for adult asthma. Lancet. 2020;395(10221):371-383.
5. Gauvreau GM, Sehmi R, Ambrose CS, Griffiths JM. Thymic stromal lymphopoietin: its role and potential as a therapeutic target in asthma. Expert Opin Ther Targets. 2020;24(8):777-792.
6. Roan F, Obata-Ninomiya K, Ziegler SF. Epithelial cell-derived cytokines: more than just signaling the alarm. J Clin Invest. 2019;129(4):1441-1451
7. Comberiati P, Katial RK, Covar RA. Bronchoprovocation Testing in Asthma: An Update. Immunol Allergy Clin North Am. 2018;38(4):545-571.
8. Chapman DG, Irvin CG. Mechanisms of airway hyper-responsiveness in asthma: the past, present and yet to come. Clin Exp Allergy. 2015;45(4):706-19.
9. Ziegler SF, Roan F, Bell BD, Stoklasek TA, Kitajima M, Han H. The biology of thymic stromal lymphopoietin (TSLP). Adv Pharmacol. 2013;66:129-55.
10. Kaur D, Doe C, Woodman L, Heidi Wan WY, Sutcliffe A, Hollins F, Brightling C. Mast cell-airway smooth muscle crosstalk: the role of thymic stromal lymphopoietin. Chest. 2012;142(1):76-85.
11. Comeau MR, Ziegler SF. The influence of TSLP on the allergic response. Mucosal Immunol. 2010;3(2):138-47.
12. Brutsche MH, Downs SH, Schindler C, Gerbase MW, Schwartz J, Frey M, Russi EW, Ackermann-Liebrich U, Leuenberger P; SAPALDIA Team. Bronchial hyperresponsiveness and the development of asthma and COPD in asymptomatic individuals: SAPALDIA cohort study. Thorax. 2006;61(8):671-7.
13. Robinson DS. The role of the mast cell in asthma: induction of airway hyperresponsiveness by interaction with smooth muscle? J Allergy Clin Immunol. 2004;114(1):58-65.
14. Sears MR, Greene JM, Willan AR, Wiecek EM, Taylor DR, Flannery EM, Cowan JO, Herbison GP, Silva PA, Poulton R. A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. N Engl J Med. 2003;349(15):1414-22.
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1. Calzetta L, Aiello M, Frizzelli A, Bertorelli G, Ritondo BL, Rogliani P, Chetta A. The Impact of Monoclonal Antibodies on Airway Smooth Muscle Contractility in Asthma: A Systematic Review. Biomedicines. 2021;9(9):1281.

L'iperreattività delle vie aeree (AHR) rappresenta un tratto distintivo della fisiopatologia dell'asma e la muscolatura liscia delle vie aeree (ASM) è il tessuto effettore implicato nell'insorgenza dell'AHR. L'ASM esercita anche azioni pro-infiammatorie e immunomodulatorie, secernendo un'ampia gamma di citochine e chemochine. Nella patogenesi dell'asma, la sovraespressione di diversi mediatori infiammatori di tipo 2, tra cui IgE, IL-4, IL-5, IL-13 e TSLP, è stata associata all'iperreattività dell'ASM, e tutti questi mediatori possono essere bersaglio di anticorpi monoclonali umanizzati (mAbs). Pertanto, lo scopo di questa review è stato quello di valutare sistematicamente le evidenze della letteratura sugli mAbs per il trattamento dell'asma in relazione al loro impatto sul tono contrattile dell'ASM. Omalizumab, mepolizumab, benralizumab, dupilumab e tezepelumab sono risultati efficaci nel modulare la contrattilità dell'ASM e nel prevenire l'AHR, ma dalla letteratura non sono stati identificati studi disponibili sull'impatto di reslizumab sull'ASM. Omalizumab, dupilumab e tezepelumab possono modulare direttamente l'ASM nell'asma, bloccando specificamente l'interazione tra IgE, IL-4, e TSLP, e i loro recettori, localizzati sulla superficie delle cellule ASM. Al contrario, mepolizumab e benralizumab hanno un impatto prevalentemente indiretto contro l'AHR, il loro target sono gli eosinofili e altre cellule effettrici immunomodulatorie che promuovono i processi infiammatori. L'AHR è stato suggerito come il principale bersaglio trattabile attraverso la medicina di precisione nei pazienti affetti da asma eosinofila, pertanto, sono necessari studi testa a testa ben disegnati per confrontare l'efficacia di quei mAb che mirano direttamente alla contrattilità dell'ASM in modo specifico contro l'AHR nell'asma grave, ovvero omalizumab, dupilumab e tezepelumab.

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2. Koefoed HJL, Zwitserloot AM, Vonk JM, Koppelman GH. Asthma, bronchial hyperresponsiveness, allergy and lung function development until early adulthood: A systematic literature review. Pediatr Allergy Immunol. 2021;32(6):1238-1254.

Premessa: Non è chiaro in quali periodi della vita si sviluppino i deficit di funzionalità polmonare, e se questi siano influenzati da fattori di rischio come l'asma, l'iperreattività bronchiale (BHR) e le comorbilità allergica. L'obiettivo di questa review sistematica era identificare le associazioni temporali di asma, BHR e comorbilità allergica con lo sviluppo della funzionalità polmonare delle grandi e piccole vie aeree dalla nascita fino al picco di funzionalità nella prima età adulta.

Metodi: Abbiamo cercato su MEDLINE, EMBASE, Web of Science e CINAHL gli articoli pubblicati prima del 01.01.2020 sui fattori di rischio e sulle misurazioni della funzionalità polmonare delle grandi e piccole vie aeree. Gli studi dovevano riportare la funzionalità polmonare in qualsiasi momento o intervallo di tempo dalla nascita fino al picco di funzionalità polmonare (età 21-26 anni) e includere almeno un fattore di rischio candidato.

Risultati: Dei 45 lavori identificati, 44 erano basati su studi di coorte e 1 era uno studio clinico con follow-up. Asma, respiro sibilante, BHR e sensibilizzazione allergica nelle prime fasi della vita a più allergeni sono stati associati a una minore crescita della funzionalità polmonare di grandi e piccole vie aeree durante la prima infanzia rispetto alle popolazioni di controllo. Lo sviluppo della funzionalità polmonare dopo l'infanzia in soggetti con asma o wheezing persistente, anche se ha continuato a crescere a un livello inferiore, è stato in gran parte parallelo a quello dei soggetti non affetti fino al raggiungimento del picco di funzionalità.

Implicazioni cliniche e ricerche future: I deficit nella crescita della funzionalità polmonare si sviluppano nella prima infanzia e i bambini affetti da asma, BHR e da una (poli)sensibilizzazione alle IgE nei primi anni di vita sono a rischio. Questo periodo è probabilmente una finestra critica di opportunità per identificare i soggetti a rischio e fornire un trattamento mirato a prevenire le sequele a lungo termine sulla funzionalità polmonare.

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3. Porsbjerg CM, Sverrild A, Lloyd CM, Menzies-Gow AN, Bel EH. Anti-alarmins in asthma: targeting the airway epithelium with next-generation biologics. Eur Respir J. 2020;56(5):2000260

Le terapie con anticorpi monoclonali hanno migliorato significativamente gli outcome dei pazienti con asma severo, tuttavia permane un significativo carico di malattia. I trattamenti biologici disponibili, inclusi gli anticorpi monoclonali anti-IgE, anti-IL-5, anti-IL-5Rαanti-IL-4Rαriducono il tasso di esacerbazioni nelle popolazioni in studio di solo circa il 50%. Inoltre non esistono attualmente trattamenti efficaci per i pazienti con asma severa di tipo 2-low. Il target degli attuali farmaci biologici sono le vie immunologiche a valle della cascata infiammatoria di tipo 2, il che potrebbe spiegare il motivo per cui le esacerbazioni siano solo in parte soppresse. Per esempio, l'infiammazione delle vie aeree di tipo 2 comprende diversi segnali infiammatori e non soltanto l'IL-5. Clinicamente, questo può essere osservato dal fatto che il FeNO, determinato dall'IL-13, non subisce variazioni durante il trattamento con l'anti-IL-5 nonostante una riduzione degli eosinofili, e che gli eosinofili non vengano modificati durante il trattamento con anti-IL-4Rαnonostante una riduzione del FeNO. La vasta risposta infiammatoria che include le citochine IL-4, IL-5 e IL-13 e che determina infiammazione eosinofilica, produzione di muco e broncospasmo, caratteristiche tipiche delle esacerbazioni, ha inizio con il rilascio delle allarmine (la linfopoietina timica stromale (TSLP), la IL-33 e la IL-25) a livello dell'epitelio delle vie aeree in risposta a diversi trigger. Il ruolo centrale e a monte della cascata infiammatoria di queste citochine ha fatto sì che siano forti potenziali target terapeutici per prevenire le esacerbazioni e migliorare la funzionalità polmonare nei pazienti con asma di tipo 2 high e low. Questo articolo descrive gli effetti delle allarmine e discute il ruolo potenziale degli anticorpi anti-allarmine nel contesto dei biologici già esistenti. Inoltre vengono discussi i fenotipi clinici dei pazienti che potrebbero beneficiare di questi trattamenti e in che modo i biomarker potrebbero aiutare a identificare i potenziali responder.

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4, Peters MC, Wenzel SE. Intersection of biology and therapeutics: type 2 targeted therapeutics for adult asthma. Lancet. 2020;395(10221):371-383

L'asma è una patologia caratterizzata da un'ostruzione reversibile del flusso aereo e che si manifesta clinicamente con respiro sibilante, mancanza di fiato e tosse. L'aumento dell'attività delle citochine di tipo 2 delle vie aeree, tra cui l'interleuchina-4 (IL-4), l'IL-5 e l'IL-13, è un meccanismo biologico ormai consolidato nell'asma. I corticosteroidi per via inalatoria sono stati il fondamento del trattamento dell'asma, in gran parte perché sono in grado di ridurre l'infiammazione di tipo 2 delle vie aeree. Tuttavia, i corticosteroidi per via inalatoria o sistemica sono inefficaci in molti pazienti con asma ed esistono poche opzioni terapeutiche per i pazienti con asma resistente agli steroidi. Sebbene i meccanismi dell'asma refrattaria ai corticosteroidi siano probabilmente numerosi, lo sviluppo di una nuova classe di agenti biologici che ha come bersaglio l'infiammazione di tipo 2 delle vie aeree ha fornito un nuovo modello per il trattamento di alcuni pazienti con asma refrattaria ai corticosteroidi. L'obiettivo di questo lavoro è quello di riassumere i nuovi farmaci di tipo 2, ponendo l'accento sul razionale biologico e sull'efficacia clinica di questa nuova classe di farmaci per l'asma

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5. Gauvreau GM, Sehmi R, Ambrose CS, Griffiths JM. Thymic stromal lymphopoietin: its role and potential as a therapeutic target in asthma. Expert Opin Ther Targets. 2020;24(8):777-792.

Introduzione: La linfopoietina timica stromale (TSLP), una citochina epiteliale (allarmina), è un regolatore centrale della risposta immunitaria agli agenti aerei ambientali, come allergeni, virus e agenti inquinanti, che avvia una cascata infiammatoria a valle. Esistono prove inconfutabili che la TSLP svolge un ruolo importante nella patologia dell'asma e sono in fase di sviluppo terapie che mirano a bloccarne l'attività.

Aree trattate: Sono stati esaminati gli studi condotti sull'uomo e sulle cellule umane, in gran parte pubblicati su PubMed nel periodo gennaio 2010-ottobre 2019, che hanno indagato i meccanismi immunitari innati e adattativi della TSLP nell'asma, rilevanti per l'infiammazione di tipo 2 (eosinofila/allergica) e l'infiammazione non di tipo 2 (non eosinofila/non allergica), e il ruolo della TSLP come mediatore tra le cellule immunitarie e le cellule strutturali delle vie aeree. Vengono inoltre discussi i dati clinici degli studi che valutano il blocco di TSLP.

Expert opinion: La posizione della TSLP al vertice della cascata infiammatoria la rende un promettente bersaglio terapeutico nell'asma. La terapia sistemica con tezepelumab, un anticorpo monoclonale anti-TSLP, ha dato finora risultati positivi negli studi clinici, riducendo le esacerbazioni e i biomarkers infiammatori nei pazienti in tutto lo spettro degli endotipi infiammatori. La somministrazione di anti-TSLP per via inalatoria è una alternativa attualmente in fase di valutazione. La sicurezza e l'efficacia a lungo termine del blocco della TSLP devono essere approfondite.

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6. Roan F, Obata-Ninomiya K, Ziegler SF. Epithelial cell-derived cytokines: more than just signaling the alarm. J Clin Invest. 2019;129(4):1441-1451.

La linfopoietina timica stromale (TSLP), l'IL-33 e l'IL-25 sono regolatori centrali dell'immunità di tipo 2, che guida una vasta gamma di risposte allergiche. Spesso definite come 'allarmine' che vengono rilasciate dall'epitelio della barriera in risposta a insulti esterni, queste citochine derivate dalle cellule epiteliali erano inizialmente ritenute in grado di agire solo nelle fasi iniziali dell'infiammazione allergica. In effetti, la TSLP può condizionare le cellule dendritiche ad avviare risposte di tipo 2 e la IL-33 può influenzare la suscettibilità all'asma attraverso il suo ruolo nella produzione dell'ambiente immunitario nei polmoni perinatali. Tuttavia, la TSLP, la IL-33 e la IL-25 regolano un ampio spettro di popolazioni di cellule immunitarie innate e sono particolarmente potenti nell'elicitare e attivare le cellule linfoidi innate di tipo 2 (ILC2) che possono agire in tutta l'infiammazione allergica. Dati recenti suggeriscono che un asse TSLP/ILC possa mediare la resistenza agli steroidi nell'asma. La recente identificazione di sottoinsiemi di cellule Th2 di memoria che sono caratterizzate da un'elevata espressione dei recettori per TSLP, IL-33 e IL-25, sostiene il ruolo di queste citochine nelle esacerbazioni allergiche. Vi è quindi un crescente interesse per lo sviluppo di biologici che abbiano come bersaglio TSLP, IL-33 e IL-25. Questa review fornisce una panoramica su TSLP, IL-33 e IL-25 e sullo sviluppo di anticorpi che hanno come bersaglio queste citochine derivate dalle cellule epiteliali.

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7. Comberiati P, Katial RK, Covar RA. Bronchoprovocation Testing in Asthma: An Update. Immunol Allergy Clin North Am. 2018;38(4):545-571

L'iperreattività bronchiale (BHR) è definita come un'aumentata risposta broncocostrittiva agli stimoli delle vie aeree. Fa parte delle caratteristiche fondamentali dell'asma, come la limitazione variabile o reversibile del flusso aereo e l'infiammazione delle vie aeree. Sebbene la BHR sia considerata un segno distintivo fisiopatologico dell'asma, occorre riconoscere che questa proprietà delle vie aeree è dinamica, poiché la sua gravità e persino la sua presenza possono variare nel tempo con l'attività della malattia, con i fattori scatenanti o l'esposizione specifica e con il trattamento. Inoltre, è importante riconoscere che esiste una componente che non riflette una specifica entità di malattia.

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8. Chapman DG, Irvin CG. Mechanisms of airway hyper-responsiveness in asthma: the past, present and yet to come. Clin Exp Allergy. 2015;45(4):706-19.

L'iperreattività delle vie aeree (AHR) è stata a lungo considerata una caratteristica fondamentale dell'asma. Lo sviluppo della misurazione dell'AHR, quarant'anni fa, ha dato il via a molti importanti contributi alla comprensione dell'asma e di altre malattie delle vie aeree. Tuttavia, la comprensione dell'AHR nell'asma rimane complicata dalla moltitudine di potenziali meccanismi sottostanti che, in realtà, è probabile che abbiano contributi diversi nei singoli pazienti. Pertanto, la presente review affronta lo stato attuale della comprensione dei principali meccanismi che si ipotizza contribuiscano all'AHR e sottolinea il modo in cui i test AHR stanno iniziando a evidenziare anomalie distinte associate a popolazioni di pazienti clinicamente rilevanti. In questo modo, ci proponiamo di fornire una base su cui la ricerca futura possa iniziare ad attribuire determinati meccanismi a specifici modelli di broncocostrizione e, di conseguenza, far corrispondere i fenotipi di broncocostrizione ai fenotipi clinici. Riteniamo che questo approccio non solo sia alla nostra portata, ma che porterà a una migliore comprensione meccanicistica dei fenotipi dell'asma e, auspicabilmente, a una migliore informazione sullo sviluppo di una terapia mirata al fenotipo.

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9. Ziegler SF, Roan F, Bell BD, Stoklasek TA, Kitajima M, Han H. The biology of thymic stromal lymphopoietin (TSLP). Adv Pharmacol. 2013;66:129-55.

Inizialmente considerata come fattore che promuove la crescita e l'attivazione delle cellule B, la linfopoietina timica stromale (TSLP) è ora nota per il suo impatto sia sulle cellule ematopoietiche sia quelle non ematopoietiche, tra cui le cellule dendritiche (DC), i basofili, gli eosinofili, i mastociti, cellule T CD4+, CD8+ e natural killer (NK), cellule B e cellule epiteliali. Mentre il ruolo della TSLP nella promozione delle risposte TH2 è stato ampiamente studiato nel contesto dei disturbi allergici specifici del polmone e della pelle, sta diventando sempre più chiaro che la TSLP può avere un impatto su molteplici stati patologici in più sistemi di organi, compreso il blocco delle risposte TH1/TH17 e lo sviluppo di cancro e dell'autoimmunità. Questa review evidenzia i recenti progressi nella comprensione della trasduzione del segnale della TSLP, nonché il ruolo di TSLP nell'allergia, nell'autoimmunità e nel cancro.

È importante notare che queste conoscenze sul ruolo multiforme di TSLP potrebbero potenzialmente consentire nuove approcci terapeutici a questi disturbi.

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10. Kaur D, Doe C, Woodman L, Heidi Wan WY, Sutcliffe A, Hollins F, Brightling C. Mast cell-airway smooth muscle crosstalk: the role of thymic stromal lymphopoietin. Chest. 2012;142(1):76-85.

Background: La localizzazione dei mastociti nella muscolatura liscia delle vie aeree (ASM) nell'asma è importante nello sviluppo alterato della fisiologia delle vie aeree. La linfopoietina timica stromale (TSLP) è espressa dalle cellule strutturali delle vie aeree. Non è chiaro se abbia un ruolo nel crosstalk tra queste cellule. Abbiamo cercato di definire l'espressione della TSLP nel tessuto bronchiale in tutto lo spettro di gravità dell'asma e di indagare l'espressione e la funzione della TSLP e del recettore della TSLP (TSLPR) in colture primarie di ASM e mastociti da soli e in co-coltura.

Metodi: L'espressione della TSLP è stata valutata nel tessuto bronchiale di 18 soggetti con asma da lieve a moderata, 12 con asma grave e 9 soggetti sani di controllo. L'espressione di TSLP e TSLPR nelle colture primarie di mastociti e di ASM è stata valutata mediante immunofluorescenza, citometria a flusso e test di immunoassorbimento enzimatico, la sua funzione è stata valutata mediante imaging del calcio. È stato esaminato il ruolo della TSLP nella proliferazione, sopravvivenza, differenziazione, funzione di sintesi e contrazione dei mastociti e della ASM.

Risultati: L'espressione della TSLP è aumentata nella muscolatura liscia delle vie aeree nella malattia lieve-moderata. TSLP e TSLPR erano espressi dai mastociti e dalla ASM ed erano funzionali. L'attivazione dei mastociti da parte della TSLP aumenta la produzione di un'ampia gamma di chemochine e citochine, ma non influisce sulla proliferazione, sulla sopravvivenza o sulla contrazione dei mastociti o della ASM.

Conclusioni: L'espressione di TSLP da parte dell'epitelio bronchiale e dell'ASM è aumentata nell'asma. TSLP promuove la funzione di sintesi dei mastociti, ma non contribuisce ad altre conseguenze funzionali del crosstalk mastocita-ASM.

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11. Comeau MR, Ziegler SF. The influence of TSLP on the allergic response. Mucosal Immunol. 2010;3(2):138-47

L'esposizione agli allergeni avviene innanzitutto sulle superfici corporee a diretto contatto con l'ambiente, come la pelle, le vie respiratorie e il tratto gastrointestinale e prove convincenti suggeriscono che le risposte infiammatorie allergiche siano profondamente influenzate dai prodotti delle cellule epiteliali situate in questi siti. Uno di questi prodotti è la linfopoietina timica stromale (TSLP), che è in grado di influenzare diverse linee cellulari coinvolte nelle reazioni allergiche. In questa review si discutono i recenti lavori che hanno permesso di comprendere il ruolo della TSLP nelle risposte infiammatorie allergiche aberranti e protettive, nonché la regolazione, l'associazione con la malattia, le fonti e le funzioni di questa importante citochina.

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12. Brutsche MH, Downs SH, Schindler C, Gerbase MW, Schwartz J, Frey M, Russi EW, Ackermann-Liebrich U, Leuenberger P; SAPALDIA Team. Bronchial hyperresponsiveness and the development of asthma and COPD in asymptomatic individuals: SAPALDIA cohort study. Thorax. 2006;61(8):671-7.

Premessa: L'iperreattività bronchiale (BHR) è una caratteristica comune dell'asma. Tuttavia, la BHR è presente anche in individui asintomatici e il suo significato clinico e prognostico non è chiaro. Abbiamo ipotizzato che la BHR possa avere un ruolo nello sviluppo della broncopneumopatia cronica ostruttiva (BPCO) e dell'asma.

Metodi: Nel 1991 i sintomi respiratori e la BHR alla metacolina sono stati valutati in 7126 dei 9651 partecipanti allo studio di coorte SAPALDIA. Undici anni dopo, 5825 di questi partecipanti sono stati rivalutati, e 4852 di essi hanno eseguito test spirometrici. La BPCO è stata definita come un rapporto FEV1/FVC di ,0.70.

Risultati: Nel 1991 il 17% dei partecipanti presentava BHR, di cui il 51% era asintomatico. Undici anni dopo, la prevalenza di asma, respiro sibilante e respiro affannoso in soggetti precedentemente asintomatici con o senza BHR era, rispettivamente, del 5,7% contro il 2,0%, dell'8,3% contro il 3,4% e del 19,1% contro l'11,9% (tutti p< 0,001). Differenze analoghe sono state osservate per la tosse cronica (5,9% vs 2,3%; p= 0,002) e la BPCO (37,9% vs 14,3%; p< 0,001). LA BHR ha conferito un odds ratio (OR) aggiustato di 2,9 (95% CI 1,8-4,5) per il respiro sibilante al follow-up tra i partecipanti asintomatici. L'OR aggiustato per la BPCO era di 4,5 (95% CI 3,3-6,0). La BHR silente era associata a un declino significativamente accelerato del FEV1 di 12 (5-18), 11 (5-16), e 4 (2-8) ml/anno nei fumatori attuali, negli ex fumatori e nei non fumatori, rispettivamente, nel SAPALDIA 2.

Conclusioni: La BHR è un fattore di rischio per un declino accelerato del FEV1 e per lo sviluppo di asma e BPCO, indipendentemente dall'atopia. I fumatori attuali con BHR presentano una perdita di FEV1 particolarmente elevata.

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Iper-reattività bronchiale in asma severo: l'altra faccia della medaglia

13. Robinson DS. The role of the mast cell in asthma: induction of airway hyperresponsiveness by interaction with smooth muscle? J Allergy Clin Immunol. 2004;114(1):58-65.

In uno studio recente, la differenza tra asma e bronchite eosinofila (una condizione caratterizzata da tosse ma non da iperresponsività delle vie aeree o da ostruzione del flusso aereo) era l'infiltrazione da parte dei mastociti nel muscolo liscio delle vie aeree (ASM) da parte dei mastociti. I mastociti producono una serie di mediatori lipidici, chemochine, citochine ed enzimi che possono interagire con le cellule ASM e causare iperreattività agli stimoli costrittivi e proliferazione, e le ASM attivate possono produrre il fattore delle cellule staminali e altre chemochine, citochine e fattori di crescita che possono agire nel reclutamento, differenziazione e mantenimento dei mastociti. L'infiltrazione dei mastociti nelle vie aeree nell'asma è T-cellula dipendente, e le citochine TH2 provenienti dai linfociti T e da altre fonti agiscono nell'espansione dei mastociti da precursori circolanti e tissutali. I dati recenti sulle interazioni tra mastociti e ASM suggeriscono che questo potrebbe essere un importante fattore di iperreattività delle vie aeree nell'asma. Il motivo per cui ciò si verifica nell'asma e come si mantiene resta da stabilire.

Articolo in lingua originale

Iper-reattività bronchiale in asma severo: l'altra faccia della medaglia

14. Sears MR, Greene JM, Willan AR, Wiecek EM, Taylor DR, Flannery EM, Cowan JO, Herbison GP, Silva PA, Poulton R. A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. N Engl J Med. 2003;349(15):1414-22.

Background: L'esito dell'asma infantile nell'adulto è stato descritto in coorti ad alto rischio, ma pochi studi basati su popolazione hanno riportato i fattori di rischio per la persistenza e la riacutizzazione.

Metodi: Sono stati valutati i bambini nati dall'aprile 1972 al marzo 1973 a Dunedin, Nuova Zelanda, ripetutamente dai 9 ai 26 anni di età con questionari, test di funzionalità polmonare, test di provocazione bronchiale e test allergologici.

Risultati: All'età di 26 anni, il 51,4% dei 613 partecipanti allo studio con dati respiratori completi aveva riportato respiro sibilante (wheezing) in più di una valutazione. Ottantanove partecipanti allo studio (14,5%) avevano un respiro sibilante che persisteva dall'infanzia ai 26 anni di età, mentre 168 (27,4%) avevano avuto una remissione, ma 76 (12,4%) hanno successivamente avuto una ricaduta entro i 26 anni. La sensibilizzazione agli acari della polvere di casa è risultata predittore della persistenza del respiro sibilante (odds ratio, 2,41; P=0,001) e della riacutizzazione (odds ratio, 2,18; P=0,01), così come dell'iperreattività delle vie aeree (odds ratio per la persistenza, 3,00; P<0,001; odds ratio per la ricaduta, 3,03; P<0,001).

Il sesso femminile è risultato predittore per la persistenza del respiro sibilante (odds ratio, 1,71; P=0,03), così come il fumo all'età di 21 anni (odds ratio, 1,84; P=0,01). Quanto più precoce è l'età di insorgenza, maggiore è il rischio di ricaduta (odds ratio, 0,89 per ogni anno di aumento dell'età di insorgenza; P<0.001). La funzione polmonare è risultata costantemente inferiore nei soggetti con wheezing persistente rispetto a quelli senza wheezing persistente.

Conclusioni: In una coorte di nascita non selezionata, più di un bambino su quattro ha avuto un respiro sibilante che è persistito dall'infanzia all'età adulta o che ha recidivato dopo la remissione. I fattori predittori per persistenza o recidiva sono risultati sensibilizzazione agli acari della polvere domestica, iperreattività delle vie aeree, sesso femminile, fumo ed età precoce di insorgenza. Questi risultati, insieme alla persistente funzione polmonare bassa, suggeriscono che gli esiti dell'asma nell'adulto possono essere determinati principalmente nella prima infanzia.

Articolo in lingua originale

Iper-reattività bronchiale in asma severo: l'altra faccia della medaglia

15. Leuppi JD, Salome CM, Jenkins CR, Anderson SD, Xuan W, Marks GB, Koskela H, Brannan JD, Freed R, Andersson M, Chan HK, Woolcock AJ. Predictive markers of asthma exacerbation during stepwise dose reduction of inhaled corticosteroids. Am J Respir Crit Care Med. 2001;163(2):406-12.

Per determinare i fattori predittivi di una mancata riduzione dei corticosteroidi inalatori (ICS), in 50 soggetti con asma ben controllata (età 43,7 [18-69]; 22 maschi) che assumevano una dose mediana di 1,000 mg di ICS/die (100-3,600 mg/die), gli ICS sono stati dimezzati ogni 8 settimane. L'iperreattività delle vie aeree (AHR) a un test di provocazione bronchiale (BPT) con istamina è stata misurata al basale. AHR al BPT con mannitolo, spirometria, ossido nitrico esalato (eNO) e, in 31 soggetti, cellule infiammatorie dell'espettorato sono stati misurati al basale e a intervalli mensili. Trentanove soggetti hanno avuto un'esacerbazione dell'asma. In sette soggetti è stato interrotto con successo l'uso degli ICS. Utilizzando un'analisi di sopravvivenza di Kaplan-Meier, i predittori significativi di un fallimento della riduzione degli ICS sono stati l'iperreattività all'istamina e al mannitolo al basale (p= 0,039), e l'iperreattività al mannitolo durante la fase dello studio di riduzione della dose (p= 0,02). I soggetti di età superiore ai 40 anni tendevano a essere più a rischio di fallimento della riduzione della dose di ICS (p= 0,059). La risposta al mannitolo e la percentuale di eosinofili dell'espettorato erano significativamente maggiori prima di una riduzione dell'ICS rispetto all'ultima riduzione dell'ICS andata a buon fine, mentre non c'erano differenze significative nei sintomi, nella spirometria o nell'eNO. Questi risultati suggeriscono che la documentazione dell'AHR o degli eosinofili dell'espettorato del paziente può essere utile per guidare la riduzione delle dosi di ICS.

Articolo in lingua originale

16. Yan K, Salome C, Woolcock AJ. Rapid method for measurement of bronchial responsiveness. Thorax. 1983 Oct;38(10):760-5.

Viene descritto un metodo rapido e semplice per misurare la reattività bronchiale all'istamina inalata. Il metodo è stato utilizzato per ottenere curve di dose risposta in 50 soggetti atopici con sintomi respiratori e nasali variabili. La dose cumulativa di istamina che ha provocato una caduta del 20% nel volume espiratorio forzato di un secondo (PD20-FEV1) variava tra 0*046 e a più di 39 umol e correlava con la gravità dei sintomi. La riproducibilità di PD20-FEV1, determinata da misurazioni in doppio in 15 soggetti con vari gradi di reattività bronchiale è risultata soddisfacente. Quando il PD20-FEV1, ottenuto con questo metodo rapido, è stato confrontato con quello ottenuto con il metodo del dosimetro, non è stata riscontrata alcuna differenza significativa. La dose erogata con questo metodo si è dimostrata cumulativa.

Articolo in lingua originale

Iper-reattività bronchiale in asma severo: l'altra faccia della medaglia

17. Cockcroft DW, Ruffin RE, Dolovich J, Hargreave FE. Allergen-induced increase in non-allergic bronchial reactivity. Clin Allergy. 1977 Nov;7(6):503-13

L'iperreattività bronchiale non allergica è una caratteristica della maggior parte dei pazienti con asma. Abbiamo misurato la reattività bronchiale non allergica all'istamina e alla metacolina inalate in tredici soggetti asmatici prima e dopo l'inalazione degli allergeni in laboratorio. L'inalazione dell'allergene ha prodotto risposte asmatiche precoci lievi (caduta del 19-40% del FEV1) in tutti e tredici i soggetti, risposte asmatiche tardive (caduta del 17-29% del FEV1) in quattro e risposte asmatiche tardive equivoche (caduta del 5-11% del FEV1) in cinque soggetti. Dopo l'inalazione dell'allergene, la reattività bronchiale non allergica è aumentata in sette soggetti per un massimo di 7 giorni. I sette includevano tutti e quattro i soggetti con risposte asmatiche tardive certe e tre dei cinque con risposte asmatiche tardive equivoche. Si conclude che gli allergeni peggiorano l'asma, in parte attraverso meccanismi non allergici, e che evitare gli allergeni è importante per ridurre l'iperreattività bronchiale non allergica.

Articolo in lingua originale

biomedicines

MDPI

Systematic Review

The Impact of Monoclonal Antibodies on Airway Smooth Muscle Contractility in Asthma: A Systematic Review

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Abstract: Airway hyperresponsiveness (AHR) represents a central pathophysiological hallmark of asthma, with airway smooth muscle (ASM) being the effector tissue implicated in the onset of AHR. ASM also exerts pro-inflammatory and immunomodulatory actions, by secreting a wide range of cytokines and chemokines. In asthma pathogenesis, the overexpression of several type 2 inflammatory mediators including IgE, IL-4, IL-5, IL-13, and TSLP has been associated with ASM hyperreactivity, all of which can be targeted by humanized monoclonal antibodies (mAbs). Therefore, the aim of this review was to systematically assess evidence across the literature on mAbs for the treatment of asthma with respect to their impact on the ASM contractile tone. Omalizumab, mepolizumab, benralizumab, dupilumab, and tezepelumab were found to be effective in modulating the contractility of the ASM and preventing the AHR, but no available studies concerning the impact of reslizumab on the ASM were identified from the literature search. Omalizumab, dupilumab, and tezepelumab can directly modulate the ASM in asthma, by specifically blocking the interaction between IgE, IL-4, and TSLP, and their receptors are located on the surface of ASM cells. Conversely, mepolizumab and benralizumab have prevalently indirect impacts against AHR by targeting eosinophils and other immunomodulatory effector cells promoting inflammatory processes. AHR has been suggested as the main treatable trait towards precision medicine in patients suffering from eosinophilic asthma, therefore, well-designed head-to-head trials are needed to compare the efficacy of those mAbs that directly target ASM contractility specifically against the AHR in severe asthma, namely omalizumab, dupilumab, and tezepelumab.

Keywords: airway hyperresponsiveness; airway smooth muscle; monoclonal antibodies; severe asthma

Citation: Calzetta, L.; Aiello, M.; Frizzelli, A.; Bertorelli, G.; Ritondo, B.L.; Rogliani, P.; Chetta, A. The Impact of Monoclonal Antibodies on Airway Smooth Muscle Contractility in Asthma: A Systematic Review. *Biomedicines* **2021**, *9*, 1281. https://doi.org/10.3390/biomedicines9091281

Academic Editor: Hiroshi Wakao

Received: 1 September 2021
Accepted: 17 September 2021
Published: 21 September 2021

1. Introduction

Asthma is a chronic, heterogeneous, inflammatory airway disorder consisting of a generally variable airflow limitation and several clinical symptoms including chest tightness, cough, wheezing, and shortness of breath [1]. Airway hyperresponsiveness (AHR) represents a central pathophysiological hallmark of asthma, underpinned by predominant inflammatory traits involving different phenotypes and endotypes [2,3]. Since airway smooth muscle (ASM) is the effector tissue that strives to shorten and constricts the bronchial lumen in response to stimuli, it is mainly implicated in the onset of AHR [4]. Thus, traditionally ASM has been considered as the main effector of AHR exclusively for its contractile properties [5]. However, it is now established that ASM may also have proinflammatory and immunomodulatory functions, by secreting a wide range of cytokines and chemokines [6], thus contributing to structural alterations associated with the disease [7].

Particularly, type 2 (T2)-high asthma is a complex endotype associated with high type 2 inflammatory markers including immunoglobulin E (IgE), interleukins (IL)-4, IL-5,

and IL-13 [8], and it is characterized by AHR, eosinophilia, and excessive airway mucus production [9,10].

Although inhaled corticosteroids (ICSs) represent the mainstay of asthma management [11] and are clinically effective in most patients [12], 5–10% of the total asthmatic population remain uncontrolled despite using high doses of ICSs and then systemic corticosteroids [13], a condition associated with a high risk of severe exacerbations, hospitalization, and mortality [12,14].

Over the last decade, improved knowledge regarding the complex pathophysiology of asthma has led to the development of new treatment options [15], and the current Global Initiative for Asthma (GINA) recommendations [11] consider patients with severe asthma associated with T2-high phenotypes/endotypes suitable for add-on biologic therapies [2].

In asthma pathogenesis, a number of inflammatory mediators of the T helper 2-dependent reaction play a pivotal role in a complex signaling environment that contributes to AHR: several experimental studies on isolated airway preparations and in vivo animal models have demonstrated an association between enhanced ASM hyperreactivity and overexpression of IgE [16], IL-4 [17,18], IL-5 [19], IL-13 [18,20,21], and TSLP [22,23].

The approved humanized monoclonal antibodies (mAbs) for the treatment of asthma are targeted against IgE (omalizumab), IL-5 (mepolizumab and reslizumab), IL-5 receptor α (IL-5Rα; benralizumab), and IL-4/IL-13 (dupilumab) [24–27]. Recently, the Biologics License Application (BLA) for the anti-thymic stromal lymphopoietin (TSLP) tezepelumab has been accepted and granted priority review for the treatment of asthma from the US Food and Drug Administration (FDA) [28]. Considering the immunomodulatory effect of these mAbs, it may be assumed that these drugs may have significant direct and indirect effects on ASM. Therefore, the aim of this article was to systematically review the evidence across literature on current mAbs for the treatment of asthma with respect to their impact on ASM contractile tone.

2. Methods

2.1. Review Question

The question of this systematic review was to assess if current mAbs for the treatment of asthma may have an impact on ASM contractility and AHR.

2.2. Search Strategy and Study Eligibility

The protocol of this synthesis of the current literature has been submitted to the international prospective register of systematic reviews (PROSPERO, submission ID: 270261), and performed in agreement with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocol (PRISMA-P) [29], with the relative flow diagram shown in Figure 1. This study satisfied all the recommended items reported by the PRISMA-P checklist [29].

The PICO (Patient problem, Intervention, Comparison, and Outcome) framework was applied to develop the literature search strategy and question, as previously reported [30]. Namely, the "Patient problem" included asthma; the "Intervention" regarded the administration of mAbs; the "Comparison" was performed with respect to baseline or placebo (PBO); the assessed "Outcome" was the impact on ASM contractility and AHR.

A comprehensive literature search was performed for in vitro, ex vivo, and clinical studies evaluating the impact of current mAbs for asthma on ASM contractility and AHR.

The search was performed on ClinicalTrials.gov (accessed on 15 August 2021), Cochrane Central Register of Controlled Trials (CENTRAL), Embase, EU Clinical Trials Register, MEDLINE, Scopus, and Web of Science in order to provide relevant studies available with no time limit up to 5 July 2021.

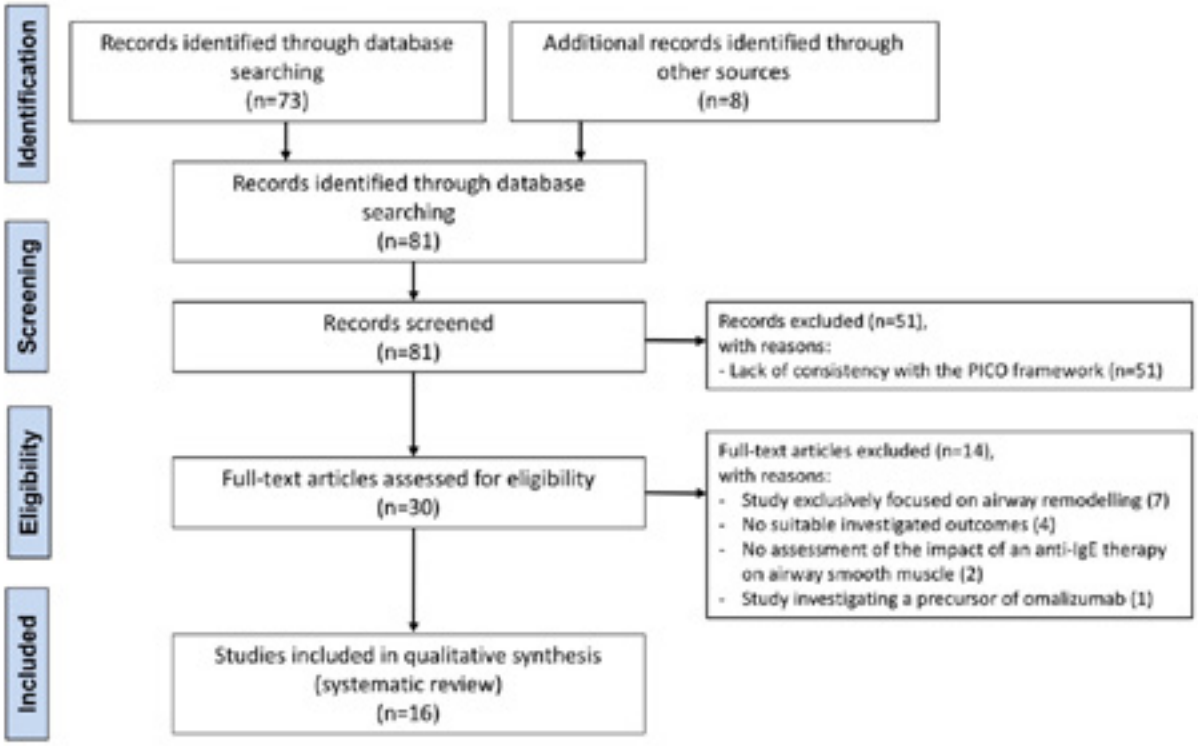

Figure 1. PRISMA flow diagram for the identification of the studies included in the systematic review concerning the impact of mAbs for the treatment of asthma on the airway smooth muscle. IgE: immunoglobulin E; mAbs: monoclonal antibodies; PICO: Patient problem, Intervention, Comparison, and Outcome; PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analysis.

The research string was as follows: ("dupilumab"[Supplementary Concept] OR "dupilumab"[All Fields] OR ("tezepelumab"[Supplementary Concept] OR "tezepelumab" [All Fields]) OR ("mepolizumab"[Supplementary Concept] OR "mepolizumab"[All Fields]) OR ("benralizumab"[Supplementary Concept] OR "benralizumab"[All Fields]) OR ("reslizumab"[Supplementary Concept] OR "reslizumab"[All Fields]) OR ("omalizumab" [MeSH Terms] OR "omalizumab"[All Fields] OR "omalizumab s"[All Fields])) AND ((("airway"[All Fields] OR "airway s"[All Fields] OR "airways"[All Fields]) AND ("muscle, smooth"[MeSH Terms] OR ("muscle"[All Fields] AND "smooth"[All Fields]) OR "smooth muscle"[All Fields] OR ("smooth"[All Fields] AND "muscle"[All Fields]))) OR (("airway"[All Fields] OR "airway s"[All Fields] OR "airways"[All Fields]) AND ("hyperresponsive"[All Fields] OR "hyperresponsiveness"[All Fields] OR "hyperresponsivity"[All Fields])) OR (("isolate"[All Fields] OR "isolate s"[All Fields] OR "isolated"[All Fields] OR "isolates"[All Fields] OR "isolating"[All Fields] OR "isolation and purification"[MeSH Subheading] OR ("isolation"[All Fields] AND "purification"[All Fields]) OR "isolation and purification"[All Fields] OR "isolation"[All Fields] OR "isolations"[All Fields]) AND ("bronchi"[MeSH Terms] OR "bronchi"[All Fields] OR "bronchus"[All Fields])) OR (("isolate"[All Fields] OR "isolate s"[All Fields] OR "isolated"[All Fields] OR "isolates"[All Fields] OR "isolating"[All Fields] OR "isolation and purification"[MeSH Subheading] OR ("isolation"[All Fields] AND "purification"[All Fields]) OR "isolation and purification"[All Fields] OR "isolation"[All Fields] OR "isolations"[All Fields]) AND ("airway"[All Fields] OR "airway s"[All Fields] OR "airways"[All Fields]))). Citations of previously published relevant reviews were examined to select further pertinent studies (if any) [31].

Two reviewers independently checked the relevant studies identified from the literature search. The studies were selected in agreement with PICO and any difference in opinion about eligibility was resolved by consensus.

2.3. Data Extraction

Data from included studies were extracted in agreement with Data Extraction for Complex Meta-anALysis (DECiMAL) recommendations [32], and checked for study references and characteristics, number and characteristics of the analyzed patients or donors or animals with age and gender, type of analyzed samples, treatments and comparators with doses of medications, smoking habits, forced expiratory volume in 1 s (FEV_1), and outcome measurements to evaluate the impact on ASM.

2.4. Endpoints

The endpoint of this systematic review was to assess the impact of current mAbs for the treatment of asthma on ASM contractility and AHR.

2.5. Strategy for Data Analysis

Data from original papers were extracted and reported via qualitative synthesis.

3. Results

3.1. Study Characteristics

Of the 81 potentially relevant records identified in the initial search, 16 studies were deemed eligible for qualitative analysis (Table 1). Overall, this systematic review included data obtained from ten randomized controlled trials (RCTs) involving patients with different levels of asthma severity [33–42], five pre-clinical studies [18,25,43–45], and one observational study on patients with severe refractory asthma [46]. Among the pre-clinical studies, two were conducted ex vivo on passively sensitized human bronchi [25,45], one was carried out in vitro on human ASM cells (ASMCs) [44], one was performed both ex vivo on human isolated bronchial tissue and in vitro on ASMCs [18], and one was an in vivo murine model of chronic asthma [43]. The investigated mAbs were omalizumab [35,36,38,40,41,43–46], mepolizumab [25,34,37,39], benralizumab [25], tezepelumab [33,42], and dupilumab [18]. No available studies concerning the impact of reslizumab on the ASM were identified from the literature search.

Table 1. Main characteristics of the studies included in the systematic review.

Study, Year, and Reference	Study Characteristics	Treatment Duration	Type of Cells, Animals, Donors, or Analyzed Patients	Number of Animals, Donors, or Patients	Drugs, Doses and Regimen of Administration	Mean Age (Years)	Male (%)	Current Smokers (%)	Smoking History (Pack-Years)	Post-Bronchodilator FEV_1 (% Predicted)	Investigated Outcome
Diver et al., 2021 (CASCADE trial) [42]	RCT	Planned 28 wks	Patients with uncontrolled moderate to severe asthma	116	Tezepelumab (210 mg, every 4 wks; SC injection)	50.4	44.0	0.0	NA	69.1	AHR to mannitol
Sverrild et al., 2021 (UPSTREAM trial) [33]	RCT	12 wks	Patients with uncontrolled asthma	40	Tezepelumab (700 mg, every 4 wks; IV infusion)	41.0	42.0	0.0	NA	88.7	AHR to mannitol
Calzetta et al., 2020 [25]	Ex vivo, prospective, randomized, negative- and positive- controlled, blind, parallel-group study	Overnight	Passively sensitized subsegmental bronchi (4–6 mm) from patients undergoing lobectomy for lung cancer (with normal serum IgE levels < 100 IU/mL and normal preoperative lung function parameters)	16	Benralizumab (1 µg/mL–100 µg/mL) vs. mepolizumab (1 µg/mL–100 µg/mL)	50.0	50.0	25.0	24.4	93.1	AHR to His, EFS, and QS and assessment of treatment effect on cAMP levels
Manson et al., 2020 [18]	Ex vivo and in vitro study	1 day	Human small airways and primary ASMCs	33	Dupilumab (1 µM)	69.0	27.0	36.4	NA	NA	AHR to His and EFS
Tajiri et al., 2014 [46]	Prospective, single-arm, observational study	48 wks	Patients with severe refractory asthma	31	OMA (every 2–4 wks, dosing based on body weight and baseline total serum IgE; SC injection)	55.0	32.3	0.0	≤10	93.5	AHR to methacholine
Kang et al., 2010 [43]	In vivo study	3 months	BALB/c mice challenged with OVA (murine model of chronic asthma)	10–15 per group	Rat anti-mouse IgE mAb clone R35–92 (100 µg/200 µL in normal saline), once a month from day 38; IV injection)	8–10 wks	0.0	NA	NA	NA	AHR to methacholine
Roth et al., 2010 [44]	In vitro study	24 h	Primary ASMCs isolated from allergic asthma donors	6	OMA (0.1, 0.5, 1.0 µg/mL)	33.3	66.7	NA	NA	69.0	IL-4, IL-6, IL-8, and TNF-α secretion and synthesis by ASMCs and IgE receptor expression in ASMCs

Table 1. *Cont.*

Study, Year, and Reference	Study Characteristics	Treatment Duration	Type of Cells, Animals, Donors, or Analyzed Patients	Number of Animals, Donors, or Patients	Drugs, Doses and Regimen of Administration	Mean Age (Years)	Male (%)	Current Smokers (%)	Smoking History (Pack-Years)	Post-Bronchodilator FEV_1 (% Predicted)	Investigated Outcome
Haldar et al., 2009 [34]	RCT	1 year	Patients with refractory eosinophilic asthma and a history of recurrent exacerbations	61	Mepolizumab (750 mg every month; IV infusion) vs. PBO	49.0	52.5	0.0	NA	77.9	AHR to methacholine
Berger et al., 2007 [45]	Ex vivo study	1 h	Passively sensitized medium bronchi and small airways obtained from non-atopic and non-asthmatic patients	10	OMA (60, 120, 180 µg/mL)	64.4	100.0	NA	24.5	86.9	ASM contractile responses to His and Dermatophagoides pteronyssinus
Prieto et al., 2006 [35]	RCT	12 wks	Patients with mild to moderate allergic asthma	34	OMA (150–300 mg every 4 wks or 225–375 mg every 2 wks; SC injection) vs. PBO	31.0	47.1	0.0	NA	100.7	AHR to AMP
Djukanovic et al., 2004 [36]	RCT	4 months	Patients with mild to moderate asthma	45	OMA (150–300 mg every 4 wks or 225–375 mg every 2 wks; SC injection) vs. PBO	26.0	46.0	0.0	NA	85.0	AHR to methacholine
Flood-Page et al., 2003 [37]	RCT on	20 wks	Patients with mild asthma	24	Mepolizumab (750 mg, 3 doses; IV infusion) vs. PBO	30.5	70.8	0.0	NA	83.5	AHR to His
Noga et al., 2003 [38]	Sub-study conducted as part of a large multicentre RCT [47,48]	16 wks	Patients with moderate to severe allergic asthma	35	OMA (at least 0.016 mg/kg/IgE IU/mL, every 4 wks; SC injection) vs. PBO	54.3	36.5	NA	NA	79.5	AHR to acetylcholine
Leckie et al., 2000 [39]	RCT	1 day	Patients with mild allergic asthma	24	SB-240563 (2.5 or 10.0 mg/kg, single dose; IV infusion) vs. PBO	27.9	100.0	0.0	NA	88.4	AHR to His
Boulet et al., 1997 [40]	RCT	10 wks	Patients with mild allergic asthma	20	rhuMAb-E25 (1.0 mg/kg; IV infusion) vs. PBO	27.0	60.0	0.0	≤10	92.2	AHR to methacholine
Fahy et al., 1997 [41]	RCT	9 wks	Patients with mild allergic asthma	18	rhuMAb-E25 (0.5 mg/kg; IV infusion) vs. PBO	31.5	NA	NA	NA	94.5	AHR to methacholine

AHR: airway hyperresponsiveness; AMP: adenosine monophosphate; ASMC: airway smooth muscle cell; cAMP: cyclic adenosine monophosphate; CT: computed tomography; EFS: electrical filed stimulation; FEV_1: forced expiratory volume in the 1st second; His: histamine; IgE: immunoglobulin E; IL-: interleukin-; IV: intravenous; NA: not available; OMA: omalizumab; OVA: ovalbumin; PBO: placebo; QS: quick stretch; RCT: randomized controlled trial; SC: subcutaneous; TNF-α: tumor necrosis factor-alpha; wks: weeks.

3.2. Omalizumab

Omalizumab is a humanized mAb that blocks the interaction between IgE and high-affinity receptor FcεRI on inflammatory cells, including mast cells and basophils. Omalizumab is the first mAb approved by the European Medicines Agency (EMA) and FDA for the treatment of patients ≥6 years old with persistent severe allergic asthma, high levels of blood IgE, and at least a sensitization to a perennial allergen [49].

Roth et al. [44] performed an in vitro study to investigate the impact of omalizumab administered at 0.1 μg/mL, 0.5 μg/mL, and 1.0 μg/mL on primary human ASMCs isolated from allergic asthma donors and stimulated with IgE 1 μg/mL. Treatment with omalizumab dose-dependently inhibited the IgE-induced overexpression of IL-6 and IL-8, by eliciting a significant ($p < 0.05$) reduction when administered at 0.5 μg/mL and 1.0 μg/mL for IL-6, and at 1 μg/mL for IL-8. Omalizumab also suppressed the IgE-induced synthesis of IL-6 and IL-8 messenger RNAs (mRNAs), with a significant ($p < 0.05$) effect achieved when administered at 1.0 μg/mL [44]. Protein secretion of tumor necrosis factor-alpha (TNF-α) was downregulated by omalizumab as well, with a significantly lower effect on ASMCs from asthmatic donors than on control cells, whereas the reduction in TNF-α mRNA synthesis was not different compared to control [44]. When omalizumab was administered at 1.0 μg/mL, TNF-α release and synthesis were significantly ($p < 0.05$) decreased. Protein and mRNA expression of IL-4 was significantly ($p < 0.05$) reduced with omalizumab 1.0 μg/mL [44]. In ASMCs, the expression of high- and low-affinity IgE receptors was not significantly ($p > 0.05$) modulated by omalizumab, but as suggested by the authors, this could be the result of different cell types and culture environment, or different lengths of the observation period [44].

Kang et al. [43] investigated the effects of anti-IgE therapy on the AHR in a murine model of chronic asthma. The administered mAb was clone R35–92, a purified rat anti-mouse IgE mAb showing efficacy in murine asthma models that parallels the results reported for omalizumab in allergic asthmatic patients [50]. Upon chronic exposure to ovalbumin (OVA), mice developed a sustained AHR to methacholine and showed increased levels of α-smooth muscle actin (α-SMA) [43]. Treatment with anti-IgE therapy significantly ($p < 0.05$) inhibited the AHR [43].

An ex vivo study [45] investigated the impact of omalizumab administered at 60 μg/mL, 80 μg/mL, and 120 μg/mL on specific and nonspecific AHR, in both proximal and distal human airways passively sensitized with serum from asthmatic donors. One-hour treatment with OMA 120 μg/mL significantly ($p < 0.05$) reduced the contractile response to cumulative concentrations of histamine (His) in small airways [45]. Concentration-dependent curves indicated that omalizumab 60 μg/mL and 120 μg/mL maximally inhibited the His-induced AHR in small airways and medium bronchi, respectively [45]. At all concentrations, omalizumab significantly ($p < 0.05$) suppressed the specific contractile response to Dermatophagoides pteronyssinus in both medium bronchi and small airways [45].

In an RCT [41] performed on patients with mild allergic asthma, the anti-IgE mAb rhuMAb-E25 (later named omalizumab) delivered at a dose of 0.5 mg/kg for nine visits did not significantly improve the provocative concentration of methacholine causing a 20% FEV_1 (PC_{20}) compared to PBO. However, 24 h after the second allergen challenge, PC_{20} was significantly ($p < 0.05$) higher (0.45 mg/mL [95%CI 0.06–14.9]) than that on the day after the first challenge, with respect to baseline (0.13 mg/mL) [41].

In the same year, Boulet et al. [40] reported that in patients with mild allergic asthma, the anti-IgE mAb rhuMAb-E25 administered at 1.0 mg/kg during six visits slightly, although significantly ($p < 0.05$), improved methacholine PC_{20}.

Prieto et al. [35] conducted an RCT to evaluate the effects of omalizumab administered at 150–300 mg every 4 weeks or at 225, 300, or 375 mg every 2 weeks on bronchoconstriction induced by methacholine and adenosine 5′-monophosphate (AMP) in patients with mild to moderate allergic asthma. In the omalizumab group, the PC_{20} for AMP significantly ($p < 0.001$) increased from 14.32 mg/mL (95%CI 9.72–21.12) to 54.07 mg/mL (95%CI 37.67–79.69) after 4 weeks of treatment and to 53.78 mg/mL (95%CI 36.48–79.28) at 12 weeks [35]. The im-

provement in AMP PC_{20} was significantly ($p < 0.05$) greater in the patients treated with omalizumab than in those receiving PBO after 4 weeks of treatment, with the mean difference between the two groups being 1.52 doubling concentrations (95%CI 0.25–2.79) [35]. Changes in AMP PC_{20} values were not significantly different between omalizumab and PBO after 12 weeks [35]. Following cessation of treatment, PC_{20} AMP values returned to pretreatment values [35]. The PC_{20} for methacholine increased in the OMA group from 1.27 mg/mL (95%CI 0.82–1.98) to 2.10 mg/mL (95%CI 1.35–3.27) after 12 weeks of treatment and the mean increase doubling the concentrations was not significant [35]. Changes in methacholine PC_{20} values were not significantly different between the OMA and PBO groups, neither when doubling the concentrations [35]. After cessation of OMA, PC_{20} methacholine parameters returned to pretreatment values [35].

A prospective observational study [46] evaluated the efficacy of 48 weeks of treatment with omalizumab on the AHR in patients with severe refractory asthma, despite the use of multiple controller medications. Omalizumab dosing was based on body weight and baseline total serum IgE levels [46]. Omalizumab did not change the airway sensitivity and reactivity to methacholine [46]. According to the authors, these results could have been affected by the limited data coming from computed tomography (CT) and methacholine responsiveness tests, since not all patients could undergo these measurements due to poor lung function and difficulty in breath-holding [46].

After 16 weeks of treatment with omalizumab administered at 150–300 mg every 4 weeks or at 225–375 mg every 2 weeks, AHR to methacholine inhalation was not significantly improved in mild to moderate asthmatic patients with sputum eosinophilia, although at baseline, patients in the PBO group were less responsive to methacholine than the omalizumab group (PC_{20} 0.54 vs. 1.01, respectively) [36]. As stated by the authors of the study, this evidence might not only be indicative of the physiological complexity of AHR, but it might also suggest that IgE and eosinophils, which were significantly decreased with omalizumab, may not mediate methacholine responsiveness in mild to moderate asthma [36].

A sub-study [38] of a previous multicenter RCT [47,48] showed that in patients with moderate to severe allergic asthma, treatment with omalizumab administered at 2- to 4-weeks intervals based on body weight and total IgE at screening, improved the AHR to acetylcholine by inducing a significant ($p < 0.05$) increase in PC_{20} compared to PBO. Three months after completing the therapy, no difference was detected between the treatment groups [38].

3.3. Mepolizumab

Mepolizumab is the first humanized anti-IL-5 mAb approved by EMA and FDA for patients ≥6 years old with severe eosinophilic asthma that remains uncontrolled despite GINA step 4 therapy. Mepolizumab targets circulating IL-5 preventing the interaction with the IL-5Rα on the surface of eosinophils, a condition necessary for the development and survival of eosinophils themselves [49].

In passively sensitized medium bronchi, a procedure that reproduces ex vivo the AHR characteristic of asthma, mepolizumab administered at concentrations ≥3 μg/mL significantly ($p < 0.05$) prevented the His-mediated AHR and at ≥10 μg/mL, the ASM contractility was significantly ($p < 0.05$) reduced at the same level of that detectable in control tissues [25]. At least 100 μg/mL of mepolizumab were necessary to significantly ($p < 0.05$) reduce the potency of His (delta negative logarithm of the half-maximal effective concentration [pEC_{50}]: 0.65 ± 0.22) [25]. In particular, mepolizumab suppressed the AHR induced by His administered at concentrations eliciting 50%, 70%, and 90% of the maximal effect (EC_{50}, EC_{70}, and EC_{90}; E_{max}), by reaching an E_{max} of −108.29 ± 32.16%, and significantly ($p < 0.05$) relaxed the bronchial contractile tone induced by electrical field stimulation (EFS) delivered at frequencies inducing 50%, 70%, and 90% of the maximal effective frequency (EF_{50}, EF_{70}, and EF_{90}), in a concentration-dependent manner [25]. At least 30 μg/mL of mepolizumab was necessary to significantly ($p < 0.05$) reduce the

hyperresponsive myogenic tone induced by quick stretch, compared to positive control tissues. Mepolizumab prevented the depletion of cyclic adenosine monophosphate (cAMP) induced by passive sensitization and restored the physiological levels when administered at 10 μg/mL [25]. Interestingly, the inhibition of AHR by mepolizumab was significantly ($p < 0.05$) correlated with the increased cAMP concentrations [25].

Leckie et al. [39] conducted an RCT in mild asthmatic patients to investigate the effects of a single intravenous infusion of SB-240563 (later known as mepolizumab) administered at doses of 2.5 mg/kg or 10.0 mg/kg. Compared to PBO, mepolizumab did not significantly modulate the AHR to His before and after allergen challenges, therefore the authors of the study suggested that blood and sputum eosinophilia might not represent the prerequisite for AHR in relation to allergen exposure and that several other cell types might be involved in these responses [39].

Flood-Page et al. [37] extended the work by Leckie et al. [39] by conducting an RCT on the impact on AHR of three intravenous doses of mepolizumab 750 mg in patients suffering from mild asthma. The study found no significant difference between mepolizumab and PBO groups in reducing the AHR induced by His [37]. According to the authors, this evidence could be explained by the fact that mepolizumab only moderately reduced bronchial tissue eosinophilia and did not modulate the ongoing eosinophilic degranulation within the bronchial mucosa [37].

Haldar et al. [34] conducted an RCT of patients with refractory eosinophilic asthma and a history of recurrent severe exacerbations to assess the impact of mepolizumab 750 mg on the AHR induced by methacholine [34]. After 12 weeks of treatment, mepolizumab did not induce an improvement of the AHR [34].

3.4. Benralizumab

Benralizumab is a humanized afucosylated mAb directed against IL-5Rα and not against the circulating IL-5. The complex of mAb/IL-5Rα promotes the apoptosis of eosinophils via antibody-dependent cell-mediated cytotoxicity, a mechanism involving natural killer cells leading to active peripheral eosinophils depletion. Benralizumab is approved by the EMA for adult patients and by the FDA for subjects aged ≥12 years old as an add-on treatment for uncontrolled severe eosinophilic asthma [49].

In passively sensitized human medium bronchi, benralizumab administered at ≥1 μg/mL significantly ($p < 0.05$) reduced the E_{max} elicited by the concentration-response curve to His and when administered at 100 μg/mL, the contractility was reduced to a significantly ($p < 0.05$) lower level than that detectable in the negative control tissues [25]. At concentrations ≥10 μg/mL, benralizumab significantly ($p < 0.05$) reduced the potency of His (pEC_{50}: 0.65 ± 0.20) [25]. Specifically, the anti-IL-5Rα mAb suppressed the AHR induced by His administered at EC_{50}, EC_{70}, and EC_{90}, by reaching an E_{max} of −134.14 ± 14.93% [25]. Moreover, benralizumab inhibited the contractile response to EFS delivered at EF_{50}, EF_{70}, and EF_{90}, in a concentration-dependent manner [25]. Benralizumab also suppressed the hyperresponsive myogenic tone induced by quick stretch and when administered at 10 μg/mL, the contractile response was reduced to a level significantly ($p < 0.05$) lower than that detectable with negative controls [25]. Benralizumab restored the physiological levels of cAMP, and interestingly, inhibition of the AHR was significantly ($p < 0.05$) correlated with the increased cAMP concentration [25]. As suggested by the authors, the improvement in the level of cAMP may represent a central mechanism to which the inhibition of the IL-5/IL-5Rα pathway may converge, thus leading to the AHR prevention [25].

3.5. Dupilumab

Dupilumab, the last approved mAb for the treatment of asthma, acts against the α chain of IL-4 receptor (IL-4R) that is common to both IL-4R and IL-13R. IL-4 and IL-13 are crucial in type 2 inflammation, leading to AHR [49].

Recently, Manson et al. [18] demonstrated that in human isolated small airways, IL-4 and IL-13 increased the potency of His and pretreatment with dupilumab administered

at 1 μM significantly ($p < 0.05$) abolished the responses to both IL-4 (pEC_{50}: 6.7 ± 0.1 vs. 6.1 ± 0.2) and IL-13 (pEC_{50}: 7.1 ± 0.1 vs. 6.3 ± 0.1). In ASMCs, dupilumab blocked the increase in the E_{max} of His-induced intracellular calcium mobilization caused by IL-13 and IL-4 [18].

3.6. Tezepelumab

Tezepelumab, recently accepted and granted priority review from the FDA for the treatment of asthma [28], is a first-in-class anti-TSLP mAb that reduces asthma exacerbations and airway inflammation, and improves pre-bronchodilator FEV_1 in patients with uncontrolled asthma, regardless of baseline blood eosinophil count [49].

The UPSTREAM RCT [33] investigated the effect of tezepelumab 700 mg on the AHR induced by mannitol in patients with uncontrolled asthma. After 12 weeks of treatment, tezepelumab administered at 700 mg induced a numerical improvement in the provoking dose of mannitol causing a 15% reduction in FEV_1 (PD_{15}) compared to PBO, and a most pronounced effect was detected in subjects affected by eosinophilic asthma [33]. At the end of the treatment period, the proportion of patients without AHR to mannitol was significantly ($p < 0.05$) higher in the tezepelumab group than in the PBO group (nine vs. three) [33].

Similarly, the very recent CASCADE RCT [42] demonstrated that in patients with uncontrolled moderate to severe asthma, tezepelumab 210 mg administered for 28 weeks significantly ($p < 0.05$) reduced the AHR to mannitol vs. PBO, both in terms of absolute change in PD_{15} (197.4 mg [95% CI 107.9–286.9] vs. 58.6 mg [95% CI −30.1–147.33], respectively), and in doubling dose units (1.41 mg [95% CI 0.84–1.99] vs. 0.57 mg [95% CI 0.01–1.13], respectively). A numerically higher proportion of patients in the tezepelumab group showed a negative mannitol test compared to PCB at the end of the treatment period (13 [43.0%] vs. 7 [25%]) [42].

4. Discussion

The evidence resulting from this systematic review indicates that omalizumab, dupilumab, and tezepelumab may modulate AHR via direct action on ASMCs and indirect influence on eosinophilic inflammation and parasympathetic activity; conversely, mepolizumab and benralizumab seem to prevent AHR prevalently by reducing airway inflammation and vagal firing (Figure 2).

ASMCs are capable of expressing the heterodimeric TSLP receptor (TSLPR), consisting of the IL-7 receptor-α chain (IL-7Rα) and TSLPR subunit [22], the heterodimeric IL-4 and IL-13 receptor (IL-4R and IL-13R) complexes which share the common subunit IL-4Rα [51], and the high-affinity receptor for IgE, also known as FcεRI [52]. By contrast, the heterodimeric IL-5 receptor (IL-5R), consisting of an α subunit specific only to IL-5 binding and the common receptor β subunit (βc), is not present on the surface of ASMCs [53].

Eosinophils, which are central to the pathogenesis of allergic and non-allergic asthma [54,55], express high levels of surface IL-5Rα chain [56], as well as TSLPR [57], the IL-4Rα chain common to IL-4R and IL-13R [58,59], and the high-affinity receptor FcεRI [60]. In addition, further inflammatory cells, including dendritic cells, lung epithelial cells, lymphocytes, mast cells, monocytes, stromal cells, and type 2 innate lymphoid cells have been shown to express the heterodimers IL-4Rα/IL-13Rα1 [61,62] and TSLPR [63–65], while basophils and mast cells represent the two main cell populations expressing IL-5R and FcεRI [66,67].

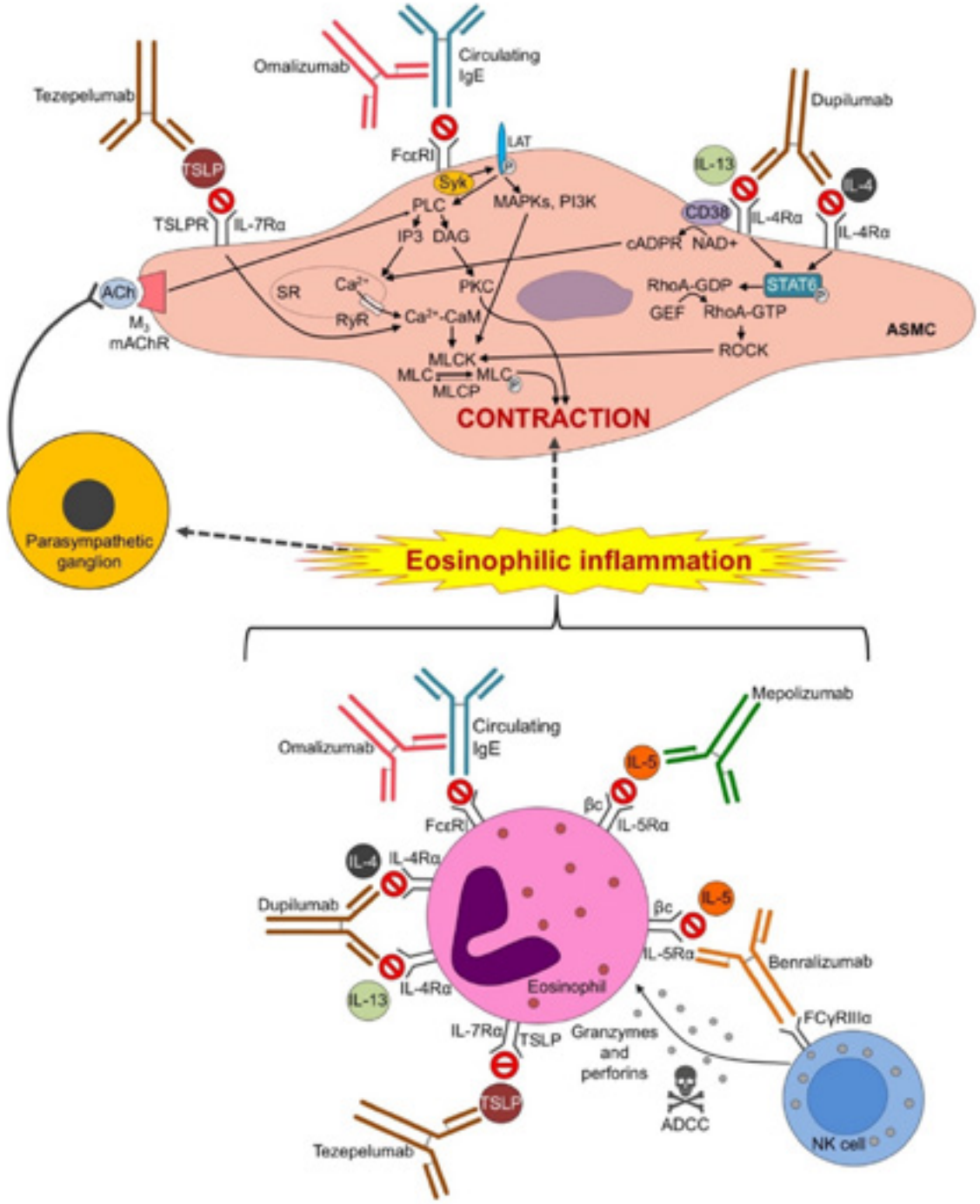

Figure 2. Direct and indirect mechanisms of action against AHR of the mAbs resulting from this systematic review. Parasympathetic ganglia are innervated by preganglionic nerve fibers carried by the vagus nerves and they are found in abundance throughout the walls of medium bronchi. ACh: acetylcholine; ADCC: antibody-dependent cell cytotoxicity; ASMC: airway smooth muscle cell; Ca^{2+}: calcium; CaDPR: Cyclic adenosine 5′-diphosphate ribose; CaM: calmodulin; DAG: diacylglycerol; FDA: Food and Drug Administration; GDP: guanosine diphosphate; GEF: guanine nucleotide-exchange factor; IgE: immunoglobulin E; IL-n: interleukin-n; IL-nR: interleukin-n receptor; IP3: inositol trisphosphate; LAT: linker for T-cell activation; mAbs: monoclonal antibodies; MAPK: mitogen-activated protein kinase; mAChR: muscarinic acetylcholine receptor; MLC: Myosin light chain; MLCK: myosin light chain kinase; MLCP: Myosin light chain phosphatase; NAD+: Nicotinamide adenine dinucleotide; NK: natural killer; PKC: protein kinase C; PLC: phospholipase C; RhoA: Ras homolog family member A; ROCK: Rho-associated protein kinase; RyR: ryanodine receptor; SR: sarcoplasmic reticulum; STAT6: signal transducer and activator of transcription 6; TSLP: thymic stromal lymphopoietin; TSPLR: TSLP receptor.

Therefore, the anti-IgE omalizumab, the anti-IL-4/IL-13 dupilumab, and the anti-TSLP tezepelumab are supposed to have the ability to directly modulate the ASM contractility and AHR in asthma, by specifically blocking the interaction between the ligands and FcεRI, IL-4Rα, and TSLPR respectively, and indirectly by targeting eosinophils and further inflammatory cells, thus leading to a reduction in the inflammatory cascade.

Conversely, mepolizumab and reslizumab, which selectively block circulating IL-5, and benralizumab, which prevents the adhesion of IL-5 to the IL-5Rα chain, may indirectly modulate the contractile properties of ASMCs by targeting eosinophils and other immunomodulatory effector cells responsible for promoting inflammatory processes of asthma.

The direct action of omalizumab, dupilumab, and tezepelumab on the ASM is mediated by complex intracellular signaling pathways (Figure 2). Omalizumab is known to recognize and sequester free serum IgE, thereby causing the high-affinity receptor FcεRI to be down-regulated [68]. In the absence of omalizumab, the FcεRI signaling cascade requires crosslinking of multivalent antigen bound to IgE, leading to the activation of Syk kinase [69]. Prevalent notion suggests that Lyn kinase is necessary to phosphorylate immunoreceptor tyrosine activating motifs (ITAMs) on FcεRI in order to provide a docking site for activated Syk, however, evidence from electron microscopy and biochemical investigations showed that Lyn dissociates from FcεRI as soon as the latter is stimulated [70,71]. Activated Syk mediates the phosphorylation of the linker for activation of T cells (LAT) that in turn activates phospholipase C (PLC) [69]. PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP-2) to inositol-1,4-5-triphosphate (IP3) and diacylglycerol (DAG) [72], and in turn IP3 mediates calcium mobilization from the sarcoplasmic reticulum (SR) [73]. In ASMCs, the fast and transient increase in intracellular calcium activates the calcium/calmodulin-sensitive myosin light chain (MLC) kinase (MLCK), followed by phosphorylation of the regulatory MLC and subsequent ASM contraction [74]. Interestingly, evidence that the IgE-induced MLCK expression is also mediated by multiple signaling pathways including mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3K) was provided by Balhara et al. [75].

Dupilumab prevents IL-4 and IL-13 signaling by blocking the IL-4Rα chain common to the IL-4R and IL-13R complexes [76]. Recent evidence has demonstrated that both cytokines directly contribute to the development of AHR at the level of ASMCs [18]. IL-4 and IL-13 upregulate RhoA-GDP by inducing the phosphorylation of signal transducer and activator of transcription 6 (STAT6) [21,77]. RhoA-GDP is activated by Rho guanosine nucleotide exchange factor (GEF), which promotes the exchange of GDP for GTP and the active RhoA-GTP stimulates Rho kinase (ROCK) to inhibit MLCP [78,79] thereby triggering ASM contraction. Another mechanism by which IL-13 is able to modulate ASM contractility has been postulated by Deshpande et al. [80,81]: IL-13 increases the expression of CD38, a cell surface hydrolase and cyclase highly expressed in asthmatic ASMCs, which catalyzes the production of cyclic adenosine diphosphate-ribosyl cyclase (cADPR), an activator of the ryanodine receptor (RyR).

Tezepelumab selectively blocks human TSLP from interacting with the heterodimeric TSLPR complex [82]. Compared with healthy controls, TSLP is highly expressed in the airways of asthmatic patients [83,84]. In vitro evidence has demonstrated that TSLP stimulates ASMCs to enhance intracellular calcium responses to contractile agonists, thus suggesting TSLP to be a potential mediator of ASM contractility [22].

Only recently, an ex vivo study provided evidence that targeting the IL-5/IL-5R pathway is effective against AHR in passively sensitized human medium bronchi [25]. Passive airway sensitization reproduces ex vivo an IgE-dependent AHR in presence of further serum factors and promotes the adhesion of eosinophils to parasympathetic nerves, thereby stimulating their degranulation with subsequent major basic protein (MBP) release. MBP causes the loss of function of the M_2 muscarinic acetylcholine (ACh) receptor (mAChR) present on postganglionic parasympathetic nerves, resulting in an excessive ACh release [85]. It has been suggested that benralizumab and mepolizumab prevent the AHR to

His and EFS by indirectly inhibiting endogenously released intermediaries of bronchoconstriction [25]. Indeed, His indirectly promotes the release of ACh from parasympathetic nerves [86], while EFS mediates the sensitization of vagal parasympathetic fibers causing the release of ACh [87,88] The interaction between ACh and M_3 mAChRs expressed on post-junctional ASMCs stimulates PLC to produce diacylglycerol (DAG), which in turn activates phosphokinase C to promote ASM contraction [89].

The present systematic review supports evidence for the direct efficacy of omalizumab, dupilumab, and tezepelumab, and the indirect beneficial effect of mepolizumab and benralizumab in modulating the contractility of the ASM and preventing AHR. Omalizumab generally improved AHR in vitro and in vivo, although surprisingly, several RCTs [35,36,41] conducted on mild to moderate asthmatic patients and one observational study [46] on severe refractory asthma reported no beneficial effect of omalizumab against AHR. Nevertheless, these clinical studies were performed on strictly selected and limited groups of patients, therefore these results should be taken with caution.

In vitro and ex vivo evidence indicates that dupilumab effectively reduced the AHR to His [18], whereas tezepelumab improved the ASM contractile response to mannitol in an RCT [33]. Furthermore, both benralizumab and mepolizumab prevented the AHR to His, parasympathetic activation, and mechanical stress, an effect correlated with improved levels of cAMP in hyperresponsive airways [25]. Results from RCTs conducted on patients with mild asthma [37,39] and refractory eosinophilic asthma [34] reported that mepolizumab did not prevent AHR induced by His and methacholine, but the stringent inclusion criteria of the studies might not be representative of broader asthmatic populations.

Interestingly, several studies conducted in vitro on ASMCs, in experimental animal models of asthma or in vivo in humans confirmed the beneficial role of omalizumab [43,46,90–95], benralizumab [53], and mepolizumab [34] in reversing airway remodeling. Specifically, OMA reduced collagen and fibronectin deposition in ASMCs [91,92], down-regulated ASM-associated components, mainly myosins and actins [93,95], and reduced the proliferation of ASMCs [92]. In severe asthmatics, omalizumab improved airway wall thickness and the luminal area at the right apical segmental bronchus [46,90] and reduced the number of circulating fibrocytes, which act as precursors of bronchial myofibroblasts [94]. Benralizumab reduced the number of tissue myofibroblasts and the ASM mass [53], while mepolizumab reduced airway wall thickness and total wall area [34].

AHR has been suggested to be a main treatable trait towards precision medicine in patients suffering from eosinophilic asthma [96,97], and together with the post-bronchodilator reversibility test, it is recommended as a tool in asthma diagnosis, classification severity, and control monitoring [98]. Appropriately designed head-to-head RCTs are needed to compare the efficacy of those mAbs directly targeting ASM contractility specifically against AHR in severe asthma, namely omalizumab, dupilumab, and tezepelumab.

Among the currently available mAbs, omalizumab has the greatest amount of data concerning the impact on ASM contractility, as it was first, and for a long time the only available add-on biologic therapy for the treatment of severe asthma [99]. To the best of our knowledge, no study covered any aspect of AHR and ASM contractility in therapy with the currently approved mAb reslizumab.

The limitations of this systematic review are related to the intrinsic characteristics of the included studies: in vitro and ex vivo studies are based on models of asthma, and RCTs have been generally carried out in small and selected populations of asthmatic patients.

In conclusion, the development of biological therapies has led to a significant step forward in the treatment of severe asthma; nevertheless, to date little is still known about the real clinical impact of mAbs against AHR. Future research of currently available and upcoming mAbs for the treatment of severe asthma should address this clinical issue as an important feature of long-term disease management.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Conflicts of Interest: The authors declare no conflict of interest.

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Received: 15 October 2020 | Revised: 31 March 2021 | Accepted: 1 April 2021

DOI: 10.1111/pai.13516

ORIGINAL ARTICLE

WILEY

Asthma, bronchial hyperresponsiveness, allergy and lung function development until early adulthood: A systematic literature review

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Editor: Ömer Kalaycı

Abstract

Background: It is unclear in which periods of life lung function deficits develop, and whether these are affected by risk factors such as asthma, bronchial hyperresponsiveness (BHR) and allergic comorbidity. The goal of this systematic review was to identify temporal associations of asthma, BHR and allergic comorbidity with large and small lung function development from birth until peak function in early adulthood.

Methods: We searched MEDLINE, EMBASE, Web of Science and CINAHL for papers published before 01.01.2020 on risk factors and lung function measurements of large and small airways. Studies were required to report lung function at any time point or interval from birth until peak lung function (age 21-26) and include at least one candidate risk factor.

Results: Of the 45 papers identified, 44 investigated cohorts and one was a clinical trial with follow-up. Asthma, wheezing, BHR and allergic sensitization early in life and to multiple allergens were associated with a lower lung function growth of large and small airways during early childhood compared with the control populations. Lung function development after childhood in subjects with asthma or persistent wheeze, although continuing to grow at a lower level, largely tracked parallel to non-affected individuals until peak function was attained.

Clinical implications and future research: Deficits in lung function growth develop in early childhood, and children with asthma, BHR and early-life IgE (poly)sensitization are at risk. This period is possibly a critical window of opportunity to identify at-risk subjects and provide treatment aimed at preventing long-term sequelae of lung function.

KEYWORDS
asthma, allergy, bronchial hyperresponsiveness, growth, lung function, small airways

1 | INTRODUCTION

Peak lung function is normally attained around the age of 22 for males and slightly earlier for females,[1] after which lung function remains stable for some years during a plateau phase before beginning to decline.[2,3] Children with asthma may reach a lower maximum lung function in adulthood,[3-6] putting them at risk for development of future COPD. Different patterns of impaired lung function development from childhood to adulthood have been described in children with asthma, such as 'normal growth', 'normal growth and early decline', 'reduced growth' and 'reduced growth and early decline'.[3] Growth of the lungs may not only be impaired during early childhood, but also throughout adolescence and early adulthood. Next to the growth of the large airways, growth of the small airways may be important, as accumulating evidence suggests that many lung diseases, including asthma and COPD, start in the small airways.[7] Therefore, better knowledge on the predictors, place (small versus large airways) and timing of the development of low lung function may set the stage for future preventative measures aimed at improving lung function growth.

So far, conflicting results have been reported on lung growth in asthmatic children. Some studies suggested no association of mild or transient asthma with reduced lung growth in the first years of life,[5,8] whereas in another study, more severe asthma and persistent wheeze were associated with reduced lung growth throughout childhood and adolescence.[4] The presence of asthma, the timing of asthma onset, persistence and severity of symptoms and the presence of allergic comorbidity may be important determinants of the maximally attained FEV_1 in early adulthood (Figure 1).[5,8-12] Moreover, it has not been systematically assessed whether these risk factors also relate to measures of small airway function growth. Thus, an important question remains when and where the lung function deficits develop: in the first years of life, in childhood, adolescence or early adulthood?

To identify the factors associated with lung function growth and their significance during different periods of development, this systematic literature review investigated current literature on the temporal associations of asthma and allergy with lung function growth of small and large airways during childhood and adolescence up to the maximum lung function in early adulthood. Asthma is heterogeneous disease with varying degrees of symptoms, comorbidities and clinical biomarkers. Candidate risk factors were therefore selected with the aim of capturing a valid representation of potential factors associated with lung function growth in subjects with asthma or allergy. In addition to asthma and wheezing in early life, bronchial hyper-responsiveness (BHR), a hallmark of asthma, was included. Furthermore, we included allergic sensitization, rhinitis and blood eosinophils as candidate risk factors for a lower lung function growth from infancy until peak lung function in early adulthood.

2 | METHODS

This systematic review (PROSPERO registration number: CRD42020172531) was conducted in accordance with guidelines reported in the Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA).[13]

Key Message

Asthma, wheezing, bronchial hyperresponsiveness (BHR) and allergic sensitization are associated with a lower lung function growth of large and small airways during early childhood. Lung function development after childhood largely tracks parallel to non-asthmatic individuals.

2.1 | Search strategy

We searched MEDLINE using the PubMed search engine, EMBASE, Web of Science (Clarivate) and CINAHL (EBSCO) for papers published before 01.01.2020 with search terms as outlined in Table 1 and Appendix S1. In addition to papers screened in MEDLINE, 52 papers, retrieved from backward citation searching, were reviewed for eligibility of which 2 studies were selected for inclusion (Figure 2).

2.2 | Study selection

Abstracts of all papers were screened independently by two researchers (HJLK and AMZ). Subsequently, full-text papers were assessed for eligibility. In case of disagreement, the study was assessed by a third independent researcher (GHK). Papers were required to contain relevant primary data on studies performed in humans (inclusion criteria; see Table 2). We included longitudinal studies that provided data on temporal associations between candidate risk factors and lung function. This entails that in studies with lung function measured at one time point, the ascertainment of candidate risk factors (eg asthma diagnosis) had to precede the measurement of lung function. In studies with multiple measurements of lung function, concurrent ascertainment of a candidate risk factor and lung function testing was permitted. We investigated the following candidate risk factors: asthma diagnosis, wheezing, BHR, markers related to allergy (rhinitis, specific IgE, skin prick tests) and blood eosinophils within asthmatic populations, non-asthmatic patients or in general population-based cohorts. These studies needed to report lung function at a point between infancy until maximum lung function was attained (age 21-26). Studies presenting a mean lung function of subjects that had an age range >2 years were excluded to avoid aggregating lung function data from subjects at different stages of development. In studies reporting findings from two or more cohorts, in which not all cohorts matched the inclusion criteria, relevant data were extracted only from cohorts that matched our inclusion criteria. Letters to editors were not included in this systematic review as this format would not allow us to verify the extensive inclusion criteria or perform a complete quality analysis. Backward

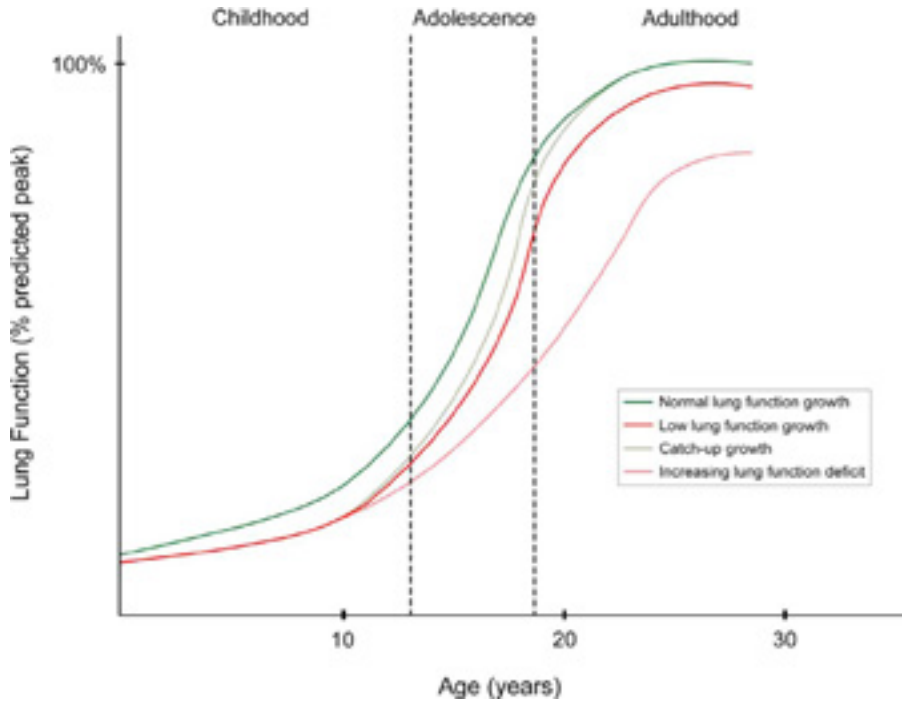

FIGURE 1 Lung function growth from childhood to adulthood. The green line represents normal lung function growth and levels. The red line represents low lung function growth and levels. The light green represents subjects with low lung function levels in early childhood and catch-up growth in adolescence and early adulthood. The pink line represents children with lower lung function levels in childhood and reduced growth until early adulthood. Figure 1 is a conceptual illustration based on Agustí et al[70]

TABLE 1 Search strategy using PubMed

Search strategy
We searched PubMed using the following key terms:
PubMed (MESH terms)
Lung Volume Measurements/Respiratory Function Tests/Spirometry/Lung/growth and development/Allergy and Immunology/Hypersensitivity/Eosinophils/Eosinophilia/Immunoglobulin E/Asthma/Respiratory Hypersensitivity/Rhinitis, Allergic/Predictive Value of Tests/Cohort Studies/Case-Control Studies/Child/Infant/Adolescent/Young Adult/Age Distribution
Title and abstract search
lung growth/pulmonary growth/lung function meas*/spiromet*/plethysmography/forced oscillation technique*/lung clearance index/multiple breath washout/lung function*/allerg*/asthma*/hypersensit*/hypperresponsiv*/eosinophil/follow-up/followup/longitudinal/cohort/case-control/trajector*/pattern*/child*/infan*/prenatal*/fetal/pediatr*/paediatr*/school/preschool/adolscen*/teenager*/young adult*/younger adult*/young people/younger people / early life/early age/young age*/younger age*
For the full strategy and searches performed in EMBASE, CINAHL and Web of Science, please see Appendix S1

citation search was performed by screening references (using title and abstract) in all full-text assessed papers for possible inclusion.

2.3 | Study population

The aim of this systematic review was to study the development of lung function in subjects with asthma or allergy compared with a non-affected population. We investigated lung function development between the ages of 0 and 26 as this period comprises lung growth from birth until peak lung function in early adulthood. Subjects could be derived from both hospital and population-based cohorts. As asthma and allergies are highly heterogeneous conditions, different candidate risk factors were chosen that characterize these. These risk factors could be defined at a specific point in time (eg asthma at age 6) or could be based on longitudinal phenotype modelling. Comparison of lung function between affected and non-affected subjects could be performed within the same population, within a separate general population-based cohort or by utilizing standard reference values. Studies with outcome parameters derived from spirometry, forced oscillation technique (FOT), multiple-breath washout (MBW) and body plethysmography were included. Separate analyses were performed for outcome parameters reflecting the large airways (eg FEV_1: forced expiratory volume in one second, FVC: forced vital capacity, FEV_1/FVC) and the small airways (eg FEF_{25-75}: forced expiratory flow at 25%-75% of FVC, sRaw: specific airway resistance, MMEF: maximal mid-expiratory flow, R_5: resistance at 5 Hz, f_{res}: resonance frequency). We classified sRaw and

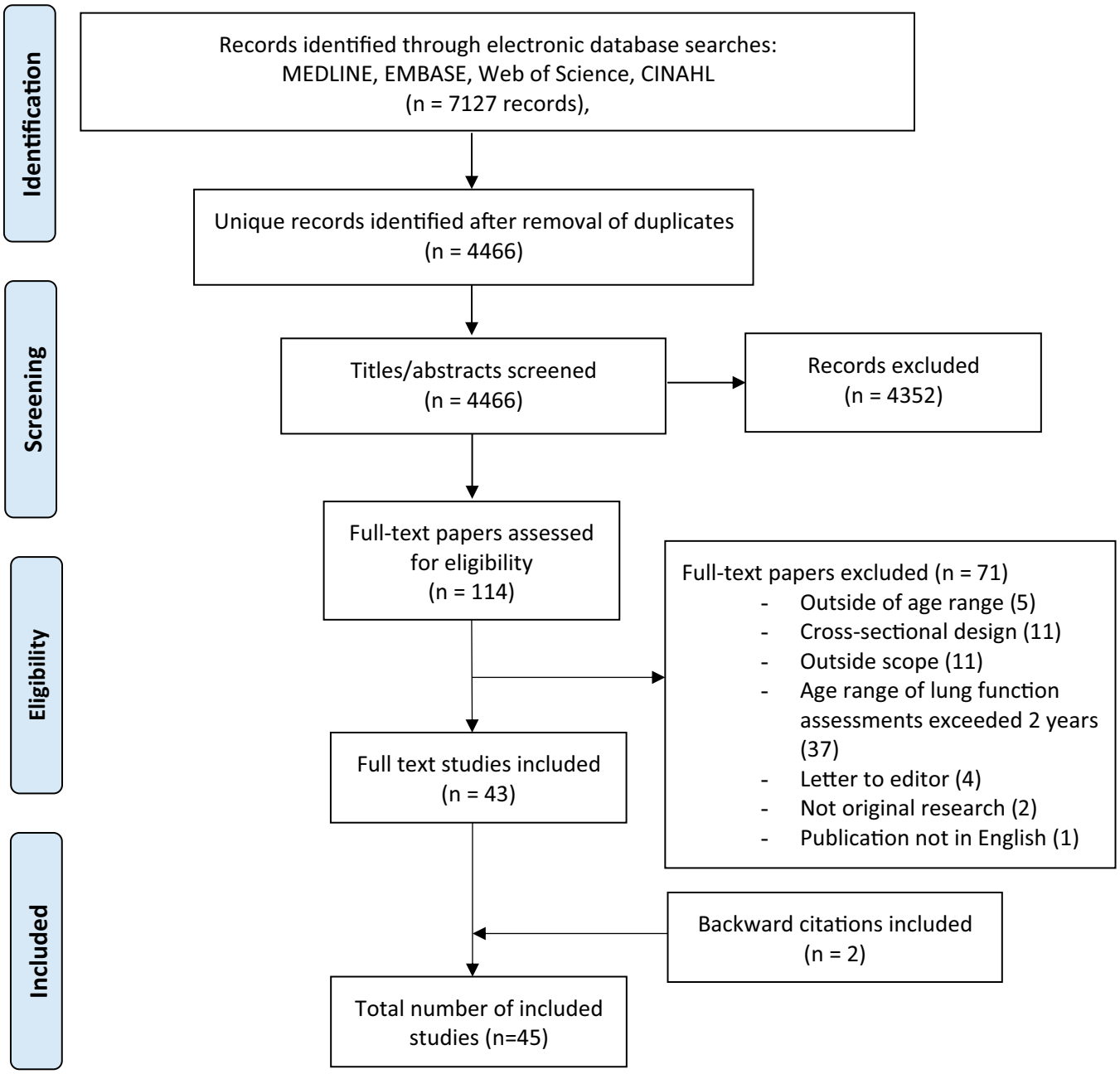

FIGURE 2 Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram

MMEF as small airway parameters, although large airway obstruction could also affect this outcome, thereby making it a mixed parameter. VmaxFRC (maximum forced expiratory flow at functional residual capacity) derived from rapid chest compression in infancy was reported if the study met requirements of lung function testing later during development.

2.4 | Data extraction

Information on study design, candidate risk factors and lung function outcomes was collected from included papers. Results were grouped according to which type of lung function outcome was presented: estimated lung function trajectories using, for example, latent class analysis (LCA), calculated change in lung function over time (growth) and lung function levels at specific time points. If included studies provided sex-stratified associations, findings were included in the same manner in review. Definitions used for periods of development and phenotype development are provided in Appendix S3. Quality assessment of included studies was performed using a modified Newcastle-Ottawa Quality Assessment Scale for cohort studies [14,15] (see Appendix S2). Information relating to quality assessment was collected from the included paper, the supplementary data or the official cohort profile. All studies with 6 or more stars were classified as high quality, while studies with 4-5 stars were classified as moderate. In the quality assessment, the following criteria were reviewed:

TABLE 2 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Longitudinal cohort studies and clinical trials with observational follow-up	Letter to editors, meeting abstracts, case reports and literature reviews
Age of subjects 0-26 y	>2-yr age range for mean lung function measurement
Subjects from population-based cohorts or hospital-based cohorts	Ascertainment of risk factor not preceding lung function measurement (cross-sectional studies only)
Papers published before 01.01.2020	
Publications written in English	
Predictors of outcome: • Asthma/Wheezing • BHR • Allergic sensitization (IgE and SPT) • Rhinitis • Blood eosinophils	
Lung function derived from: • Spirometry • Forced oscillation technique • Multiple-breath washout • Body plethysmography	

Abbreviations: BHR, bronchial hyper-responsiveness; IgE, immunoglobulin E; SPT, skin prick test.

1. Representativeness of the exposed cohort (eg non-selected general population-based birth cohorts)
2. Selection of the non-exposed cohort (selection from the same cohort as exposed subjects or a separate cohort).
3. Ascertainment of exposure/candidate risk factor (eg structured interview vs. self-reported observations).
4. Comparability of cohorts (degree of study control for the following confounders: age, height and sex)
5. Duration of follow-up (studies with more than 2-year follow-up were awarded a star)
6. Adequacy of follow-up (degree of follow-up and description of subjects lost to follow-up)

3 | RESULTS

3.1 | Search results

The literature search strategy identified 7127 records (Figure 2). After removal of duplicate records, 4466 records were reviewed using title and abstract. Of these, 114 full-text papers were assessed for eligibility resulting in 43 included studies. Backward citations from selected papers yielded an additional 2 studies bringing the total number of papers in the final analysis to 45.

3.2 | Characteristics of studies

Of the 45 selected papers, 38 were population-based,[4,5,16-51] 6 were clinical/hospital-based or high-risk cohorts,[52-57] and one was a clinical trial with observational follow-up.[3] In total, 23 different cohorts were identified (Figure S1), of which 15 were birth cohorts (Tables 3,4,S1). Seven studies reported lung function trajectories (Table 3). These studies mainly captured differences in lung function levels that remained stable throughout development. Next, 14 studies reported associations with lung function growth during a defined period until early adulthood (Table 4), while 33 papers reported associations with lung function levels (Table S1).

Separate lung function trajectories that capture differences in growth in an affected population relative to a control group were identified in two studies.[3,47] Small airway parameters were included in 22 studies,[5,17,21,22,25-29,31,33,35-38,41,44,48,49,52,53,56] of which $FEF_{25\%-75\%}$ was the most frequently used. Of the 45 included studies, one had a moderate level of quality, while the remaining had a high level (Appendix S2). Due to the overall high quality, differences in quality were not considered when reporting or interpreting findings from included studies. Different strategies in ascertainment of exposure, that is candidate risk factor, contributed to the greatest variation in quality amongst selected studies. The most frequent biases were use of parental questionnaires and observations to ascertain the presence of a risk factor.

3.3 | Asthma and wheezing

3.3.1 | Lung function trajectories

Asthma and wheezing

Asthma and/or wheezing were associated with a lower-than-normal lung function trajectory from childhood until adolescence [45,46] and until early adulthood.[43,45,47,48] The trajectories identified differences in lung function level over time but not growth rate during development. Asthma in childhood was associated with lower lung function trajectories for both small and large airways until early adulthood.[48]

TABLE 3 Studies on lung function trajectories

First author	Cohort	Type and age lung function measurement(s)	End-points	Predictors of outcome	Main findings (S: significant, NS: non-significant)
Schultz[46]	BAMSE (n = 1425) Population-based birth cohort	Spirometry at 8 and 16 y	Lung function trajectory: Low Normal/high Large airways: FEV_1	Asthma/wheeze	S: early wheeze and asthma ever till age 8 were associated with a low FEV_1 trajectory (<25th percentile at 8 and 16 y of age). Prevalence of early wheeze: 25% in low trajectory and 12% in normal/high trajectory. Prevalence of asthma: 23% in low trajectory and 14% in normal/high trajectory. NS: Current wheeze at age 8 was not associated with a low FEV_1 trajectory
				Allergic sensitization (IgE)	NS: allergic sensitization at age 8 was not associated with a low FEV_1 trajectory (<25th percentile at 8 and 16 y of age).
Belgrave[45]	MAAS (n = 1046) Population-based birth cohort ALSPAC (n = 1390) Population-based birth cohort	MAAS: spirometry at 5, 8, 11 and 16 y ALSPAC: Spirometry at 8, 15 and 24 yars	Lung function trajectory: Persistently high Normal Below average Persistently low Large airways: FEV_1	Asthma, wheeze	S: asthma and wheeze throughout the follow-up period were associated with a persistently low FEV_1 trajectory (see appendix of original paper for exact data)
				BHR (yes/no)	S: BHR (ALSPAC at ages 15 and 24 and MAAS at ages 5, 8, 11 and 16) was associated with a persistently low FEV_1 trajectory.
				Allergic sensitization (skin prick test)	S: allergic sensitization in early childhood in MAAS was associated with a persistently low FEV_1 trajectory. NS: allergic sensitization in adolescence (MAAS) or at age 7 (ALSPAC) was not associated with a persistently low FEV_1 trajectory
McGeachie[3]	CAMP (n = 684) Randomized controlled trial with extended follow-up in asthmatic patients	Spirometry (age 5/12-26/30)	Lung function trajectory: Normal growth Reduced growth Normal growth, early decline Reduced growth, early decline Large airways: FEV_1	BHR severity	S: more severe BHR (at inclusion) was associated with a reduced FEV_1 growth pattern (OR for reduced growth compared with normal growth: 0.61 per unit change in log-transformed mg per mL)
				Allergic sensitization (skin prick test)	S: subjects with a 'reduced growth and early decline' trajectory had a greater number of positive skin prick tests at enrolment compared with subjects with a 'normal growth' trajectory (OR for ≥3 positive skin tests vs. <3:2.42)
Rasmussen[23]	Dunedin, New Zealand (n = 788) Population-based birth cohort	Spirometry at 18 and 26 y	Lung function trajectory: Consistently normal Variable Consistently low Large airway: (FEV_1/VC)	Asthma	S: asthma reported at any time during the study (between age 9 and 26) was associated with a consistently low FEV_1/VC trajectory between ages 18 and 26. Prevalence of asthma: males: 68% in consistently low and 30% in consistently normal, females: 82% in consistently low and 30% in consistently normal
				BHR (yes/no)	S: BHR at age 9 was associated with a consistently low FEV_1/VC trajectory. Prevalence of BHR: males: 55% in consistently low and 17% in consistently normal, females: 57% in consistently low and 14% in consistently normal
				Allergic sensitization (skin prick test): House dust mite Cat Atopy IgE	S: allergic sensitization to house dust mite or to cat at age 21 was associated with a consistently low FEV_1/VC trajectory. Prevalence of house dust mite sensitization: males: 57% in consistently normal and 78% in consistently low, females: 51% in consistently normal and 90% in consistently low. Prevalence of sensitization to cat: males: 28% in consistently normal and 48% in consistently low, females: 25% in consistently normal and 50% in consistently low. Higher levels of IgE at ages 11 and 21 were also associated with a consistently low trajectory in females. Age 11 IgE: consistently normal 4.6 (ln), consistently low 5.6 (ln). Age 21 IgE: consistently normal 3.7 (ln), consistently low 5.0 (ln). NS: atopy at age 13 or 21 (at least one SPT ≥2 mm) was not associated with a consistently low FEV_1/VC trajectory. Sensitization to house dust mite and cat at age 13 was not associated with a consistently low FEV_1/VC trajectory

(Continues)

TABLE 3 (Continued)

First author	Cohort	Type and age lung function measurement(s)	End-points	Predictors of outcome	Main findings (S: significant, NS: non-significant)
Berry[43]	TCRS (n = 599) Population-based birth cohort	Spirometry at 11, 16, 22, 26 and 32 y	Lung function trajectory: Persistently low Normal Large airways: FEV_1/FVC	Asthma	S: asthma between the ages of 6 and 32 (survey age 6, 11, 22, 26 and 32) was associated with a persistently low FEV_1/FVC trajectory. Prevalence of asthma ranged from 7.7% to 18.0% in the normal trajectory and from 26.4% to 43.9% in the persistently low trajectory
Karmaus[48]	IoW birth cohort (n = 1157) Population-based birth cohort	Spirometry at 10, 18 and 26 y	Lung function trajectory: low high Large airways: FVC, FEV_1, FEV1/FVC: low high Lung function trajectory: low medium high Small airways: FEF_{25-75}	Asthma	Males S: asthma at ages 4, 10, 18 and 26 was associated with a low FEV_1/FVC and FEF_{25-75} trajectory. Asthma at ages 4, 10 and 26 was associated with a low FEV_1 trajectory. NS: asthma at ages 4, 10, 18 and 26 was not associated with a low FVC trajectory. Asthma at age 16 was not associated with a low FEV_1 trajectory Females S: asthma at ages 10, 18 and 26 was associated with a low FEV_1/FVC and FEF_{25-75} trajectory. Asthma at age 18 was associated with a low FEV_1 trajectory (see appendix of original paper for exact data). NS: asthma at age 4 was not associated with a low FEV_1/FVC or FEF_{25-75} trajectory. Asthma at ages 4, 10 or 26 was not associated with a low FEV_1 trajectory
				Allergic sensitization (skin prick test)	Males S: allergic sensitization at age 4 was associated with a low FEV_1/FVC trajectory (RR 1.64). Females S: Allergic sensitization at age 4 was associated with a low FEV_1, FVC and FEF_{25-75} trajectory (RR 1.32)
Bui[47]	TAHS (n = 2438) Population-based birth cohort	Spirometry at 7, 13, 18, 45, 50 and 53 y	Lung function trajectory: Persistently high Average Below average Persistently low Early below average, accelerated decline Early low, accelerated growth, normal decline Large airways: FEV_1	Asthma	S: childhood asthma (during the first 7 y of life) was associated with the persistently low (OR 1.7 compared with the average trajectory) and the early below average, accelerated decline (OR 3.1 compared with the average trajectory) FEV_1 trajectory. NS: Childhood asthma was not associated with early low, accelerated growth, normal decline or persistently high FEV_1 trajectory
				Allergic rhinitis	S: allergic rhinitis (during the first 7 y of life) was associated with the early below average, accelerated decline FEV_1 trajectory (OR 2.0 compared with the average trajectory). NS: allergic rhinitis was not associated with the other lung function trajectories.
				Food allergy	NS: food allergy (during the first 7 y of life) was not associated with a lung function trajectory

Note: In papers reporting significant associations without providing estimates, these estimates were recorded as missing in the results. All lung function outcomes are pre-salbutamol unless otherwise specified.

Abbreviations: BHR, bronchial hyper-responsiveness; FEF_{25-75}, forced expiratory flow at 25%-75% of FVC; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; IgE, immunoglobulin G; n, based on number of subjects with lung function measurement relevant to analysis; NS, not significant; OR, odds ratio; RR, risk ratio; S, significant; VC, vital capacity.

TABLE 4 Studies on lung function growth

First author	Cohort	Age lung function measurement(s)	End-points	Predictors of outcome	Main findings (S: significant, NS: non-significant)
Hallberg[30]	BAMSE (n = 1957) Population-based birth cohort	Spirometry at 4 and 8 y	Lung function growth (4-8 y) Large airways: PEF	Asthma phenotypes: Never Transient Persistent Late onset	S: Transient asthma (−5.8 L/min) had a lower growth in PEF compared with never asthma. NS: persistent or late-onset asthma was not associated with growth in PEF
Hallberg[41]	BAMSE (n = 2355) Population-based birth cohort	Spirometry at 8 and 16 y	Lung function growth (8-16 y) Large airways: FEV_1, FVC, FEV_1/FVC Small airways: FEF_{50}	Asthma phenotypes: Never Early transient Early persistent Late onset	S: early persistent (FEV_1 −262 mL, FEF_{50} −668 mL) and late-onset asthma (FEV_1 −124 mL, FEF_{50} −350 mL) had lower growth in FEV_1 and $FEF_{50,}$ compared with never asthma. Early transient asthma had lower growth in FEF_{50} (−221 mL) NS: early transient asthma was not associated with growth in FEV_1. No asthma phenotypes were associated with growth in FEV_1/FVC
Bisgaard[53]	$COPSAC_{2000}$ (n = 336) High-risk birth cohort (children from asthmatic mothers)	Raised volume rapid thoracic compression in neonatal period. Spirometry at age 7	Lung function growth (0-7 y) Large airways: FEV_1, FVC, FEV_1/FVC Small airways: FEF_{50}	Asthma Blood eosinophils	S: asthma at the age of 7 was associated with a lower growth in FEV_1 (Z-score −0.62) and FEF50 (Z-score −0.69) from infancy until the age of 7 compared with no asthma NS: blood eosinophils (at age 6) were not associated with lung function growth between the ages of 0 and 7
Hallas[56]	$COPSAC_{2000}$ (n = 367) High-risk birth cohort (children from asthmatic mothers)	Raised volume rapid thoracic compression in neonatal period. Spirometry half-yearly between 5 and 7 y and at 13 y. Whole-body plethysmography half-yearly between 3 and 7 and at 13 y	Lung function growth (0-13 y) Large airways: FEV_1 Small airways: MMEF, sRaw	Asthma phenotypes: Ever asthma, Never asthma Asthma remission Asthma and allergic sensitization (skin prick test, IgE)	NS: asthma during the first 13 y of life was not associated with a lower lung function growth compared with never asthma. Asthma remission was not associated with catch-up growth up until the age of 13 NS: asthma and concurrent allergic sensitization (at age 13) was not associated with a lower lung function growth ($FEV_{1,}$ MMEF or sRaw) from 1 month until age 13 compared with asthma and no allergic sensitization
Duijts[44]	ALSPAC (n = 7278) Population-based birth cohort	Spirometry at ages 9 and 15	Lung function growth (9-15 y) Large airways: FEV_1, FEV_1/FVC Small airways: FEF_{25-75}	Wheezing phenotypes: Transient early Prolonged early Intermediate onset Late onset Persistent Never/infrequent	S: Prolonged early (FEV_1/FVC −0.23 SDU, FEF_{25-75} − 0.10 SDU) and persistent (FEV_1/FVC −0.27 SDU, FEV_1 −0.13 SDU) wheezing had lower growth in FEV_1/FVC and FEF_{25-75} compared with never/infrequent wheezing NS: transient, intermediate-onset or late-onset wheezing was not associated with a different growth in FEV_1/FVC and FEF_{25-75} compared with never/infrequent wheezing. No association between wheezing phenotypes and growth in FEV_1 was found

(Continues)

TABLE 4 (Continued)

First author	Cohort	Age lung function measurement(s)	End-points	Predictors of outcome	Main findings (S: significant, NS: non-significant)
Belgrave[38]	MAAS (n = 1051) Population-based birth cohort	Whole-body plethysmography at ages 3, 5, 8 and 11	Lung function growth (3-11 y) Small airways: sRaw	Wheezing phenotypes: No wheezing Transient early Late onset Persistent	S: Persistent wheezing had a larger increase in sRaw (0.011 kPa/s^{-1} /y) over time compared with no wheezing. NS: transient and late-onset wheezing were not associated with change in sRaw between ages 3 and 11
				Allergic sensitization phenotypes (skin prick test and IgE): Non-atopic Dust mite Non-dust mite Multiple early Multiple late	S: The multiple early (0.011 kPa/s^{-1} /y) and multiple late (0.008 kPa/s^{-1} /y) trajectories had a larger increase in sRaw compared with non-atopic
Sherrill[16]	Dunedin, New Zealand (n = 696) Population-based birth cohort	Spirometry at 9, 11, 13 and 15 y	Lung function growth (9-15 y) Large airways: FEV_1, VC, FEV_1/VC	Wheezing phenotypes: Severe wheezing Moderate wheezing Occasional wheezing Non-wheezing	S: moderate wheezing (between the ages of 3 and 15) (−0.053 L/y) had a lower FEV_1 growth compared with non-wheezing. Occasional wheezing (0.031 L/y) had a greater VC growth compared with non-wheezing. Severe (0.499%/y) and moderate wheezing (0.303% /y) had a higher growth in FEV_1/VC compared with non-wheezing. NS: severe and occasional wheezing were not associated with FEV_1 growth. Severe and moderate wheezing were not associated with VC growth
				BHR severity: Hyper-responsive Mildly responsive Non-responsive Consistently responsive Remission New responders Consistently non-responsive	S: mild (−0.032 L/y) and hyper-responsive (−0.045 L/y) BHR were associated with a lower FEV_1 growth compared to non-responders. Mildly responsive BHR (−0.023 L/y) was associated with a lower VC growth. Hyper-responsive BHR (−0.394%/y) was associated with a lower growth in FEV_1/VC. Consistent responders and new responders had a lower FEV_1 and FEV_1/VC growth compared with never responders (means not provided). NS: hyper-responsive BHR was not associated with a VC growth. Mildly responsive BHR was not associated with FEV_1/VC growth. Subjects who went into remission did not have a different lung function growth compared with consistently non-responsive BHR
Sears[4]	Dunedin, New Zealand (n = 613) Population-based birth cohort	Spirometry at 9, 11, 13, 15, 18, 21 and 26 y	Lung function growth (9-26 y) Large airways: FEV_1/FVC	Wheezing phenotypes: Persistent from onset Relapse Remission Intermittent Transient Never wheeze	NS: Growth in FEV_1/FVC was not different for any wheezing phenotypes compared with never wheezing

(Continues)

TABLE 4 (Continued)

First author	Cohort	Age lung function measurement(s)	End-points	Predictors of outcome	Main findings (S: significant, NS: non-significant)
Arshad[37]	IoW birth cohort (n = 181 at age 18) Population-based birth cohort	Spirometry at 10 and 18 y	Lung function growth (10-18 y) Large airways: FEV_1, FVC Small airways: FEF_{25-75}	Asthma groups: Persistent Remission	Males S: Remission of asthma (2.6 L) was associated with a higher growth in FEV_1 (between 10 and 18 y) compared with persistent asthma (2.4 L). Remission of asthma (2.7 L) was associated with a higher growth in FEF_{25-75} compared with persistent asthma (2.1 L). NS: No asthma groups were associated with growth in FVC. Females NS: Remission of asthma was not associated with a difference in lung function growth compared to subjects with persistent asthma
Kurukulaaratchy[33]	IoW birth cohort (n = 418, male 186, female 232) Population-based birth cohort	Spirometry at 10 and 18 y	Lung function growth (10-18 y) Large airways: FEV_1, FVC, FEV_1/FVC Small airways: FEF_{25-75}	Asthma groups: Adolescent-onset Never-asthma	Males NS: subjects with adolescent-onset asthma did not have a different growth in lung function compared with never asthma. Females S: subjects with adolescent-onset asthma (1.36 L) had a lower growth in FEV_1 (between 10 and 18 y compared with never asthma (1.52 L). NS: asthma groups were not associated with growth of FVC, FEV_1/FVC and FEF_{25-75}
Morgan [5]	TCRS (n = 826) Population-based birth cohort	Partial expiratory flow volume manoeuvre at age 6. Spirometry at ages 11 and 16	Lung function growth (6-16 y) Large airways: VmaxFRC Small airways: FEF_{25-75}	Wheezing phenotypes: Never Transient early Late onset Persistent	NS: None of the wheezing phenotypes had a different lung function growth compared with never wheezing
Jędrychowski[21]	Krakow, Poland (n = 1001) Population-based cohort	Spirometry at 9 and 11	Lung function growth (9-11 y) (binary: slow lung function growth (SLFG)=lowest quintile of growth) Large airways: FEV_1, FVC Small airways: FEF_{25-75}	Asthma/wheezing phenotypes: Healthy New cases Continued Remission	S: Continued asthma between the age of 9 and 11 was associated with a higher prevalence of SLFG (FEV_1 OR 3.46, FVC OR 3.40 and FEF_{25-75} OR 5.84) compared with healthy subjects. New symptoms of asthma between the age of 9 and 11 were associated with SLFG for FEV_1 (OR 1.46) between the age of 9 and 11. Remission of asthma symptoms was associated with SLFG for FEF_{25-75} (OR 2.63). NS: remission of asthma symptoms was not associated with SLFG for FEV_1 or FVC
Nakadate[18]	Ibaraki, Japan (n = 325) Population-based cohort	Spirometry at 10 and 14	Lung function growth (10-14 y) Large airways: FEV_1, FVC Small airways: FEF_{50}, FEF_{25}	Asthma categories: Category A: asthma at first survey (10 y) Category B: bronchitis or pneumonia at first survey (10 y) Category C: no asthma, bronchitis or pneumonia at any time during follow-up	S: Category A (−79 mL/s year) was associated with a lower annual growth in FEF_{25} compared with Category C (8 mL/s/y). NS: category A was not associated with a different growth in FEV_1, FVC or FEF_{50} compared with Category C

(Continues)

TABLE 4 (Continued)

First author	Cohort	Age lung function measurement(s)	End-points	Predictors of outcome	Main findings (S: significant, NS: non-significant)
Weiss[17]	Boston, USA (n = 602) Population-based cohort	Spirometry annually during the 13-y of follow-up starting at enrolment (age 5-9)	Lung function growth (5-9 to 18-22 y) Large airways: FEV_1, FVC Small airways FEF_{25-75}	Asthma categories: Active asthma Inactive asthma No asthma	Males S: active asthma (−4.18% /y) was associated with a lower growth in FEF_{25-75}% predicted compared with no asthma. Active asthma (2.45% /y) was associated with a higher growth in FVC % predicted compared with no asthma. NS: No association was seen for growth in FEV_1. Females S: active asthma (−2.12% /y) was associated with a lower growth in FEV_1% predicted compared with no asthma. Active asthma (−5.75% /y) was associated with a lower growth in FEF_{25-75}% predicted compared with no asthma. NS: no significant association was seen for growth in FVC. NS: inactive asthma was not associated with differences in lung function growth compared with no asthma

Note: In papers reporting significant associations without providing estimates, these estimates were recorded as missing in the results. All lung function outcomes are pre-salbutamol unless otherwise specified.

Abbreviations: BHR: bronchial hyper-responsiveness; FEF_{25-75}: forced expiratory flow at 25% and 75% of FVC; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; IgE: immunoglobulin G; MMEF: maximal mid-expiratory flow; n: based on number of subjects with lung function measurement relevant to analysis; NS: not significant; PEF: peak expiratory flow; S: significant; sRaw: specific airway resistance; VC: vital capacity; VmaxFRC: maximum forced expiratory flow at functional residual capacity.

3.3.2 | Lung function growth

Asthma and wheezing

Asthma and/or wheezing before the age of 7 [30,53] and later in childhood [21] were associated with a lower lung function growth during childhood. Asthma and/or wheezing during childhood [18,41] and during adolescence [16,17] were associated with a lower lung function growth during adolescence. Adolescent-onset asthma was associated with a lower FEV_1 growth in females between ages 10 and 18, but not in males.[33] Interestingly, remission of asthma in males during the same period of development was associated with a greater gain in FEV_1 and FEF_{25-75} from childhood to early adulthood when compared to subjects with a persistent asthma phenotype.[37] However, another study reported that remission of asthma during childhood was not associated with catch-up growth from infancy until the age of 13.[56] Asthma and/or wheezing was significantly associated with lower growth of large and small airway parameters during childhood in three out of four studies [17,21,53] ; one study did not confirm this.[33] Lower lung function growth during childhood in subjects with asthma [56] and wheezing [38] using sRaw (ie higher sRaw) and small airways expiratory flows (higher MMEF) during childhood [56] was reported in two independent studies.

Wheezing phenotypes

A persistent wheezing phenotype was associated with a larger increase in specific airway resistance (sRaw) during childhood compared with the never-wheezing subjects.[38] Early transient,[41] prolonged early,[44] persistent [41,44] and late-onset [41] wheeze were associated with a lower lung function growth from childhood until adolescence. The association for persistent wheezing was reported in studies using both LCA and a hypothesis-driven approach combined with asthma treatment records for phenotype development. In contrast, one study found that growth of FEF_{25-75} from age 6 until 16 in subjects with any of the reported wheezing phenotypes was not significantly different from non-wheezing subjects.[5] Although phenotype definition was also defined a priori, this study differed from Hallberg et al in that persistence of wheeze was based solely on reporting during the first 6 years of life. Another study that incorporated reporting of wheeze between the ages of 9 and 26 reported no difference in the change in FEV_1/FVC between the ages 9 and 26 between subjects with any wheezing phenotype compared with the never-wheeze reference group.[4]

3.3.3 | Lung function at distinct stages of development

Asthma and wheezing

A history of asthma or wheezing before the age of 7 [22,26,30,39-41,51] and later in childhood [20,28,39,52] was associated with lower lung function levels during childhood. Childhood asthma was associated with a lower lung function in adolescence, which persisted into adulthood in two studies.[20,52] Persistence of asthma from childhood to adulthood was associated with lower FEV_1 during early adulthood.[37]

Remission of asthma during adolescence was associated with higher FEV_1/FVC level in early adulthood when compared to subjects with persistent asthma.[54] We identified no studies investigating the difference in lung function levels between subjects with asthma remission and never asthma.

Wheezing phenotypes

Transient [19,36] and persistent [36] wheezing phenotypes were associated with a lower lung function in infancy. An association between wheezing phenotype and lung function for transient wheezing was observed in studies using both LCA and a hypothesis-driven approach for phenotype development, while an association for persistent wheezing was only reported in the ALSPAC cohort using LCA. At age 3, persistent wheezing was associated with a higher sRaw (ie higher resistance) when compared to non-wheezers at age 3. At age 5, both transient and persistent wheezing phenotypes were associated with a higher sRaw.[27]

Later in childhood, (early) transient,[5,19,24,25,27,29,31,32,35,36,40,49] persistent,[5,19,24,25,27,29,31,32,35,36,40,49] prolonged early [29] and late-onset wheezing [25,29,35,40] were associated with lower lung function levels when compared to non-affected control subjects. Associations with lung function for (early) transient, late-onset and persistent wheezing were observed in studies using both a hypothesis-driven approach [5,19,24,25,27,31] and LCA.[29,32,35,36,40,49] Transient (early),[5,44] prolonged early,[44] intermediate-onset,[44] late-onset [44] and persistent [5,44] wheezing phenotypes were also associated with lower lung function levels in adolescence. Transient wheezing was the only phenotype developed using both LCA and a hypothesis-driven approach to be associated with a lower lung function level in adolescence. Granell et al found that phenotypes with early childhood-onset wheezing persisting into adolescence were associated with FEV_1/FVC and FEF_{25-75}; however, no association was seen for FEV_1.[50] In one study, a persistent wheezing phenotype was associated with lower lung function levels in adulthood.[4] Ten studies included both small and large airway parameters in their analysis with asthma and/or wheezing (including longitudinal phenotypes thereof).[5,25,28,29,33,35,36,41,49,52] All these papers found that asthma and/or wheezing were associated with both reduced large and small airway parameters.

3.4 | Bronchial hyper-responsiveness

3.4.1 | Lung function trajectories

BHR, measured in childhood, adolescence and adulthood, was associated with a lower-than-normal lung function trajectory in childhood, up to early adulthood.[23,45] One study reported that more severe BHR in childhood was associated with a reduced lung function growth trajectory based on FEV_1.[3] No studies reported the association between BHR and trajectories of the small airways.

3.4.2 | Lung function growth

More severe BHR measured in childhood and adolescence was associated with a lower growth of FEV_1, FEV_1/VC and VC from age 9 to 15 until adolescence.[16] Adolescent-onset BHR was associated with a lower growth pattern of FEV_1 in adolescence compared with subjects without BHR,[16] whereas remission of BHR was not associated with lower lung function growth until adolescence compared with subjects who were never–BHR-responsive.[16] No studies analysed the association between BHR and small airway growth.

3.4.3 | Lung function at distinct stages of development

The presence of BHR in childhood and until adulthood was associated with a lower FEV_1 and FEV_1/FVC level in early adulthood.[20,23] No studies reported associations between BHR and small airway lung function.

3.4.4 | Atopic sensitization

Lung function trajectories

Allergic sensitization between the ages of 3 and 11 was associated with a persistently low FEV_1 trajectory until adolescence,[45,48] but this association was not seen for sensitization in adolescence.[45] Another study also reported no association between allergic sensitization at age 8 and a low lung function trajectory during adolescence.[46] A higher number of positive skin prick tests in childhood were associated with a lower FEV_1 trajectory until adulthood compared to subjects with a normal trajectory.[3] Allergic sensitization in adulthood to house dust mite or to cat in adulthood was associated with a consistently lower FEV_1/VC trajectory in adulthood,[23] whereas food allergy was not associated with any lung function trajectory.[47] Allergic rhinitis was associated with an '*early below average, accelerated decline*' trajectory.[47] Allergic sensitization in early childhood was associated with both small and large airway trajectories in females.[48] In males, allergic sensitization at age 4 was only associated with a low FEV_1/FVC trajectory but not with the trajectory of the small airway parameter (ie FEF_{25-75}).[48]

Lung function growth

Sensitization to multiple allergens early in life was associated with an increase in sRaw between the ages of 3 and 11 compared with non-atopic subjects.[38] Asthma with concurrent allergic sensitization, measured at age 13, was not associated with a lower degree of lung function growth in large and small airway parameters from infancy until the age of 13, compared to subjects with asthma without allergic sensitization.[56] None of the papers assessed the role of allergic rhinitis in lung function growth.

Lung function at distinct stages of development

At age 3, a positive skin prick test was associated with a higher sRaw in non-wheezing subjects compared with the non-atopic non-wheezing group.[22] A combined wheezing and atopic phenotype in childhood was associated with a lower FEV_1 and FEV_1/FVC at age 7.[57] In two separate cohorts, sensitivity to a wide variety of allergens, including mite, pollens, cat and dog around age 10/11, was associated with a lower FEV_1 and FEV_1/FVC.[34] Early sensitivity to mite, grass and tree pollens with later onset of sensitivity to pets was associated with a lower FEV_1 at age 11 (based on sensitivity testing at ages 1, 3, 5, 8 and 11).[34] Allergic sensitization to cat dander at age 13 was associated with a lower FEV_1 level between the ages of 9 and 15.[16] In one study, atopic wheeze was associated with lower lung function parameters of both large and small airways (FEV_1, FEV_1/FVC, FEF_{75} and FEF_{25}) at age 7 compared with no wheeze.[28] For subjects with early-onset timothy grass sensitization and a dust mite sensitization trajectory (based on sensitization profiles at ages 5, 8 and 11 years), a lower FEV_1 was reported at age 11.[42] At ages 8-9, a late-onset allergic rhinitis phenotype was associated with lower FEV_1 and FEF_{25-75} compared with the reference group.[49] Atopic wheeze was associated with lower FEV_1, FEV_1/FVC, FEF_{75} and FEF_{25} at age 7 compared with no wheezing.[28] A late-onset allergic rhinitis phenotype was associated with lower large and small airway parameters (FEV_1, FEF_{25-75} and FEV_1/FVC).[49]

Blood eosinophils

Only one study reported associations of blood eosinophils with lung function outcomes. No association between blood eosinophils at age 6 and lung function growth (FEV_1, FVC, FEV_1/FVC and FEF_{50}) between 0 and 7 years was found for either large or small airway parameters.[53]

4 | DISCUSSION

4.1 | Main findings

Asthma and different patterns of wheezing are associated with a low lung function trajectory in childhood, adolescence and up to early adulthood.[43,45-48] Additionally, BHR is a strong risk factor for low lung function in childhood up to adolescence.[3,45] Most studies report this for large airways parameters (FEV_1, FEV_1/FVC), with a paucity of studies of the small airways. In asthmatic and wheezing children, reduced lung function growth appears to occur mainly in early childhood, after which lung function often tracks at a parallel, but lower level to that of non-affected individuals.[4,5,43] Allergic sensitization [45] and allergic rhinitis [47] are also associated with lower-than-normal lung function trajectories, yet results varied. The timing of allergic sensitization (preschool age) and the level of sensitization (polysensitization) appeared to be strongly predictive of low lung function growth.[3,34,38]

4.2 | Lung function development until peak function in subjects with asthma or wheezing

Many children with asthma or wheezing have a lower lung function level and lower lung function growth, and reach a lower peak lung function in early adulthood compared with a control population,[43,45-48] possibly predisposing them to COPD.[3] This is likely attributed to a lower degree of lung function growth during early childhood [30,53] after which lung function growth tracks parallel to non-asthmatic controls.[4,5] Consequently, early childhood should be identified as a key period of development in which exposure to risk factors such as asthma and wheezing play an integral role in lung function growth. Despite this, the association of adolescent-onset BHR with a lower lung function growth pattern suggests that lung function development can change after childhood as well.[16] This is further emphasized by the improvement in lung function in early adulthood in subjects with asthma remission, relative to subjects with persistent asthma.[37,54] These observations were done in mainly population-based studies that include children with mild asthma. Thus, future studies should also address lung growth in children with persistent, moderate-to-severe asthma, since evidence suggests that lung growth up to the plateau may be limited.[3] The heterogeneity of lung function development is further increased by sex-related differences,[17,33,48] and future research should therefore incorporate sex-stratified analyses to further explore these differences.

Asthma is a highly heterogeneous condition, which can present as several phenotypes with varying degrees of severity. Based on findings presented in this systematic review, a greater disease severity, manifested by earlier onset and persistence of asthma and or wheezing, was associated with lung function deficits throughout development compared with the control population. In addition to an earlier onset and persistence of symptoms, the number of exacerbations may be important as well in subjects with asthma. In this systematic review, we did not include exacerbations as a candidate risk factor for lung function growth. However, the number of exacerbations in children with asthma and wheezing has been reported to be predictive of a lower lung function throughout childhood compared to children with asthma and no exacerbations.[38,58] As such, accurate recognition of asthma exacerbations and timely intervention to treat and prevent exacerbations may be warranted to preserve optimal lung function growth.

4.3 | Preschool asthma, wheezing phenotypes and lung function

Asthma predominantly starts in preschool life, often as recurrent wheezing episodes. Different patterns of wheeze were associated with low lung function in childhood and adolescence. After the seminal publication by Martinez et al,[19] describing transient early wheeze, late-onset wheeze and persistent wheeze in early

childhood, these patterns of wheezing onset and persistence have been confirmed in other cohorts and by machine learning approac hes.[29,32,35,36,38,40,44,49,59,60] Children with an early transient wheeze had a lower lung function compared with persistent, late-onset and never-wheezing phenotypes at the age of 2 months, prior to onset of wheezing and that lung function remained at a lower level during childhood in this group,[19] with a replication study yielding conflicting results.[25] Direct comparison is made difficult by different approaches in establishing the wheezing phenotypes. Later in childhood, transient wheeze phenotypes were still associated with lower lung function levels.[5,19,24,25,27,29,31,32,35,36,40,49] This supports the hypothesis that transient wheeze early in life is likely the clinical presentation of congenitally narrow airways predisposing to wheeze, especially during viral infections. Following growth of airway calibre, wheezing resolves in most subjects; however, a lower lung function remains.

The early persistent, intermediate and late-onset wheezing phenotypes have also been associated with low lung function growth until adolescence and early adulthood.[59] Furthermore, the association of persistent,[32,59] intermediate [32,44,59] and late-onset wheezing phenotypes [32,44,59] with a later diagnosis of asthma in childhood suggests that these wheezing phenotypes have a stronger relation to asthma and reflect ongoing inflammatory airway disease. Children with persistent wheeze had a lower lung function in infancy compared with never-wheezing subjects in the SWS study,[36] but this was not replicated for persistent or late-onset wheeze phenotypes in the Tucson study.[19] A direct comparison is, however, not possible due to differences in phenotype modelling. Low lung function in early life may be a reflection of a more severe asthma phenotype with earlier onset, thereby being both causally and consequentially related to a lower lung function growth. Since almost all studies were done in general populations, it is likely that these observations reflected milder asthma, as severe asthma has a low prevalence in the general population.[61]

4.4 | Risk factors for lung function development: BHR, atopy and eosinophils

BHR is a universally recognized hallmark of asthma and has been associated with lower lung function in childhood,[62,63] adolescence [16] and adulthood,[4,20,23,64] making it a prime risk factor for adverse lung function growth. The notion of BHR as strong predictor of lower lung function growth is supported by the association of adolescent-onset BHR with a lower lung function growth pattern in that period of life.[16] In parallel, improvement in lung function growth, which may be seen as catch-up growth, was observed in adolescent subjects with BHR remission.[16] These findings suggest that lung function growth is amendable to change after childhood as well.

The use of inhaled corticosteroids (ICS) has not shown to improve lung function growth in subjects with asthma [65] ; however, a sparsity of information exists on the topic. Use of ICS amongst subjects with asthma has furthermore been associated with a lower lung function level during development in several studies.[4,23,52] However, interpretation of the association between ICS and lung function growth in a non-randomized setting is complicated as ICS use suggests a more severe asthma phenotype. There was a paucity of studies investigating the association between blood eosinophils and lung function growth. Recently published research has shown that blood eosinophils in adolescent subjects with asthma are associated with a lower lung function growth.[66] Therefore, further research should investigate the role of ICS and anti-eosinophilic treatments in the preservation of lung function development in subjects with asthma.

Allergic sensitization [45] and allergic rhinitis [47] are associated with lower-than-normal lung function trajectories, yet results varied between studies. In some studies, children sensitized to common allergens were more likely to have a lower-than-normal lung function trajectory until childhood,[45,48] adolescence [45,46,48] and early adulthood [3,48] compared to children without sensitization. The timing of allergic sensitization (preschool age) and the level of sensitization (polysensitization) appeared to be strong predictors of low lung function growth.[3,34,38] The association of early onset of sensitization or polysensitization with lower lung growth may be the result of a more atopic constitution leading to a more severe and chronic course of asthma.

Next to sensitization, allergic rhinitis was associated with a lower-than-normal lung function trajectory until adulthood.[47] The association between allergic rhinitis and adverse lung function is supported by the Norwegian ECA cohort in which lung function growth in FEV_1 and $FEF_{25\%-75\%}$ until adolescence was significantly lower in children with allergic rhinitis, atopic dermatitis and asthma compared to children with only asthma or rhinitis,[12] findings also supported by the PARIS cohort.[49] These findings suggest that there is an additive effect of allergic comorbidity on lung function deficits in children with asthma and that the contribution to airway inflammation is also present in the small airways. Consequently, allergic rhinitis should be seen as a risk factor for lower lung function growth, primarily in children with asthma. Allergic rhinitis, in addition to sharing many of the same immunologic traits of the lower airway, may also impact lung inflammation by not properly performing air humification and filtration during periods of rhinorrhoea. Given the association with lung function growth, it may be speculated that accurate recognition and treatment of allergic rhinitis in children with asthma may potentially impact long-term lung function development.

4.5 | Small airway disease

In this systematic review, we found that asthma and/or wheezing,[17,21,53] allergic sensitization [28,48,56] and allergic rhinitis [49] were associated with both large and small airway parameters. Furthermore, two studies reported lower lung function growth during childhood in subjects with asthma [56] and wheezing [38] using

sRaw and MMEF during childhood.[56] Disease of the small airways, defined as airways with a diameter of <2 mm in adults,[67] is therefore an integral part of lung function development in children with asthma and allergy. Small airway parameters should be further evaluated for their value in the clinical management of childhood respiratory disease. However, lack in definitions for small airway disease and uniform lung function testing that provides an accurate reflection of peripheral impairment complicates analysis of growth patterns.[68] We identified no papers using multiple-breath washout in this systematic literature review. Given the need to establish better methods for analysing peripheral airway damage, we recommend cohorts to analyse lung function growth until peak lung function using MBW.[68] Furthermore, impulse oscillometry (IOS) has shown to be a promising approach in assessing small airway function and future studies should develop reference values in large, population-based samples to facilitate a meaningful clinical interpretation.[69]

4.6 | Strengths and limitations

An obvious limitation of our work is that asthma definitions were highly heterogenous: asthma is difficult to define early in life, and studies defining wheezing phenotypes were therefore also addressed. Given the heterogeneity of disease definition and lung function outcomes, a formal meta-analysis was not appropriate. In addition to differences in applied definitions, international linguistic variation in the understanding of the word 'wheeze' further complicates comparison of cohorts. When establishing phenotypes, several studies used latent class analysis. This is a relatively new statistical model aimed at uncovering real-world longitudinal patterns of asthma onset and persistence. However, differences in follow-up, parental- vs. physician-confirmed wheeze and use of ICS may conceal valid representations of groups within the general population. Furthermore, small sample sizes of certain phenotypes may inhibit the ability to discern significant associations. In this review, a 2-year maximum age range for lung function data at any measurement point was an inclusion criterium, to enable analysis of lung function development in a distinct time frame. As a result, 37 studies were excluded. However, this criterion increased the validity of our findings as the association between exposure and lung function is analysed within a certain period of development. Furthermore, it improved our ability to compare the selected studies.

4.7 | Critical appraisal and directions for future research

Lung function from childhood tracks until early adulthood, especially in children with a low initial lung function. Remission or re-emergence of asthma and BHR may, however, impact lung function growth in adolescence, and catch-up growth in adolescence is a possibility. Despite this, the degree and pace of lung growth at different periods of development and age at which peak lung function is achieved is subject to individual variation. Future research should therefore aim to investigate growth using both large and small airway parameters until individual peak lung function is achieved, while stratifying for sex. This research should be performed not only in population-based studies, but also in clinical cohorts of children with asthma. Since 11% of children with moderate-to-severe asthma met the lung function criterium for COPD at age 26,[3] future studies should try to identify children at risk for COPD and develop novel therapeutic approaches to preserve and enhance lung growth in childhood.

ACKNOWLEDGEMENTS

We would like to thank Sjoukje van der Werf for her assistance in developing search strategies for the included databases.

AUTHOR CONTRIBUTION

HJLK: Research design; database searches; screening; full-text review for eligibility; data analysis and interpretation; manuscript—drafting. AMZ: Research design; database searches; screening; and full-text review for eligibility; critical revisions of the drafted article. GHK: Research design; research supervision; data analysis and interpretation; critical revisions of the drafted article. JMV: research supervision; data analysis and interpretation; critical revisions of the drafted article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Koefoed HJL, Zwitserloot AM, Vonk JM, Koppelman GH. Asthma, bronchial hyperresponsiveness, allergy and lung function development until early adulthood: A systematic literature review. *Pediatr Allergy Immunol*. 2021;32:1238–1254. https://doi.org/10.1111/pai.13516

Published in final edited form as:
Lancet. 2020 February 01; 395(10221): 371–383. doi:10.1016/S0140-6736(19)33005-3.

Intersection of biology and therapeutics: type 2 targeted therapeutics for adult asthma

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Abstract

Asthma is a disease of reversible airflow obstruction characterised clinically by wheezing, shortness of breath, and coughing. Increases in airway type 2 cytokine activity, including interleukin-4 (IL-4), IL-5, and IL-13, are now established biological mechanisms in asthma. Inhaled corticosteroids have been the foundation for asthma treatment, in a large part because they decrease airway type 2 inflammation. However, inhaled or systemic corticosteroids are ineffective treatments in many patients with asthma and few treatment options exist for patients with steroid resistant asthma. Although mechanisms for corticosteroid refractory asthma are likely to be numerous, the development of a new class of biologic agents that target airway type 2 inflammation has provided a new model for treating some patients with corticosteroid refractory asthma. The objective of this Therapeutic paper is to summarise the new type 2 therapeutics, with an emphasis on the biological rationale and clinical efficacy of this new class of asthma therapeutics.

Introduction

Asthma is a chronic airway disease that inflicts between 300 million and 400 million people worldwide.[1] A diagnosis of asthma requires verifying the presence of reversible airflow obstruction,[2] which is accomplished by showing either airflow limitation that improves following bronchodilator administration or worsening airflow obstruction in the setting of airway provocation.[3] The disease is characterised by coughing, shortness of breath,

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MCP and SEW made equal contributions to the work. Both authors searched the literature, wrote and edited the manuscript, and created figures. MCP and SEW are the guarantors of the paper, taking responsibility for the work from inception to publication. Both authors reviewed and approved the final manuscript.

Declaration of interests

MCP reports personal fees from Merck, and grants from AstraZeneca, Boehringer Ingelheim, Genentech, GlaxoSmithKline (GSK), Sanofi Genzyme-Regeneron, and TEVA Pharmaceuticals Industries, outside the submitted work. SEW reports grants and personal fees from Sanofi, AstraZeneca, GSK, grants from Novartis, and personal fees from Pieris Pharmaceuticals. She also reports grants from AstraZeneca, Boehringer-Ingelheim, Genentech, GSK, Sanofi Genzyme-Regeneron, and Teva Pharmaceuticals Industries, outside the submitted work.

chest tightness, and wheezing.[4] These symptoms result from impaired airway inflammatory responses that cause mucus hypersecretion, bronchial hyperresponsiveness, and activation of airway granulocytes.[5] Mouse models of asthma were used to identify pivotal roles for the cytokines interleukin-4 (IL-4), IL-5, and IL-13 in driving the pathophysiological features of allergic asthma.[6,7-9] Because T-helper-2 (Th2) cells were believed to be the principle source of these signalling molecules they were originally named Th2 cytokines,[10] but other cells, including innate lymphocytes, can also produce these proteins, and the research community has since migrated to the broader term of type 2 (or T2) cytokines. Confirming the pathological role of these factors in human asthma would take nearly 25 years as initial human trials of targeted therapies returned negative results.[11-13] In fact, establishing the efficacy of these cytokines for asthma in humans required a convergence of two concepts. First, that asthma was a complex heterogeneous disease, and second, that biologic therapies needed to target the population of asthma patients with elevated type 2 cytokine activity in their airways.[14-16]

Type 2-high asthma

Inspired by observations that allergic inflammation in mice was driven by Th2 cytokine activity[8,9] and that these cytokines were measured at high concentrations in the lungs of patients with asthma,[17-20] multiple monoclonal antibodies were developed to inhibit type 2 inflammation. Unfortunately, the first clinical trials testing the inhibition of IL-5 (and IL-4) with these antibodies were profoundly disappointing.[11-13] Proving the efficacy of type 2 cytokine inhibition would have to wait until a new insight emerged, namely that Th2 inflammation was not a causal disease mechanism in all patients with asthma. Furthermore, multiple immune cells other than Th2 cells have been increasingly recognised as able to produce IL-4, IL-5 and IL-13, including several innate immune cells such as basophils, mast cells, and type 2 innate lymphoid cells,[21-24] with potentially differing regulatory mechanisms than those observed for adaptive immune Th2 cells (figure 1). This concept prompted the community to refer to these factors as type 2 cytokines and their downstream effects (or signatures) as type 2 inflammation. Additionally, measuring the protein concentrations of type 2 cytokines proved difficult, thus necessitating the need for downstream or associated biomarkers to identify the subgroup of patients with type 2-high asthma. Eosinophil cell counts in the blood and sputum, fraction exhaled nitric oxide (FeNO), periostin concentrations, and measurements of airway type 2 cytokine gene expression have now all been used successfully as surrogate biomarkers for airway type 2 inflammation.[25-28] Through the use of these biomarkers, only a subset (40–70%) of asthma patients clearly show increases in airway type 2 inflammation (type 2-high), with the remaining subgroup demonstrating low to normal type 2 inflammatory measures (type 2-low).[14,16,25,29-32]

By recognising that not all asthma is the same, studies of type 2 cytokine inhibition began to target patients with elevations in these type 2 biomarkers. For example, an anti-eosinophilic medication (anti-IL-5 monoclonal antibody) did not meet its primary or secondary endpoints in all-comers trials, but clinical efficacy became apparent when targeted to patients with increased blood and sputum eosinophil counts.[33,34] This success was followed by studies of an anti-IL-13 antibody in which responses to therapy were greater in those patients

with elevated serum periostin and exhaled FeNO, than in those patients without these elevations.[31] With this new realisation regarding the heterogeneity of asthma, multiple type 2 biologics were tested with eosinophil counts or other type 2 biomarkers as predictors of type 2-high asthma in patients that met criteria for severe asthma. The majority of these targeted trials proved efficacious and led to the development of a growing list of type 2 biologic agents. To date, there are four approved drugs that directly inhibit type 2 cytokines. Three of these agents, mepolizumab, benralizumab, and reslizumab target the IL-5 cytokine or its receptor (IL-5RA), whereas the fourth agent, dupilumab, targets IL-4RA, which is the primary signalling receptor for IL-4 and IL-13.

Omalizumab

Although patients and asthma physicians are excited about the type 2-targeted biologics, the first approved biologic for asthma (omalizumab) was infact targeted to immunoglobulin E (IgE). Multiple reviews have discussed in detail the use of omalizumab as a treatment for asthma.[35,36] Free or circulating IgE binds to high-affinity IgE receptors (FcεRI) expressed on the surface of basophils and mast cells, leading to their cellular activation. Omalizumab is a monoclonal antibody that binds to circulating IgE and inhibits the binding of IgE to FcεRI. The most consistent clinical benefit of this treatment is a reduction in asthma exacerbations. Importantly, the biomarkers initially used in the early omalizumab trials (IgE and the presence of specific IgE) have not been proven to be effective at predicting clinical response, and retrospective analysis suggests that type 2 biomarkers, including blood eosinophils and the amount of exhaled FeNO, are more effective.[37] Thus, although not initially thought of as a type 2-targeted drug, there is strong overlap with the type 2-high phenotype; yet the drug has never been studied in this population. In a pooled analysis of 25 randomised controlled trials (in patients who met total and specific IgE criteria only), omalizumab reduced the number of patients with asthma exacerbation from 26% in the placebo group to 16% in the omalizumab treatment group over 48 weeks.[38] Although the effect size is less than the benefit seen with anti-IL-5 or anti-IL-4RA therapies, some key differences in the trial designs need to be highlighted between the major omalizumab trials and those with the newer type 2 biologic agents.[39-41] Principally, the omalizumab trials did not exclusively restrict participation to those patients with eosinophilic asthma and the inclusion criteria for these trials did not include a requirement to have had an asthma exacerbation in the previous year. In fact, many of the registered trials for omalizumab were completed in patients who would not meet more recent definitions for severe asthma.[42,43] Therefore, directly comparing the effect sizes between omalizumab versus the newer type 2 biologic agents is challenging.[44]

Eosinophils and IL-5 inhibition

Eosinophils are granulocytes that release a variety of proinflammatory mediators, including proinflammatory cytokines following activation, major basic protein, eosinophil peroxidase, eosinophil cationic protein, eosinophil-derived neurotoxin, and galactin-10 or Charcot-Leyden crystals (figure 1).[7] Some patients with asthma show increases in airway eosinophilia, which has been appreciated for over 100 years.[45,46] This observation prompted a considerable amount of research focused on understanding the role of

eosinophils as mediators of asthmatic disease. There is now an understanding that these granulocytes instigate airway dysfunction through degranulation and the release of reactive oxygen species that promote airway epithelial-barrier dysfunction (figure 1).[47] Although traditionally characterised as innate cells, new findings also suggest that eosinophils are directly involved as pivotal orchestrators of the type 2 immune response.[48] Together these studies support a crucial role for eosinophils as drivers of type 2 immune-inflammatory responses in asthma.

IL-5 is required for eosinophil maturation, survival, and the translocation of these cells from the bone marrow into the systemic circulation. Therefore, inhibiting IL-5 signalling was an obvious therapeutic target in asthma. IL-5 signals via an IL-5 specific receptor, IL5RA, and a signal-transducing β receptor that is shared with the cytokines IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF).[49] When used in patients with evidence of eosinophilic inflammation, medications that inhibit binding of the ligand to its IL5RA receptor reduce systemic eosinophil counts, decrease basement membrane thickening, reduced airway tissue remodelling,[50] and might promote airway mucus plug formation.[51]

The first of these agents to be tested was the IL-5 ligand-directed IgG1 antibody, mepolizumab. As previously noted, the initial mepolizumab trials were disappointing, questioning the role of eosinophils as active mediators of asthmatic disease.[11,12] However, the results were statistically significant when mepolizumab was directed to biomarker-defined eosinophilic asthma.[30,33,34,52,53] The initial trial targeted patients with eosinophilic asthma using sputum eosinophil cell counts greater than 3%,[33] and the pivotal DREAM study[30] used a combination of sputum eosinophil percentages (>3%) or cell count in the blood (≥300 cells per μL) to define eosinophilic asthma. However, counting sputum cells is technically challenging and subsequent phase 3 trials used more convenient blood eosinophil cell measurements. Specifically, in the first phase 3 clinical trial, mepolizumab (Subcutaneous, 100 mg every 4 weeks) met its primary outcome by decreasing asthma exacerbation by 53% when compared with placebo in patients with blood eosinophil counts of more than 150 cells per μL at screening or 300 cells per μL or higher during the previous year (figure 2A). Small but significant improvements in forced expiratory volume in 1 s (FEV_1) of 98 mL (figure 2B) and an asthma control questionnaire-5 result of 0·42 (which did not reach a clinically significant difference) were also noted.[53] Using similar eosinophilic inclusion criteria, in a study of patients who were dependent on systemic corticosteroids, mepolizumab treatment also improved the likelihood of decreasing systemic-oral-prednisone dosing, with patients given mepolizumab showing a 2·39 greater increase in reducing oral prednisone treatment compared with patients given placebo.[52] Impressively, despite this corticosteroid dose reduction, patients given mepolizumab also achieved better lung function and improved asthma control questionnaire scores when compared with placebo.[52]

The success of mepolizumab was duplicated with reslizumab, a similar anti-IL-5 monoclonal antibody (IgG4). The inclusion criteria for the reslizumab trials differed slightly from the mepolizumab studies with the inclusion of patients on a medium-to-high dose of inhaled corticosteroids and a slightly higher blood-eosinophil threshold of 400 cells per μL

or more. Despite these differences, reslizumab decreased asthma exacerbation by 54% when given intravenously (3 mg/kg every 4 weeks),[54] with similar improvements in FEV_1 (120 mL) and symptom scores (asthma control questionnaire-7 score of 0·25) when compared with placebo (figure 2A, B).[54]

The third IL-5 pathway inhibitor to show clinical efficacy was benralizumab. Unlike mepolizumab or reslizumab, benralizumab is an IgG1 monoclonal antibody targeting the α subunit of the IL-5 receptor. One unique aspect of benralizumab is that it lacks a fucose molecule in the constant segment (Fc fragment) of the monoclonal antibody. This afucosylation results in the enhanced affinity of benralizumab for the human Fcγ receptor that is expressed on cytotoxic cells, such as natural killer cells, macrophages, and neutrophils.[55] As such, benralizumab has a unique capacity to induce antibody-mediated cellular toxicity resulting in prolonged eosinophil depletion when compared with monoclonal antibodies that directly bind to a ligand. Pharmacodynamically, this characteristic means that benralizumab can be dosed at 30 mg every 4 weeks for the first 3 months and then every 8 weeks thereafter. However, despite these theoretical benefits, the clinical efficacy of benralizumab was similar to that observed in the ligand-antibody trials (figure 2A, B). Specifically, in patients with eosinophilic asthma (defined by ≥300 cells per μL), benralizumab decreased asthma exacerbation by 28% in one trial and 51% in the other when compared with placebo.[56,57] Small improvements in FEV_1 (159 mL, 116 mL) (figure 2B) and symptoms were also shown. In addition, in an oral corticosteroid reduction trial in which patients were only required to have blood eosinophil counts of 150 cells per μL or more, patients treated with benralizumab were able to decrease oral-prednisone doses by 75% compared with 25% in the placebo group.[58] The percentage of patients able to decrease oral prednisone dose by more than half after starting benralizumab (48%) was greater than the fraction of patients on mepolizumab (37%).[52,58] However, the clinical or statistical meaning of this difference is difficult to compare.

Overall, these findings support the clinical efficacy of all three IL-5 pathway antagonists as they showed similar effect sizes in the primary outcome for reducing asthma exacerbation (table 1). Small improvements in FEV_1, asthma symptoms, and quality of life were also seen, as was a reduction in systemic corticosteroid dependency.[52,56-58] However, reslizumab in subcutaneous form did not show a reduction in systemic corticosteroid dependency (NCT02501629). Although the improvements in FEV_1 were statistically significant when compared with placebo, the overall improvement of between 98 mL and 159 mL was relatively modest. Conversely, these medications show consistent and relatively robust effects on asthma exacerbations, with overall decreases of 35–55% when compared with the placebo group (figure 2A, B; table 1). Importantly, the effect of anti-IL-5 therapy on asthma exacerbation was sustained even after multiple years of treatment.[59,60]

Adverse effects of IL-5 inhibition

The most severe adverse reaction observed with the IL-5 inhibitors was anaphylaxis. This reaction was more common with the intravenously administered reslizumab than the other IL-5 antagonists and occurred in 0·3% of patients[61]—the US Food and Drug Administration (FDA) has given the drug a black box warning in this regard (table 2). This frequency is

similar to that seen with the subcutaneous anti-IgE monoclonal antibody (omalizumab).[62] Hypersensitivity reactions were slightly less frequent with the subcutaneous medications of mepolizumab and benralizumab, but such events did occur, and prescribers should be able to manage anaphylaxis and hypersensitivity while administering these medications.[60,63]

An unexpected observation was that two serious herpes zoster infections occurred in patients given mepolizumab, but none in the participants given placebo (table 2). The association between herpes zoster infections and mepolizumab treatment has been observed in subsequent observational studies, but uncertainty remains regarding the clinical impact of IL-5 inhibition on the rates of these infections.[60] Due to this uncertainty, herpes zoster vaccination might be considered in patients with a high risk of infection, but uniform vaccinations before initiating anti-IL-5 medications is not yet standard practice.

Eosinophils are commonly elevated in helminth infections[64] and although the essential role of eosinophils in the elimination of different types of parasites remains controversial, one concern of IL-5 inhibition is the potential to increase the risk of these infections. These infections, however, rarely occur in the high-income countries in which these therapies are tested, and in initial studies patients were screened to exclude participants with a parasitic infection.[53] As such, the potential risk has not been confirmed. However, the risk remains, and caution is advisable in countries where parasites are endemic.

Two additional hypothetical concerns arise when treating patients with IL-5 inhibitors. First, an inverse relationship exists between blood and mucosal eosinophil cell counts and colon cancer risk.[65,66] These findings suggest that decreasing eosinophil numbers might increase the risk of certain mucosal cancers. Second, eosinophils have a crucial role in the maintenance of adipose tissue metabolism,[23,67] and decreasing eosinophil numbers in this tissue leads to obesity and metabolic dysfunction.[67,68] Therefore, prolonged inhibition of eosinophils could lead to obesity and metabolic dysfunction, including insulin resistance. These complications could potentially be of greater concern with benralizumab as this treatment has a prolonged effect on eosinophil depletion; however, this therapy does not appear to completely eliminate tissue eosinophilia (table 2).[69,70] Furthermore, using fewer systemic corticosteroids in patients treated with these biologics could be speculated to also limit further weight gain. Certainly, long-term followup studies are needed in patients treated with anti-IL-5 therapies to address these potential concerns.

IL-4RA inhibition

The cytokines, IL-4 and IL-13, are complementary both in their biologic roles and in their signalling machinery. Namely, the primary receptor for IL-4 is IL-4RA, which upon binding with IL-4, complexes with the common γ-chain (γ_c) to signal via intracellular JAK1 or JAK3 pathways (type 1 receptor).[71] IL-13 also uses the IL-4RA receptor through a heterodimerisation with IL-13RA1 that signals via JAK1, JAK2, and TYK2 (type 2 receptor).[71,72] Thus, blocking IL-4RA inhibits the primary signalling pathways of both IL-4 and IL-13.[72] Both cytokines promote B lymphocyte class switching from IgM antibodies to IgE antibodies,[73] induce airway smooth-muscle hyper-reactivity,[8,73] and promote eosinophilic chemotaxis through expression of vascular cell adhesion molecule-1

(VCAM-1)[74] and numerous eosinophilic chemokines (figure 1). However, IL-4 is essential for promoting the differentiation of Th2 cells from T0 lymphocytes,[75] and IL-13 is a prominent driver of the airway epithelial transformations that occur in asthma.[8] Specifically, both IL-4 and IL-13 can promote goblet cell metaplasia, mucus production, subepithelial fibrosis, and basement membrane thickening in conjunction with, or independent of, IL-4 (figure 1).[6,76-78]

Because of the strong animal data supporting the role of IL-13 in driving asthma pathogenesis and the success of the anti-IL-5 medications, IL-13 inhibition was reasonably assumed to prove efficacious in asthma. Unfortunately, clinical trials that selectively targeted IL-13 did not show consistent efficacy, supporting the broader importance of both IL-4 and IL-13 in asthma.[31,79-85] Subsequently, IL-4RA became a target as it is a dual receptor for IL-4 and IL-13.

Efforts to block the IL-4 and IL-13 signalling axis with the IL-4RA inhibitors, pitrakinra and AMG 317, were initially done in all-comers trials and this unstratified approach did not show clinical efficacy (table 1).[13,86,87] However, 3 years later with the added insight of asthma heterogeneity, and secondary analysis showing the efficacy of pitrakinra in patients with eosinophilic asthma,[86] a new IL-4RA antibody was tested in an initial proof-of-concept phase 2A trial.[88] In this study, 104 patients on medium-to-high dose combination therapy (inhaled corticosteroids and long-acting β agonists) with blood eosinophil counts of 300 cells per μL or higher, or sputum eosinophils of 2% or more, were randomly assigned to drug versus placebo groups. Following a 4-week stable treatment phase, background medication was successively withdrawn, with the primary endpoint being loss of asthma control. Dupilumab treatment led to an 87% reduction in loss of asthma control compared with placebo, and improved FEV_1 and asthma symptoms despite withdrawal of background medications. This proof-of-concept study was followed by a phase 2B study of dupilumab at 200 mg or 300 mg doses given subcutaneously at 2-week or 4-week intervals. The prespecified analysis plan in this trial subdivided patients into eosinophilic (blood eosinophils ≥300 cells per μL) and non-eosinophilic (<300 cells per μL) subgroups,[89] and the primary endpoint of improvement in lung function was measured at 12 weeks in the eosinophil-high subgroup. The trial met its primary endpoint, improved FEV_1 at 12 weeks in both eosinophil-low and eosinophil-high patients, and maintained this improvement at 6 months while decreasing severe exacerbations in both subgroups at 6 months.[89] However, the treatment effect size for asthma exacerbations and FEV_1 was larger in eosinophil high patients compared with eosinophil low patients.[89] A phase 3 follow-up trial confirmed these findings, with large effect sizes seen for reducing asthma exacerbations and improving FEV_1 measurements in patients with blood eosinophil counts of 300 cells per μL or higher, and gradually diminishing responses over the follow-up period (52 weeks) in patients with lower blood eosinophil cell counts.[90] Specifically, asthma exacerbations decreased by 48% in all patients treated with dupilumab at 200 mg and 46% in those treated with 300 mg subcutaneously, and FEV_1 increased by 140 mL (200 mg dose) and 130 mL (300 mg dose).[90] In patients with blood eosinophil counts of 300 cells per μL or higher, the overall effect size was larger, with an exacerbation reduction of 66% and 67% (figure 2A) and FEV_1 improvement of 210 mL and 240 mL at the 200 mg and 300 mg doses (figure 2B; table 1). Conversely, no reduction in asthma exacerbation or improvement in FEV_1

was seen in patients with blood eosinophil counts of less than 150 cells per μL.[90] As with mepolizumab and benralizumab, dupilumab has also been shown to enable patients on systemic glucocorticoids to decrease their corticosteroid dose, with a 70% reduction of oral corticosteroids in patients treated with dupilumab compared with a 42% decrease in patients treated with placebo.[91] Unlike the steroid-sparing trials for mepolizumab and benralizumab,[52,58] dupilumab did not require blood eosinophilia as an inclusion criteria. However, patients with blood eosinophil counts of 300 cells per μL or higher at baseline were over two times as likely to reduce their corticosteroid dose by 50% than those participants with lower eosinophil numbers. Although the differences in trial design and inclusion criteria (primarily related to eosinophil counts) make directly comparing the effects on corticosteroid reduction difficult, the overall decrease in oral prednisone by 70% for patients on dupilumab was similar to the decrease observed with benralizumab (75%).[52,58,91]

Adverse effects of IL-4RA inhibition

Dupilumab is relatively well tolerated and the most common adverse events are injection site reactions. In addition, treatment with dupilumab increased the frequency of a poorly characterised conjunctivitis (about 10%) in the atopic dermatitis studies, an effect not yet seen in the asthma trials.[92] As with the anti-IL-5 monoclonal antibodies, there was a reported increase in herpes-related events, and IL-4RA inhibition also increased blood eosinophil counts after treatment, peaking at 1–2 months and typically falling back to baseline values by 3 months. The biological mechanism and clinical relevance of this increase remains unknown, but a few patients did develop eosinophil counts higher than 5000 cells per μL, and several cases of eosinophilic granulomatosis with polyangiitis have also been reported (table 2).[93] Current recommendations are to exclude patients with blood eosinophil counts greater than 1500 cells per μL at baseline. Finally, similar to the anti-IL-5 medications, theoretical concerns exist regarding increased risk of parasitic infections and potential increases in obesity and metabolic dysfunction. There is a dose effect with dupilumab, and the higher 300 mg dose is associated with a higher frequency of adverse events. Thus, the lower 200 mg dupilumab dose is recommended for the majority of patients with moderate-to-severe asthma, and 300 mg is reserved for patients with systemic corticosteroid-dependent asthma or with comorbid conditions responsive to dupilumab, such as atopic dermatitis or nasal polyposis.

Comparing clinical efficacy and differing trial designs of phase 3 trials

Comparing clinical efficacy between medications requires a randomised blinded trial design directly testing the medications in a head-to-head analysis. To our knowledge, no such trial has been done and would probably require large numbers of patients. Furthermore, because study populations and analysis plans differed greatly between the type 2 biologic clinical trials, directly comparing the treatment effect sizes for each drug is difficult.[94] For example, the threshold to discriminate eosinophilic from non-eosinophilic asthma differed substantially between the trials. The phase 3 mepolizumab asthma exacerbation trial required patients to have a blood eosinophil count of more than 150 cells per μL at the time of enrolment or 300 cells per μL in the past year,[53] whereas the reslizumab trials

required patients to show one blood eosinophil count of 400 cells per μL or higher over a 2–4 week screening period.[54] Alternatively, the phase 3 benralizumab and dupilumab studies enrolled patients with eosinophilic and non-eosinophilic asthma, and these studies used a cutoff of 300 cells per μL or higher in the blood to discriminate eosinophilic from non-eosinophilic subgroups.[57] The dupilumab trials did not exclude patients with eosinophil blood counts of less than 150 cells per μL, even though efficacy was only seen at concentrations of greater than 150 cells per μL. Furthermore, although all the trials used a similar primary outcome that measured clinical asthma exacerbations (defined as a treating physician electing to administer systemic corticosteroids for at least 3 days, or an emergency department visit, or hospitalisation for asthma), each of the trials included slight modifications to the inclusion criteria. Specifically, mepolizumab and benralizumab required a history of at least two asthma exacerbations requiring systemic corticosteroid treatment in the past year,[53] whereas reslizumab and dupilumab required at least one exacerbation treated with systemic corticosteroids in the past year. The anti-IL-5 trials primarily enrolled patients with severe asthma on a high dose of inhaled corticosteroids, whereas dupilumab was tested in a slightly less severe population in patients on both medium and high doses of this treatment.[90] These minor differences are important because restricting inclusion criteria to patients with more severe asthma improves study power to detect differences in clinical outcomes between the drug and placebo. The phase 2B and phase 3 dupilumab trials also excluded patients who were taking systemic corticosteroids before study enrolment.[90] Not surprisingly, these protocol variations resulted in robust differences in the exacerbation rates in the placebo group. The highest placebo exacerbation rate occurred in the reslizumab trials at 1·8 clinical asthma exacerbations per year, followed by the mepolizumab studies at 1·7 clinical asthma exacerbations per year, the benralizumab studies at 1·3 clinical asthma exacerbations per year (in the eosinophil-high subgroup), and the lowest rate in the dupilumab studies at 1·1 clinical asthma exacerbations per year (in the eosinophil-high subgroup).[53,54,57,90] These relatively large differences in placebo exacerbation rates amplify the complexity in comparing clinical efficacy across the type 2 biologic agents.

Acknowledging these limitations, a reasonable interpretation of the data is that any clinical differences between these biologic agents are likely to be small. For example, the effect sizes for exacerbation reduction and improvements in FEV_1 are related to starting eosinophil numbers, with a greater reduction in asthma exacerbations in the dupilumab trials (approximately 60%) than reductions of 40–50% in anti-IL-5 and anti-IL-5RA studies in patients with eosinophil counts of 300 cells per μL or more (figure 2A). Despite these differences, the confidence interval for the reduction in asthma exacerbations overlaps in all phase 3 type 2 biologic trials. Improvements in FEV_1 are comparably higher in the dupilumab studies in patients with eosinophilic asthma, with a similar overlap in confidence intervals. Specifically, in patients with eosinophil counts of 300 cells per μL or higher, dupilumab improved FEV_1 by 210–260 mL compared with 98–159 mL in the IL-5 inhibitor trials, which is an overall difference of about 100 mL (figure 2B).[53,54,56,57,90]

Fevipiprant

Although it is not a monoclonal antibody, fevipiprant is an oral medication that blocks the binding of prostaglandin D2 to its receptor, the chemoattractant receptor-homologous

molecule expressed on Th2 cells (CRTH2 or PTGDR2). As the name implies, this receptor is commonly expressed on Th2 cells, and would therefore be expected to work in patients with type 2-high asthma. However, clinical trials of fevipiprant have shown mixed results, with the most consistent finding showing a small improvement in FEV_1 measurements when compared with placebo.[95,96] This response was similar to the effect size seen with montelukast, a leukotriene receptor antagonist.[95] As an oral medication fevipiprant is relatively easy to administer, but additional data are needed to assess the added benefit of fevipiprant over other available asthma medications.

Biomarkers of treatment response

Blood eosinophil counts are a predictor of response for each of the type 2 biologic agents.[54,90,97,98] Patients with blood eosinophil counts of 300 cells per μL or higher have approximately a 50% reduction in asthma exacerbation when treated with either anti-IL-5 or anti-IL-4RA therapies, whereas the clinical benefit in patients with blood eosinophil counts of less than 300 cells per μL is considerably reduced.[90,98] However, blood eosinophilia does not uniformly predict treatment response, and not all patients with elevations in type 2 biomarkers respond to these biologic agents. For example, the benralizumab and dupilumab trials enrolled patients with eosinophilic asthma and non-eosinophilic asthma, and both studies showed decreased asthma exacerbation in patients with blood eosinophil counts between 0 cells per μL and 300 cells per μL.[56,57,90] In the benralizumab studies, inconsistent improvements were seen in those patients with blood eosinophil counts of less than 300 cells per μL across the two pivotal trials, and the patients with blood eosinophil counts below this threshold were not subdivided further. By contrast, clinically significant responses to IL-4RA antibodies were consistently observed in patients with blood eosinophil counts between 150 cells per μL and 300 cells per μL (but not with <150 cells per μL). These responses were greater in patients who also had elevated concentrations of FeNO (>24 parts per billion; ppb), although dupilumab did not benefit patients with FeNO measurements of less than 25 ppb and eosinophil counts of less than 150 cells per μL.[90] Thus, considering both FeNO values and blood eosinophil cell counts could conceivably improve the ability to predict response patterns.

Improved targeting of type 2 biologics will probably require additional biomarkers beyond those currently available. For example, eosinophils are key regulators of glucose homoeostasis[23] and changes in nutrition or intermittent fasting can change blood eosinophil counts.[99-101] Blood eosinophil counts are poor biomarkers for type 2 airway inflammation in patients with obesity, suggesting that these patients might benefit from IL-5 inhibition even when blood eosinophil counts remain low.[25,102] Finally, although blood eosinophilia is an effective predictive biomarker for treatment response (before starting treatment), it is inadequate as a monitoring biomarker to distinguish medication responders from non-responders after starting treatment. As such, there are no biomarker-based rules to identify and stop these medications in patients with a low likelihood of benefitting after treatment has been started.

Indications for treatment

The American Thoracic Society and the European Respiratory Society's definition of severe asthma is the presence of poor asthma control despite maximal treatment with high doses of inhaled corticosteroids and one additional controller medication.[42,103] The relatively small percentage of patients with severe asthma (5–10%) contributes to the majority of asthma-associated healthcare costs.[104] The cost of health care for each patient helps to justify the high expense of these new biologic treatments. Furthermore, use of blood eosinophil cell counts and FeNO values as biomarkers has initiated a more precision medicine-based approach to asthma treatment. Namely, asthma control and asthma exacerbations are likely to improve in patients with frequent asthma exacerbations and high blood eosinophil cell counts after starting on a type 2 biologic therapy. Despite this biomarker driven approach, the reduced cost to health care by preventing a single asthma exacerbation needs to be weighed against the current market value of the type 2 biologic.[105] Thus, more precise biomarkers are needed before more cost-effective therapeutics become available.

New therapies for severe non-eosinophilic or type 2-low asthma

Treatment options for patients with type 2-low severe asthma remain limited, and aggressive efforts to identify non-type 2 treatment options remain scarce. Multiple cytokines with roles that overlap with the prototypical type 2 cytokines have been tested in asthma with mixed results. An early phase 2B trial with an inhibitor of the epithelial cell-derived cytokine thymic stromal lymphopoietin (TSLP) have been positive, with tezepelumab decreasing asthma exacerbation by 60–70%.[106] By contrast, inhibitors of IL-33, a member of the IL-1 cytokine family that has a role in promoting type 2 innate lymphoid cell activation, showed some clinical efficacy but was inferior in a head-to-head analysis with dupilumab.[107] Thus, tezepelumab and IL-33 inhibitors might prove efficacious in broader patient populations that include people with type 2-high and type 2-low asthma.

The prominence of old age (>50 years old)[25] and obesity are among the phenotypic features of severe asthma[108,109] and raise the possibility that the systemic inflammation associated with ageing, obesity, and metabolic dysfunction could have effects on the airway to worsen asthma. Recent work supports this hypothesis and patients with asthma with elevated IL-6 concentrations in the plasma (IL-6-high asthma) show lower lung function and increased asthma exacerbation than patients with low amounts of IL-6 (IL-6-low asthma).[110] Targeting this systemic IL-6 inflammation has shown efficacy in the treatment of cardiovascular diseases[111] and a similar benefit might plausibly exist in patients with IL-6-high asthma.

Another interesting molecular endotype is the observation that many patients with asthma have impairments in the resolution of inflammation. Traditionally, asthma has been described as a disease of chronic airway inflammation,[2] but little attention has been dedicated to understanding how different types of inflammation (type 2 and others) are restored back to homoeostatic concentrations. Recent work has shown that in severe asthma these mechanisms of inflammation resolution might be impaired[112-114] and treatments that restore inflammation resolution pathways to homoeostatic concentrations might be beneficial.

Finally, microbial imbalances (dysbiosis) of the asthmatic airway have been implicated as a possible mechanism of disease in some patients. Initial trials testing the use of antibiotics (specifically macrolide antibiotics) for the treatment of asthma were ineffective,[115,116] but the AMAZES trial[117] showed that azithromycin reduced asthma exacerbations in adult patients with both eosinophilic and non-eosinophilic asthma. These findings raise the possibility that some patients could benefit from antibiotic treatment.[117,118] Unfortunately, no biomarker exists to identify responders from non-responders in terms of antibiotics treatment and alterations in microbial dysbiosis is unlikely to be specific for the type 2 pathway. Therefore, considerable debate remains regarding the appropriate approach for the use of antibiotics in asthma.[119]

Future directions and remaining controversies

Although the results of the clinical trials do not provide evidence that inhibition of IL-5 is superior or inferior to inhibiting IL-4RA, there are clues that heterogeneity in type 2 inflammatory-immune processes might eventually define pathobiological subgroups of patients with type 2 asthma who respond better to inhibition of one pathway versus the other. For example, airway eosinophilia can be induced by activation of Th2 cells or type 2 innate lymphoid cells. Type 2 innate lymphoid cells generate considerably more IL-5 and IL-13 than IL-4, whereas Th2-driven processes are likely to have elevations in IL-4, IL-5, and IL-13 (figure 1). Thus, if the type 2 subtype is related to type 2 innate lymphoid cells, then inhibiting IL-5 alone could be sufficient to improve disease outcomes. Conversely, in Th2-driven processes (as in allergic asthma phenotypes), inhibiting IL-4 and IL-13 could be more important for improving disease outcomes.[120] These differences might explain some of the observed differences in response patterns to IL-4RA versus IL-5 pathway-targeted therapies. For example, IL-4RA targeted therapies (and IL-13) inhibit the late asthmatic response (bronchoconstriction that recurs 3–4 h after the initial allergen challenge), whereas mepolizumab, an anti-IL-5 monoclonal antibody, was found to be ineffective against this response despite a large reduction in blood eosinophils.[11,87,121] By contrast, post-hoc analyses of anti-IL-5 trials have suggested that despite similar starting eosinophil counts, both reslizumab and benralizumab are more effective in patients whose asthma developed in adulthood (>40 years for reslizumab and >18 years for benralizumab) or in those individuals with nasal polyps, which are subgroups of asthma that show lower blood IgE concentrations.[97,122-124] In addition, although traditionally viewed as a granulocyte with minimal immunological activity, eosinophils and eosinophil-derived proteins, such as Charcot-Leyden crystals, could possibly initiate or amplify an airway type 2 immune response and have a pivotal role in airway mucus formation in some patients.[48,51] Thus, in patients with asthma where eosinophils are the key orchestrators of the type 2 immune response, or function as key propagators of airway mucus plugging, IL-5 inhibition might be superior to IL-4RA inhibition.[51] Targeted and mechanistic comparison studies could help to distinguish these potential pathobiological differences.

Intriguingly, anti-IL-5 agents have been disappointing as treatments for eosinophilic oesophagitis and atopic dermatitis (NCT03055195),[125] and two small studies have shown some efficacy for nasal polyposis.[126,127] Conversely, the blockade of IL-4RA signalling is a highly effective therapy for atopic dermatitis,[128] is FDA-approved for treating nasal

polyposis (dupilumab),[129,130] and IL-4RA has also shown promise as a therapeutic target for eosinophilic oesophagitis.[131] These studies all support immunological differences among these diseases (or subgroups), all of which are considered to have type 2 inflammatory processes that might explain the differences in clinical response patterns. Thus, although the overall efficacy is similar, the different biological or clinical characteristics of these diseases might eventually be used to better identify the most appropriate treatment for patients from the two drug classes (IL-5 and IL-4RA inhibitors). However, better biomarkers are required to match patients to the most effective treatment.

The fundamental goal of asthma research is to find a cure. Multiple biological defects are likely to contribute to the initiation and maintenance of abnormal increases in airway type 2 inflammation. Therefore, developing an asthma cure will require a deeper understanding of how airway type 2 inflammation develops and persists in airway tissue. For example, work investigating airway sputum gene expression has shown that categorising patients into type 2-high and type 2-low asthma is too simplistic.[22] Some patients show uniform and robust elevations in multiple airway type 2 gene expression networks compared with other asthmatics. These increases occur despite treatment with systemic or inhaled corticosteroids and these so-called type 2 ultra-high patients show unique clinical features such as older age (>50 years old at time of study), reduced lung function, and elevations in airway genes specific for CD11b and IRF4 double-positive type 2 dendritic cells.[22,25,132,133] These immunological findings suggest that the immune senescence that occurs during ageing could explain the increase in asthma severity seen in older patients.[108,134] Multiple other biological pathways probably have similar roles and a better understanding is needed for how type 2 inflammation develops in lung tissue.[4]

Although these new type 2 biologic agents have fundamentally changed the lives of many patients with severe asthma, questions remain regarding key clinical issues for patient care. What is the long-term safety of these medications? Are these medications disease modifying so that patients could eventually be taken off these medications? Do certain subgroups of patients respond preferentially to the different type 2 biologic agents? Will similar efficacy be observed in children? Will guidelines for their use evolve? Answers to these questions require a continued focus on identifying and understanding the molecular mechanisms that contribute to the pathogenesis of human asthma. Furthermore, the growing list of type 2 therapies will require the development of new and improved biomarkers to direct patients to medications with the highest likelihood of success.[4]

Conclusion

The emergence of type 2 biologics for the treatment of severe asthma is a welcomed and much needed advance in the management of patients with asthma. Although a cure for asthma remains elusive, many patients with severe asthma show a robust and sustained response to this new class of medication. Critical needs remain regarding better biomarkers to identify patients that are most likely to respond to these drugs and a deeper understanding for how airway type 2 inflammation develops in airway tissue. Few treatment options exist for patients with type 2-low asthma and developing new medications for this patient subgroup is essential.

Acknowledgments

This study was funded by the National Institutes of Health Grants: (K23 HL138303, P30 DK098722, U10 HL109152, AI106684-01A1, HL109152-05, 5UG1HL139098-02, GM114311-01A1).

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Search strategy and selection criteria

We evaluated the biological target and clinical efficacy of type 2 monoclonal antibodies in asthma. References for this Review were identified through searches of PubMed for articles published between Jan 1, 1950, and Oct 31, 2019 (last searched Nov 7, 2019). The search terms “Asthma/drug therapy” [MeSH], “Antibodies, monoclonal/therapeutic use” [MeSH], “Clinical Trial” [publication type], “Eosinophilia/drug therapy” [MeSH], “Asthma/immunology” [MeSH], “Th2 Cells/immunology” [MeSH], and “asthma and type-2 inflammation” [MeSH] were used and applied no language restrictions. A total of 577 items were found.

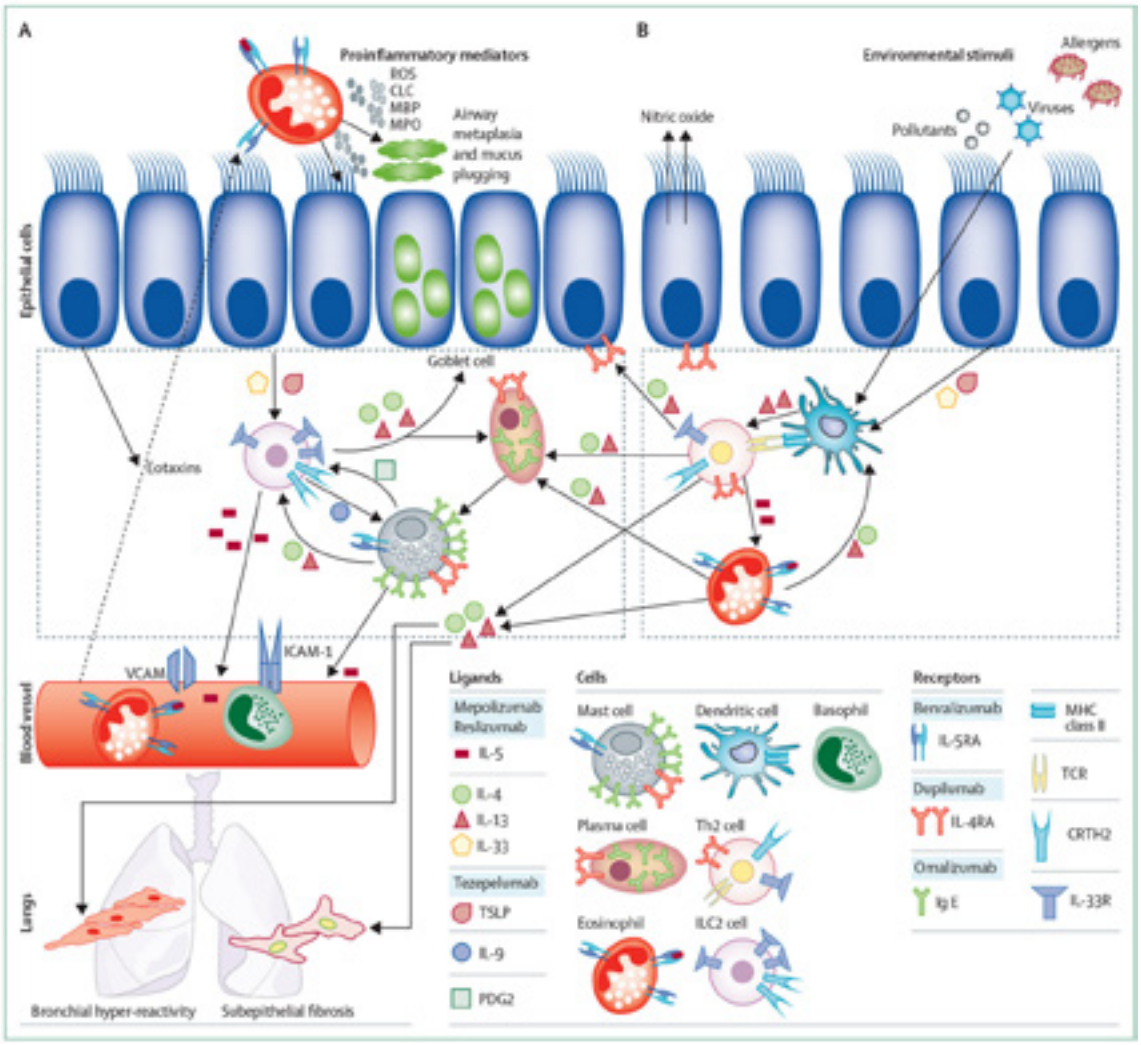

Figure 1: ILC2s and Th2 cells are key activators of airway type-2 inflammation

The type 2 cytokines are responsible for the key pathological features of asthma, including goblet cell metaplasia, mucus plugging, bronchial hyper-reactivity, and airway eosinophilia. The type 2 immune cascade is initiated by epithelial cell exposure to environmental stimuli (ie, allergens, viruses, and pollutants). Epithelial cells secrete eotaxins that promote chemotaxis of eosinophils, basophils, and T-helper-2 (Th2) cells. (A) The role of the group 2 Innate lymphoid cell (ILC2) in driving the type 2 immune response. ILC2 cells are activated through the epithelial production of IL-33 and TSLP, and in this state secrete large amounts of type 2 cytokines (IL-4, IL-5, and IL-13). ILC2 cells induce mast cell proliferation via IL-9 and assist plasma cell class switching to immunoglobulin E (IgE) through the release of IL-4 and IL-13. (B) The role of Th2 cells as propagators of the type 2 immune response. Dendritic cells process and present antigens leading to the production of type 2 cytokines by Th2 cells. ROS=reactive oxygen species. CLC=charcot-leyden crystals. MBP=myelin basic protein. MPO=myeloperoxidase.

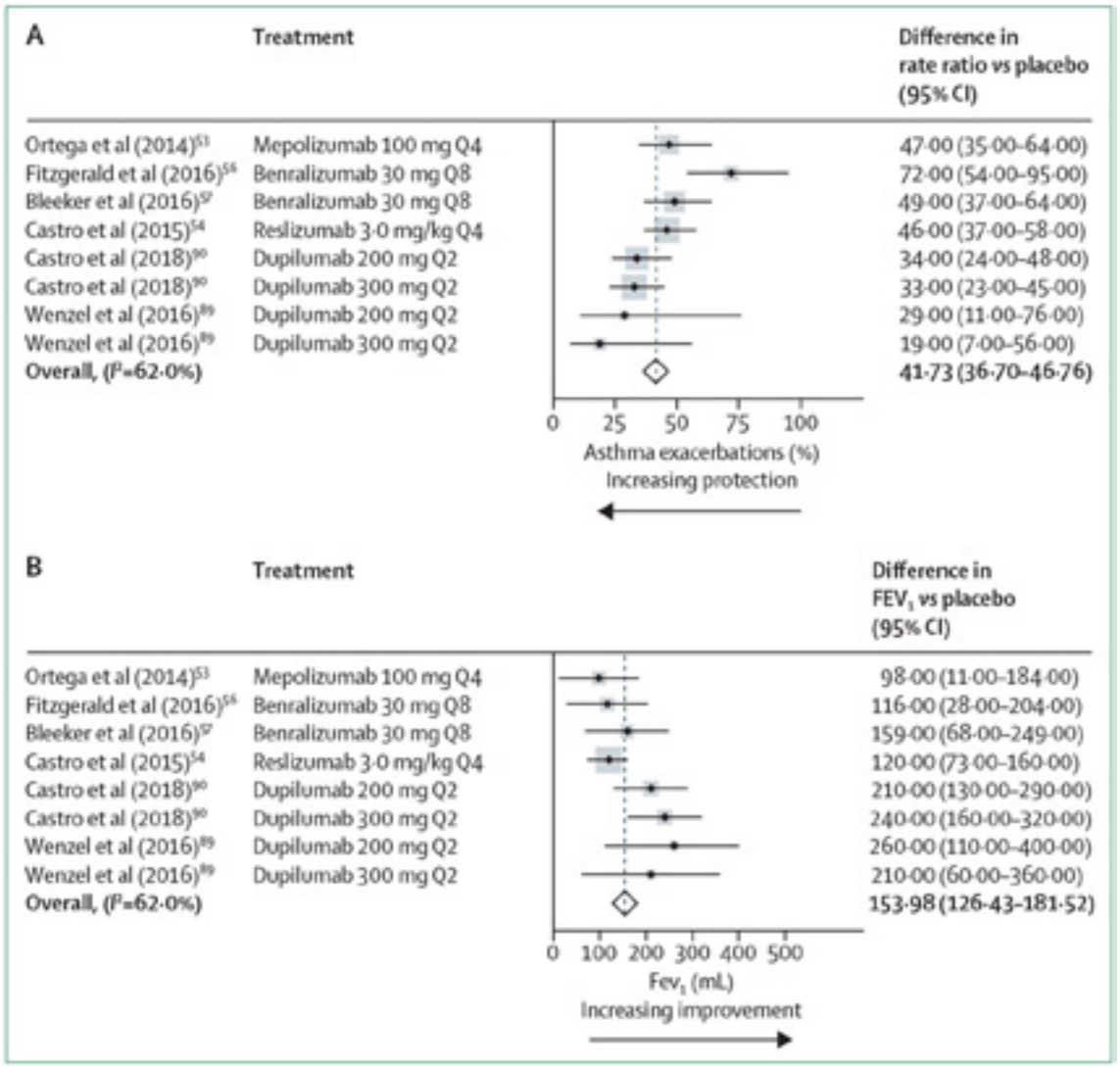

Figure 2: Forest plots showing the effect size of type 2 biologic agents in patients with eosinophilic asthma

(A) Effect of biologic agents on asthma exacerbation. (B) Effect of biologic agents on forced expiratory volumes in 1 s (FEV_1). The standardised mean difference (dashed line) and 95% CI for the combined treatment effects are shown. Q2=dose every 2 weeks. Q4=dose every 4 weeks. Q8=dose every 8 weeks.

Table 1:

Type 2 biologic medications for severe asthma

	Target	Dose	Primary treatment group	Primary benefits	Stage of development
Mepolizumab (GlaxoSmithKline, Brentford, UK)	IL-5	Subcutaneous, 100 mg, Q4 weeks	Severe eosinophilic asthma (≥150 cells per μL at screening or ≥300 cells per μL in past year)	Considerable improvement in asthma exacerbations and symptoms; mild improvement in FEV_1 and steroid sparing	FDA approved for severe eosinophilic asthma
Reslizumab (Teva Pharmaceuticals, Petah Tikva, Israel)	IL-5	Intravenous, 3·0 mg/kg, Q4 weeks	Moderate to severe eosinophilic asthma (≥400 cells per μL)	Considerable improvement in asthma exacerbations; mild improvement in FEV_1 and symptoms	FDA approved for severe eosinophilic asthma
Benralizumab (MedImmune, Gaithersburgh, USA; and AstraZeneca, Cambridge, UK)	IL-5RA	Subcutaneous, 30 mg, Q8 weeks	Severe eosinophilic asthma (≥300 cells per μL)	Considerable improvement in asthma exacerbations; mild improvement in FEV_1 and steroid sparing	FDA approved for severe eosinophilic asthma
Lebrikizumab (Genentech, San Francisco, USA; and Roche, Basel, Switzerland)	IL-13	Subcutaneous, 38–125 mg, Q4 weeks	Severe asthma with periostin concentrations ≥50 ng/mL or blood eosinophils ≥300 cells per μL	Mild improvement in asthma exacerbations	No longer in development for asthma
Pitrakinra (Amgen, Thousand Oaks, USA)	IL-4RA	Subcutaneous, 25 mg once a day or 60 mg nebulised twice a day	Atopic asthma	Modest efficacy in allergen challenge model	No longer in development for asthma
Dupilumab (Regeneron, Tarrytown, USA; and Sanofi, Paris, France)	IL-4RA	Subcutaneous, 200 or 300 mg, Q4 weeks	Moderate to severe eosinophilic asthma (>300 cells per μL)	Considerable improvement in asthma exacerbations, FEV_1, and symptoms; mild improvement in steroid sparing	FDA approved for moderate to severe eosinophil asthma or oral corticosteroid-dependent asthma
Tezepelumab (Amgen; and MedImmune)	TSLP	Subcutaneous, 70 mg Q4 weeks, 210 mg Q4 weeks, or 280 mg Q2 weeks	Moderate to severe asthma	Considerable improvement in asthma exacerbations; mild improvement in FEV_1 and symptoms	Ongoing phase 3 trial
REGN3500 (Regeneron)	IL-33	Subcutaneous, dose to be determined	Moderate to severe eosinophilic asthma (≥300 cells per μL)	Mild improvement in loss of asthma control and FEV_1	Recently completed phase 2B trial

FEV_1=forced expiratory volumes in 1 s. FDA=US Food and Drug Administration. Q2=every 2 weeks. Q4=every 4 weeks. Q8=every 8 weeks.

Table 2:

Risk for type 2 therapeutics

	Observed risks	Hypothetical risks
Mepolizumab	Herpes zoster	Parasitic infections, malignancy, obesity or metabolic dysfunction
Reslizumab	Anaphylaxis	Parasitic infections, malignancy, obesity or metabolic dysfunction
Benralizumab	Prolonged decrease in eosinophil counts	Parasitic infections, malignancy, obesity or metabolic dysfunction
Dupilumab	Eosinophilia, conjunctivitis	Parasitic infections, obesity or metabolic dysfunction, eosinophilic granulomatosis with polyangiitis

All medications report low and similar frequencies for injection site reactions (2–10%) and hypersensitivity reactions (<1–3%).

Expert Opinion on Therapeutic Targets

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/iett20

Thymic stromal lymphopoietin: its role and potential as a therapeutic target in asthma

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To cite this article: Gail M. Gauvreau, Roma Sehmi, Christopher S. Ambrose & Janet M. Griffiths (2020) Thymic stromal lymphopoietin: its role and potential as a therapeutic target in asthma, Expert Opinion on Therapeutic Targets, 24:8, 777-792, DOI: 10.1080/14728222.2020.1783242

To link to this article: https://doi.org/10.1080/14728222.2020.1783242

Published online: 27 Jun 2020.

EXPERT OPINION ON THERAPEUTIC TARGETS
2020, VOL. 24, NO. 8, 777–792
https://doi.org/10.1080/14728222.2020.1783242

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REVIEW

Thymic stromal lymphopoietin: its role and potential as a therapeutic target in asthma

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ABSTRACT

Introduction: Thymic stromal lymphopoietin (TSLP), an epithelial cytokine (alarmin), is a central regulator of the immune response to inhaled environmental insults such as allergens, viruses and pollutants, initiating a cascade of downstream inflammation. There is compelling evidence that TSLP plays a major role in the pathology of asthma, and therapies that aim to block its activity are in development.
Areas covered: We review studies conducted in humans and human cells, largely published in PubMed January 2010–October 2019, that investigated the innate and adaptive immune mechanisms of TSLP in asthma relevant to type 2-driven (eosinophilic/allergic) inflammation and non-type 2-driven (non-eosinophilic/non-allergic) inflammation, and the role of TSLP as a mediator between immune cells and structural cells in the airway. Clinical data from studies evaluating TSLP blockade are also discussed.
Expert opinion: The position of TSLP at the top of the inflammatory cascade makes it a promising therapeutic target in asthma. Systemic anti-TSLP monoclonal antibody therapy with tezepelumab has yielded positive results in clinical trials to date, reducing exacerbations and biomarkers of inflammation in patients across the spectrum of inflammatory endotypes. Inhaled anti-TSLP is an alternative route currently under evaluation. The long-term safety and efficacy of TSLP blockade need to be evaluated.

ARTICLE HISTORY
Received 16 April 2020
Accepted 12 June 2020

KEYWORDS
Alarmin; AMG 157; airway; CSJ117; epithelium; GSK2618960; inflammation; tezepelumab; TSLP; type 2

1. Introduction

Asthma is a common lower respiratory disease, generally characterized by chronic inflammation of the airways. The hallmarks of asthma include variable expiratory airflow limitation and variable symptomatology, both of which are commonly triggered by various epithelial insults such as viruses, allergens, bacteria, air pollutants and other environmental irritants. With prolonged disease, airway limitation may become persistent. Most patients with asthma have mild disease, but approximately 5–10% of patients have severe disease that requires high-dosage inhaled corticosteroids and additional medications to achieve disease control [1,2]. Furthermore, many patients with severe asthma can have disease that remains uncontrolled despite such therapy [1–3].

The inflammation associated with asthma is heterogenous and has been associated with multiple inflammatory endotypes. The most common endotypes include allergic and eosinophilic inflammation, collectively referred to as type 2 (T2) disease. Population-based studies have consistently concluded that the majority of patients with asthma have T2-driven inflammation [4–7]. Patients who are characterized as having either very low or absent signs of T2 inflammation may instead have neutrophilic or paucigranulocytic inflammation, generally in the context of inhaled corticosteroid therapy [8–10]. Within any individual patient with asthma, there may be evidence of multiple upregulated inflammatory pathways and it can be difficult to identify a single predominant endotype, which further limits the predictive value of currently available biomarkers. For patients with severe asthma whose disease is not adequately controlled by inhaled therapies, an understanding of the patient's inflammatory endotype(s) helps inform selection of the optimal add-on treatment, including targeted biologic therapies [1,2].

In recent years, the downstream effectors of allergic and eosinophilic inflammation have been the focus of severe asthma research and treatment. These have included immunoglobulin (Ig) E, sputum and blood eosinophils, interleukin (IL)-4, IL-5 and IL-13. This research has resulted in five approved biologic therapies for patients with moderate-to-severe allergic and/or eosinophilic asthma, and all have demonstrated higher efficacy in patients with eosinophilic inflammation than in those without eosinophilic inflammation [11–15]. There are currently no approved biologic treatment options for patients with moderate-to-severe asthma that is characterized by non-eosinophilic inflammation.

The immunology of thymic stromal lymphopoietin (TSLP), an epithelial cytokine, provides an opportunity for a novel approach to treat asthma inflammation. A member of a class of epithelial cytokines commonly referred to as alarmins (whose other members are IL-25 and IL-33), TSLP is released by airway epithelial cells in response to various environmental insults, including viruses, bacteria, allergens, chemical irritants and physical injury [16,17]. Functionally, TSLP is a key

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Article highlights

- There is a critical need for new therapies to treat patients with severe, uncontrolled asthma, which can be difficult to treat owing to the heterogeneity of airway inflammation.
- Thymic stromal lymphopoietin (TSLP) is a cytokine primarily expressed by the airway epithelium and released in response to environmental insults, instigating a range of downstream inflammatory processes.
- TSLP expression is increased in the airways of patients with asthma compared with healthy individuals, correlating with disease severity and lung function; polymorphisms in the *TSLP* gene are associated with asthma.
- Evidence indicates that TSLP is a key mediator of asthma pathophysiology, driving eosinophilic (allergic and non-allergic) inflammation, non-eosinophilic inflammation and structural changes to the airway, through its actions on a wide variety of adaptive and innate immune cells and structural cells.
- Clinical trials of TSLP blockade, delivered via a systemic route, have produced positive results in a broad population of patients with asthma, reducing exacerbations and multiple biomarkers of inflammation while improving lung function.

This box summarizes key points contained in the article.

instigator of the immune response to environmental insults, initiating a range of downstream inflammatory pathways. While TSLP drives a pronounced T2 inflammatory response [18–20], there is emerging evidence of TSLP involvement in non-T2 processes involving interactions with both immune and structural cell types. The considerable scope of effects mediated by TSLP is illustrated by the wide range of cell types that express the TSLP receptor (TSLPR), including hematopoietic progenitor cells, eosinophils, basophils, mast cells, airway smooth muscle cells (ASMCs), group 2 innate lymphoid cells (ILC2s), lymphocytes, dendritic cells and monocytes/macrophages [21,22]. In addition to its actions on specific cell populations, the possibility that TSLP serves as a key mediator between immune cell types and structural cells in the airway milieu is intriguing and is an area of ongoing research.

In individuals with asthma, as well as those with other inflammatory diseases such as atopic dermatitis, TSLP production appears to be dysregulated. Several studies have shown that TSLP expression is elevated in patients with asthma compared with healthy individuals in inner and outer epithelial layers of airway biopsies [23–30] and in samples of serum [31,32], sputum [33], exhaled breath condensate [34] and bronchoalveolar lavage fluid [30,35]. Furthermore, the level of TSLP expression in patients with asthma has been shown to correlate with airway obstruction and disease severity [25,29,33,35,36]. Various elements of asthma pathophysiology, including airway hyperresponsiveness, mucus overproduction and airway remodeling, are believed to be at least partly driven by TSLP via its downstream, pro-inflammatory effects involving cytokines such as IL-4, IL-5 and IL-13 [37]. The role of TSLP in asthma is underscored by genome-wide association studies that have identified associations between asthma risk and single-nucleotide polymorphisms (SNPs) in the *TSLP* gene [38–40]. These include rs1837253 [41,42], which has been shown to regulate TSLP production in nasal epithelial cells [43] and influence asthma manifestation [44]. TSLP has also been implicated in aspirin-exacerbated respiratory disease (AERD), which is characterized by asthma, chronic rhinosinusitis with nasal polyps, and intolerance of cyclooxygenase-1 inhibitors. Examination of nasal polyp tissue from individuals with AERD and those with chronic rhinosinusitis without AERD demonstrated that TSLP mRNA expression was increased with AERD and with markers of mast cell activation and prostaglandin D2 expression [45].

The compelling evidence of TSLP's role in the pathogenesis and pathology of asthma has led to the development of anti-TSLP monoclonal antibodies as a potential therapeutic option for these patients. The results of clinical studies of anti-TSLP therapy [46,47] have provided the strongest evidence to date for a major role for TSLP in asthma. The purpose of this review is to summarize the available data regarding the mechanisms of action of TSLP in human asthma across the spectrum of inflammatory endotypes, with the goal of elucidating the therapeutic potential of novel therapies that block TSLP activity. Although the biology of the TSLP pathway appears to be similar in humans and rodents, the ability to use rodent models to study the impact of blocking TSLP in humans is limited by the generally low translatability of rodent models to complex heterogeneous human disease [48,49]. As such, we have excluded studies of TSLP in animal models of asthma from this review and, instead, have emphasized observations with direct clinical relevance.

To inform the review, we conducted a literature search of the PubMed database for articles in English published between 1 January 2010 and 1 October 2019 using the search terms (TSLP[title/abstract] OR thymic stromal lymphopoietin [title/abstract]) AND asthma*[title/abstract], employing the 'Humans' species filter and excluding review articles. The results from this search were screened for relevance, i.e. whether they contained information about sites of TSLP expression, TSLP effector cells, or physiological or clinical effects of TSLP, and were supplemented by further relevant articles known to the authors. The included articles are summarized in Table 1.

2. Innate immune mechanisms of action of TSLP relevant to T2-driven (eosinophilic/allergic) inflammation in asthma

Several local effector cells play a role in propagating T2 inflammatory responses, and the interaction between the airway epithelium and these cells is an important process driving eosinophilic inflammation. Evidence that TSLP directly activates innate immune cells involved in T2 inflammatory processes in human asthma is discussed in this section, with evidence for the role of TSLP in adaptive immune cell-mediated inflammatory processes discussed in section 3. This evidence is summarized in Figures 1, 2 and 3.

2.1. TSLP and group 2 innate lymphoid cells (ILC2s)

ILC2s are lineage-negative cells, lacking antigen-recognition receptors, which provide the primary early innate cellular source of T2

Table 1. Studies of TSLP with relevance to asthma, relating to cellular sites of expression/localization, effector cells and physiological and clinical effects.

	Studies in patients or patient samples*		Studies in samples from healthy individuals or in human cell lines		
	Number of studies	References	Number of studies	References	Total studies
Cellular sites of TSLP expression/ localization‡					
Airway epithelial cells§	14	[25,26,29,30,33,50–54,71,116,122,127]	10	[18,19,55–59,90,123,125]	**24**
ASMCs	1	[24]	5	[19,93,119,123,124]	**6**
Bone marrow mesenchymal stromal cells	0	-	1	[83]	**1**
Bronchial endothelial cells	4	[25,29,30,116]	0	-	**4**
Dendritic cells	0	-	2	[100,101]	**2**
Fibroblasts	1	[116]	3	[19,123,127]	**4**
Monocytes/macrophages	3	[25,29,30]	1	[100]	**4**
Mast cells	6	[24,25,29,30,91,116]	2	[19,92]	**8**
Neutrophils	3	[29,30,116]	0	-	**3**
TSLP effector cells					
Airway smooth muscle cells	2	[24,122]	5	[60,93,119–121]	**7**
Basophils	6	[74,85–89]	2	[73,84]	**8**
Bronchial epithelial cells	1	[122]	1	[61]	**2**
Dendritic cells	5	[26,85,104,105,114]	9	[19,95–98,102,103,107,113]	**14**
Eosinophils	5	[28,36,46,81,82]	1	[80]	**6**
Fibroblasts	0		3	[125,126,128]	**3**
Hematopoietic progenitor cells	3	[74–76]	1	[73]	**4**
ILC2s	5	[27,65,67,71,72]	1	[66]	**6**
Mast cells	1	[24]	3	[18,90,93]	**4**
Monocytes/macrophages	0	-	2	[94,95]	**2**
Neutrophils	0	-	1	[115]	**1**
T cells, $CD4^+$	3	[74,104,109]	6	[19,95,98,102,108,113]	**9**
T cells, $CD8^+$	0	-	1	[110]	**1**
T cells, regulatory	2	[32,106]	0	-	**2**
Th2 cells	4	[104,105,109,114]	6	[97,98,102,103,107,113]	**10**
Th17 cells	1	[114]	1	[113]	**2**
Physiological and clinical effects of TSLP					
Airway inflammation¶	7	[25,28,35,36,46,47,134]	N/A	N/A	**7**
Airway obstruction/reduced lung function	7	[25,29,33,35,36,46,47]	N/A	N/A	**7**
Airway remodeling	3	[50,129,135]	4	[121,125,126,128]	**7**
Epithelial barrier maintenance	1	[50]	1	[61]	**2**

*Patients with asthma (studies may also include healthy controls or patients with other atopic diseases), except for two studies in patients with atopic dermatitis [85,114].

‡Cellular sites of TSLP mRNA expression and/or TSLP protein expression/localization.

§Includes bronchial, nasal and unspecified airway epithelial cells. Owing to the very large number of studies indicating that TSLP is expressed in airway epithelial cells, not all have been cited in the main text.

¶Defined as effects on eosinophil and/or neutrophil numbers in patient sputum, bronchoalveolar lavage fluid or lung tissue biopsies.

ASMC, airway smooth muscle cell; ILC2, group 2 innate lymphoid cell; N/A, not applicable; Th, T helper; TSLP, thymic stromal lymphopoietin.

cytokines that drive eosinophilic inflammation. ILC2s produce substantial amounts of T2 cytokines including IL-5, IL-13 and IL-9 following activation by alarmin cytokines such as TSLP, IL-25 and IL-33 [63–65]. This effect is enhanced in the presence of IL-2 and IL-7 [66]. TSLP can synergize with IL-25 or IL-33 to promote ILC2 production of IL-5 and IL-13 [65] and prolonged ILC2 survival [66]. Activation of ILC2s by IL-33 and TSLP results in upregulation of surface expression of c-Kit and downregulation of IL-7Rα and CRTH2, suggesting that alarmin cytokines can create heterogeneous populations of ILC2s [66]. The functions of the various populations remain to be clarified.

With respect to human asthma models, Chen *et al.* (2017) reported that, in mild asthmatics, there was a rapid and significant increase in sputum ILC2s expressing high levels of IL-5 and IL-13 within 24 hours post-allergen inhalation challenge [67]. Phenotypic analysis of ILC2s in this study showed upregulation of TSLPR on $ST2^+$ILC2s, indicating that increased responsiveness of ILC2s to TSLP within the airways may help to propagate eosinophilic inflammation. Other studies have shown that ILC2 numbers are increased in patients with severe asthma and persistent eosinophilia compared with those with mild asthma, with the greatest number of airway IL-5^+IL-13^+ILC2s observed in patients with uncontrolled eosinophilia despite treatment with high-dose oral corticosteroids [68–70]. In endobronchial biopsies from prednisone-dependent patients with severe asthma, ILC2s were found to co-localize to TSLP-immunopositive regions [25]. Similarly, the number of ILC2s in nasal biopsies have been found to correlate positively with nasal tissue TSLP levels in patients with severe asthma and chronic rhinosinusitis [27]. Liu *et al.* (2018) reported that dexamethasone treatment following *in vitro* stimulation of peripheral blood cultures from patients with severe asthma using *Aspergillus* or IL-2/IL-33 resulted in inhibition of IL-5 production by ILC2s [71]. In contrast, dexamethasone had no effect on ILC2s from the airways, indicating compartmental differences in steroid resistance in ILC2s [71]. This was attributed to higher levels of TSLP in the airways. Specifically, the study showed that the inhibitory effects of dexamethasone on airway ILC2s was reduced in the presence of TSLP and IL-7,

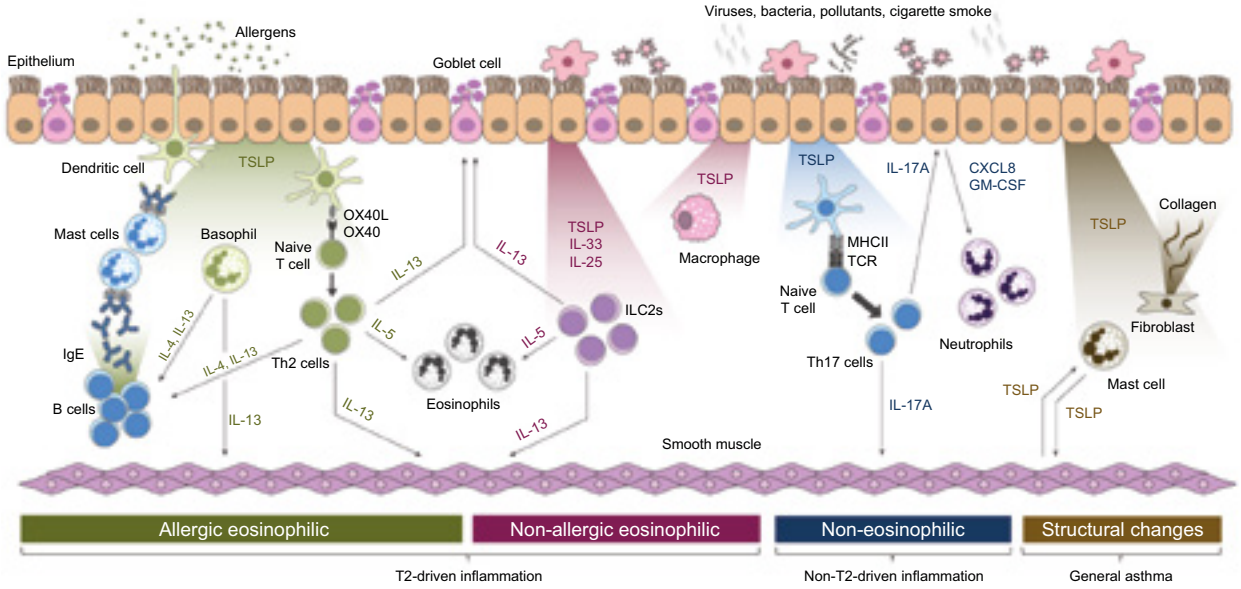

Figure 1. The role of TSLP in driving disease mechanisms in different asthma endotypes. In allergic eosinophilic inflammation, TSLP initiates pathways involving Th2 lymphocytes, basophils and mast cells to drive airway eosinophilia. In non-allergic eosinophilic inflammation, TSLP activates innate lymphocytes such as ILC2s that contribute to airway eosinophilia. The mechanisms underlying non-eosinophilic inflammation require further elucidation, but TSLP-related processes involving Th17 lymphocytes and neutrophils appear to be involved. TSLP also mediates structural mechanisms that contribute to airway remodeling, involving airway smooth muscle cells and fibroblasts. Further details of the mechanisms are provided in Figures 2–5. Figure adapted, with permission, from Brusselle G & Bracke K, Ann Am Thorac Soc. 2014;11 Suppl 5:S322–8 [62]. CXCL8, chemokine (C-X-C motif) ligand 8; GM-CSF, granulocyte-macrophage colony-stimulating factor; IgE, immunoglobulin E; IL, interleukin; ILC2, group 2 innate lymphoid cell; OX40 L, OX40 ligand; T2, type 2; Th, T helper; TSLP, thymic stromal lymphopoietin.

and this was found to be dependent on MEK and STAT5 signaling [71]. Three genes, *CBX7, MEK2* and *TRL2*, have been identified in TSLP-stimulated lymphoid cells that are resistant to dexamethasone treatment [72]. TSLP itself can induce expression of MEK2, which translocates to the nucleus and interacts with CBX7, suggesting a positive feedback regulatory pathway [71]. It is proposed that, while dexamethasone may attenuate the pro-inflammatory activity of ILC2s driven by IL-33, TSLP may have a role in conferring steroid resistance of ILC2s.

2.2. TSLP and hematopoietic progenitor cells

There is evidence to support an association between allergen-induced asthmatic responses and mobilization of eosinophil progenitor cells (EoPs) from the bone marrow. Affected tissues support local differentiation, proliferation, maturation and activation of EoPs that home to the site of allergen exposure in airway disease. TSLP has been shown to drive activation, migration and local differentiation of EoPs within the airways.

Cord-derived hematopoietic progenitor cells cultured overnight with TSLP at nanomolar levels upregulate IL-5Rα expression and then stimulate significant outgrowth of eosinophil/basophil colony-forming units (Eo/Bo-CFUs) in combination with IL-3 or GM-CSF [73]. In addition, increased eosinophilopoietic activity was evident in bronchial epithelial supernatants from patients with severe eosinophilic asthma compared with mild asthmatic and healthy controls. This activity was attenuated by a receptor-blocking antibody to TSLP [74]. At picogram levels, TSLP stimulated the outgrowth of Eo/Bo-CFUs, with additive effects in the presence of IL-5 [74]. At the mRNA level, a synergistic increase of GATA-2 and CEBPα in $CD34^+$ cells was observed in the presence of TSLP and IL-5 [74]. Collectively, these findings indicated that eosinophilopoiesis is not solely driven by IL-5, but rather is a complex process involving the interaction between local and systemically elaborated growth factors, including TSLP.

Migration of precursor cells to the airways is an important component of driving local eosinophilic inflammation. Pre-exposure to TSLP and IL-33 primes migration of progenitor cells towards the chemoattractant SDF-1α (CXCL12) [75]. This implies that the airway epithelium can locally release alarmin cytokines that enhance the migrational responsiveness of CD34+ progenitor cells. In addition, CD34+ primitive progenitor cells express TSLPR and overnight stimulation with TSLP results in a dose-dependent release of IL-5, IL-13, GM-CSF and chemokines including CCL22, CXCL8 and CCL1 [75,76]. This indicates that TSLP not only drives local maturation of eosinophil-lineage committed progenitor cells but may also promote pro-inflammatory function and migration of primitive progenitor cells.

2.3. TSLP and eosinophils

Eosinophilic inflammation is a major contributor to physiological changes and airway remodeling in asthma. Eosinophils are present and subsequently activated locally within asthmatic airways, and are increased in number when asthma is uncontrolled [77] or severe [78], while being decreased in controlled asthma [79]. Despite numerous murine studies

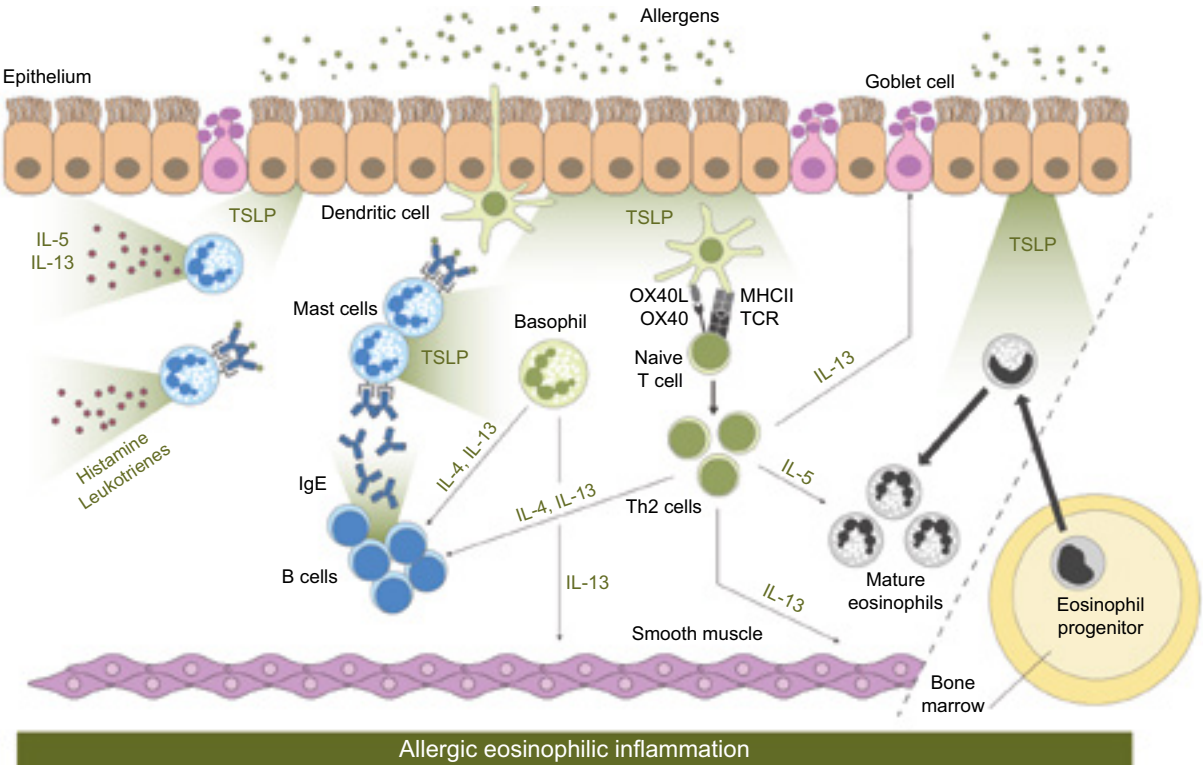

Figure 2. Immune mechanisms of TSLP in asthma relevant to allergic eosinophilic inflammation. TSLP, released in response to allergens, upregulates expression of MHCII and co-stimulatory molecules, facilitating antigen presentation by dendritic cells to $CD4^+$ naive T cells, and induces upregulation of OX40 L expression on dendritic cells, accelerating differentiation of $CD4^+$ naive T cells to Th2 cells. It is hypothesized that TSLP can also promote proliferation and differentiation of naive T cells directly. Th2 cells produce IL-4, IL-5 and IL-13, leading to IgE switching in B cells, degranulation of mast cells, airway eosinophilia, mucus hypersecretion from goblet cells, and smooth muscle contraction resulting in airway hyperresponsiveness. TSLP primes recruitment of primitive CD34+ hemopoietic progenitors from bone marrow to airway tissue and drives local differentiation to mature eosinophils. TSLP can also directly induce mast cells to produce T2 cytokines, and mast cells themselves can produce significant amounts of TSLP following IgE cross-linking. Basophils also release T2 cytokines and histamine in response to TSLP. IgE, immunoglobulin E; IL, interleukin; ILC2, group 2 innate lymphoid cell; MHCII, major histocompatibility complex class II; OX40 L, OX40 ligand; TCR, T cell receptor; Th, T helper; TSLP, thymic stromal lymphopoietin.

reporting effects of TSLP on eosinophil function, few studies have looked at the direct effect of TSLP on mature human eosinophils, much less a cross-sectional comparison with cells from patients with asthma compared with healthy controls. Human eosinophils express both TSLPR and IL-7Rα subunits, and their expression is enhanced by TNF-α and IL-3 [80,81]. TSLP promotes eosinophil viability by attenuating apoptosis and induces significant production of IL-6, eosinophil-derived neurotoxin and chemokines, including CXCL8, CXCL1 and CCL2 [80,81]. TSLP upregulates ICAM-1 and CD18 but suppresses L-selectin surface expression, indicating that it plays a role in promoting eosinophil transmigration and tissue accumulation [80]. The effects of TSLP on eosinophils are mediated through ERK, p38 MAPK and NF-κB signaling pathways [80,81]. Furthermore, TSLP can induce formation of eosinophilic extracellular traps consisting of mitochondrial DNA in association with eosinophilic cationic protein, which play an important role in innate immune responses to infectious agents leading subsequently to tissue damage in asthmatic airways [82]. These studies indicate a role for TSLP in promoting airway eosinophilia in asthma, and are supported by findings from a clinical trial in patients with mild asthma in which anti-TSLP therapy significantly reduced numbers of blood and sputum eosinophils in conjunction with a reduction in airway bronchoconstriction following allergen challenge [46]. Further support comes from correlational studies in patients with atopic asthma showing that levels of immunopositive staining for TSLP in bronchial biopsies correlated with airway eosinophilia 24 hours post-allergen challenge [28]. In contrast, levels of TSLP were inversely related to the number of eosinophils in induced sputum from patients with asthma during virus-induced exacerbations, suggesting differing mechanisms of action of TSLP in acute exacerbations versus chronic eosinophilic inflammation [36].

2.4. TSLP and basophils

Basophils play an important role in asthma as a significant source of T2 cytokines, including IL-4, IL-13 and pro-inflammatory mediators such as histamine and leukotrienes. Basophil development, homeostasis and function have been thought to be largely regulated by IL-3; however, accumulating evidence suggests that TSLP also influences basophil differentiation.

Peripheral blood-derived $CD34^+$ cells pre-incubated with IL-3 and TNF-α have enhanced sensitivity to TSLP-mediated basophil lineage commitment [73]. Additionally, mast cell-activated bone marrow mesenchymal stromal cells produce TSLP, which can enhance differentiation of $CD34^+$ progenitors into Eo/Bo-CFUs [83]. Mature basophils express TSLPR, which

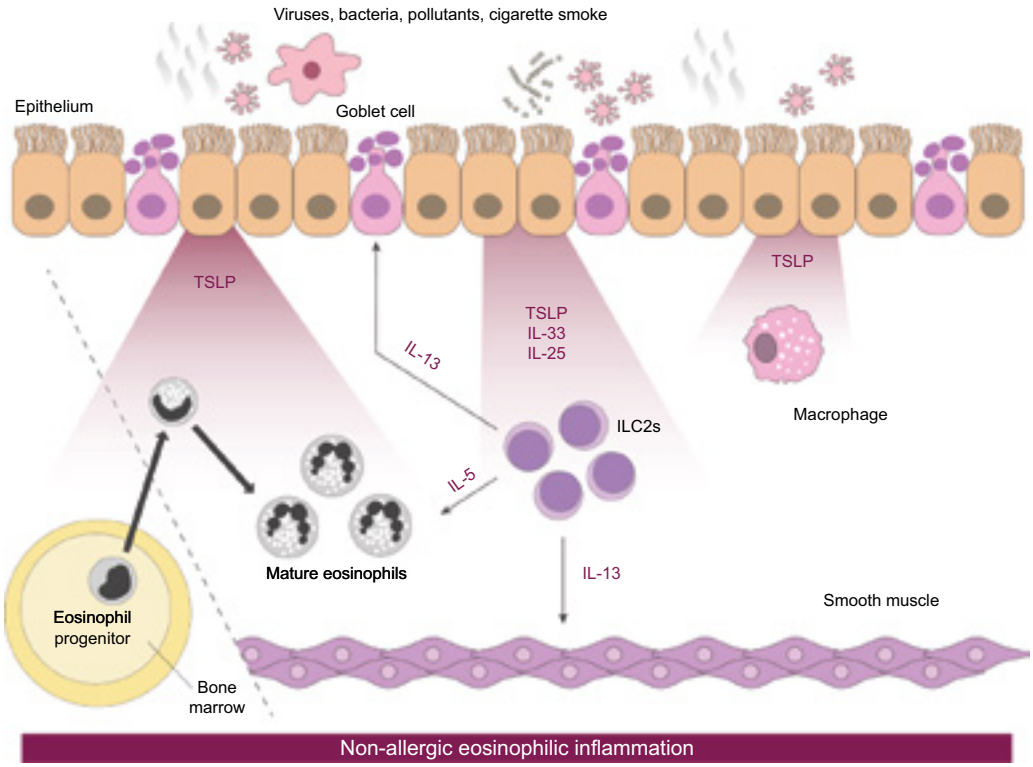

Figure 3. Immune mechanisms of TSLP in asthma relevant to non-allergic eosinophilic inflammation. Exposure to viruses, bacteria, air pollutants, cigarette smoke and other insults induces the release of TSLP and other epithelial cytokines, IL-33 and IL-25, which activate ILC2s. Activated ILC2s produce IL-5 and IL-13, leading to eosinophilia, mucus hypersecretion and airway hyperresponsiveness. TSLP may also have direct effects on eosinophils, promoting eosinophil viability by attenuating apoptosis, as well as priming recruitment of primitive CD34+ hemopoietic progenitors from bone marrow to airway tissue and driving local differentiation to mature eosinophils. Furthermore, TSLP may have effects on macrophages, although these have not yet been fully elucidated. IL, interleukin; ILC2, group 2 innate lymphoid cell; TSLP, thymic stromal lymphopoietin.

can be upregulated in the presence of IL-3 [84]. By comparison, TSLP-stimulated basophils exhibited a greater expression of the IL-33 receptor ST2, suggesting the existence of heterogeneous basophil populations [84]. Allergen stimulation of peripheral blood mononuclear cells in patients with atopic dermatitis resulted in upregulation of TSLPR on basophils and myeloid dendritic cells, which was further increased with IgE-FcεR1 cross-linking [85]. In blood samples from patients with allergic asthma, there was significant upregulation of TSLPR on basophils following direct stimulation with cross-linking anti-IgE antibody, which correlated with serum total IgE [86]. However, another study of patients with asthma reported that anti-IgE stimulation increased IL-25 and IL-33 receptor expression, but not TSLPR [87]. These studies suggest that there may be both IgE-dependent and IgE-independent mechanisms that enhance the responsiveness of basophils to TSLP. Salter *et al*. (2015) expanded on these findings to show that basophil TSLPR expression is increased significantly post-allergen challenge within the airways of individuals with mild asthma [88]. In addition, TSLP stimulation of peripheral basophils increased activation marker expression (CD203 c), T2 cytokine production, histamine release and eotaxin-induced cellular migrational responses [88]. Stimulation of basophils with TSLP also upregulates expression of the IL-25 receptor (IL-17RB) and ST2, suggesting that TSLP can enhance basophil responsiveness to other alarmin cytokines [89]. TSLP is an important mediator of basophil inflammatory function, and this axis may be a potential target to attenuate airway eosinophilia.

2.5. TSLP and mast cells

Mast cells play an important role in initiating eosinophilic and/or allergic asthma through IgE-FcεR1 cross-linking, leading to degranulation of histamine, leukotrienes and cytokines/chemokines. Evidence demonstrates that alarmin cytokines can influence mast cell function. Mast cells express TSLPR and when stimulated with TSLP, alone or in concert with IL-1β and TNF-α, produce T2 cytokines and chemokines CXCL8 and CCL1 with no effect on mast cell proliferation or survival [18,24,90]. Interestingly, mast cells themselves can produce significant amounts of TSLP following IgE cross-linking or priming with IL-4 [91,92], and a crosstalk between ASMCs and mast cells has been reported, as shown by chronically activated mast cells triggering the release of TSLP in a TNF-α-dependent pathway. In turn, ASMC-derived TSLP induced T2 cytokine production by mast cells [93]. Collectively, these studies demonstrate that TSLP can directly interact with mast cells to propagate eosinophilic and allergic inflammation, through the production of T2 cytokines. As mast cells can themselves produce TSLP, there may be an autocrine feedback loop that could be a viable target for asthma management.

2.6. TSLP and monocytes/macrophages

Macrophages are an abundant leukocyte found in alveoli, distal airspaces and conducting airways. T2 cytokines can drive differentiation of lung macrophages into alternatively activated macrophages (aAMs). However, there are few supporting studies in humans showing that effects of TSLP directly promote quiescent macrophage differentiation into aAMs. TSLP has been shown to enhance CD80 activation marker expression in blood $CD14^+$ monocytes/macrophages, indicating a role in promoting differentiation to mature macrophages [94]. In addition, cDNA taken from human monocytes cultured with TSLP and IL-7 showed upregulation of *CCL17, CCL18* and *CCL22*, thereby implicating TSLP as a promotor of subsequent migration of effector cells to the airways [95]. Further studies are needed to determine whether TSLP can influence differentiation of macrophages into aAMs in humans. Immunostaining of bronchial biopsy tissue shows that TSLP expression in tissue colocalizes to epithelial $CD68^+$ macrophages, with greater numbers detected in patients with asthma compared with disease controls or healthy individuals, which supports this postulate [25,29,30].

3. Adaptive immune mechanisms of action of TSLP relevant to T2-driven (eosinophilic/allergic) inflammation in asthma

3.1. TSLP and dendritic cells

Human myeloid dendritic cells express TSLPR [96], and TSLP stimulation can directly upregulate expression of major histocompatibility complex class II and co-stimulatory molecules CD40, CD86, CD54, CD90, CD83 and CD-LAMP, as well as chemokines CXCL8, CCL24, CCL17, CCL22 and CCL1 [19,97–99]. Interestingly, monocyte-derived dendritic cells can themselves produce TSLP upon stimulation by microbial products, suggesting that TSLP can act in an autocrine manner to further drive T2 inflammation [100,101]. Studies also implicate TSLP as being an important driver of dendritic cell-mediated T cell differentiation [102]. In the absence of IL-12, TSLP can induce expression of OX40 ligand (OX40 L) [98], and OX40 L expressed by TSLP-induced dendritic cells results in differentiation of naive $CD4^+$ T cells into $TNF\text{-}\alpha^+IL\text{-}10^-$ T helper (Th) 2 cells [98]. OX40 L expression can convert IL-10-producing regulatory Th1 cells induced by IL-12 into TNF-α-producing Th2 cells, indicating that OX40 L produced by TSLP-stimulated dendritic cells can act as a Th2-polarizing signal [98,103]. Similarly, a combination of TSLP and allergen can stimulate peripheral myeloid dendritic cells from individuals with allergic asthma to induce $CD4^+$ T-cell differentiation into Th2 cells, whereas TSLP on its own can promote polarization into Th9 cells [104]. OX40 L expression is required for the induction of Th2 but not Th9 polarization, and in contrast Th9 cells require the presence of TGF-β1 [104]. Huang *et al.* (2019) expanded on the above findings to show that exosomes produced by TSLP-activated dendritic cells expressed OX40 L, which had the capacity to promote $CD4^+$ T-cell proliferation and production of IL-4 [105]. TSLP was shown to have a priming effect on myeloid dendritic cell-mediated expansion and function of $CRTH2^+CD4^+$ Th2 memory cells but directly hindered the development of $FOXP3^+$Tregs [106,107]. These studies demonstrate that the interaction between dendritic cells and TSLP is an important triggering event that leads to the promotion of naive T-cell differentiation and polarization, and downstream T2 inflammation, in part mediated by OX40 L.

3.2. TSLP and lymphocytes

Although the majority of studies investigating TSLP and lymphocytes have focused on the indirect effect of TSLP on T-cell differentiation mediated by dendritic cells [98,102,104], there is evidence to suggest that TSLP can directly modulate human T lymphocytes. In resting $CD4^+$ T cells, there is minimal expression of TSLPR; however, following their activation, TSLPR expression levels increase significantly [108]. TSLP, in the presence of T cell receptor (TCR) stimulation or IL-4, can promote proliferation and differentiation of naive $CD4^+$ T cells into Th2 cells or memory T cells [108,109]. Similar effects are seen on $CD8^+$ T cells: TSLP can directly enhance the expansion of $CD8^+$ T cells activated with TCR stimulation [110].

The direct effects of TSLP on Tregs have not been well studied. Tregs express TSLPR and stimulation with TSLP impairs IL-10 production [106]. Suppressive Treg activity was found to be decreased in allergic asthmatics compared with non-allergic asthmatics or healthy controls in both adult and pediatric populations [32,106]. These findings suggest that TSLP has the ability to reduce the anti-inflammatory function of Tregs and hence further potentiate T2 inflammation in asthma. Collectively, these data suggest that TSLP can directly modulate T lymphocytes, leading to downstream T2 inflammation and airway eosinophilia.

4. Mechanisms of action of TSLP relevant to non-eosinophilic/non-allergic asthma

Asthma is a heterogeneous disease with numerous phenotypes. Moderate and severe asthma phenotypes have been associated with non-eosinophilic inflammation, mediated by Th17 cells and neutrophils (Figures 1 and 4). IL-17A produced by Th17 cells has been shown to have various effects in asthma pathophysiology, including stimulating bronchial epithelial cells to produce neutrophilia-promoting cytokines such as CXCL8 (IL-8) and GM-CSF [111], and promoting airway remodeling by altering the function of ASMCs [112]. However, few studies have investigated the role of TSLP in non-eosinophilic asthma. One study reported that TSLP enhanced Toll-like receptor (TLR) 3 ligand-induced production of IL-23 by dendritic cells, and induced the programming of naive $CD4^+$ T cells into Th17 cells [113]. Another study reported that TSLP-stimulated dendritic cells pulsed with ovalbumin led to Th2 and Th17 polarization, as evident by an increase in $IL\text{-}4^+$/$IL\text{-}17A^+$ T cells and upregulation of IL-4/IL-17A protein levels in co-culture supernatant. Furthermore, Th17-related cytokines, like IL-6 and IL-23, were upregulated in co-culture supernatants of TSLP-stimulated dendritic cells pulsed with ovalbumin, compared with those of lipopolysaccharide-stimulated dendritic cells [114]. These findings suggest that TSLP and TLR3 ligands promote Th17-cell differentiation under Th2 polarizing

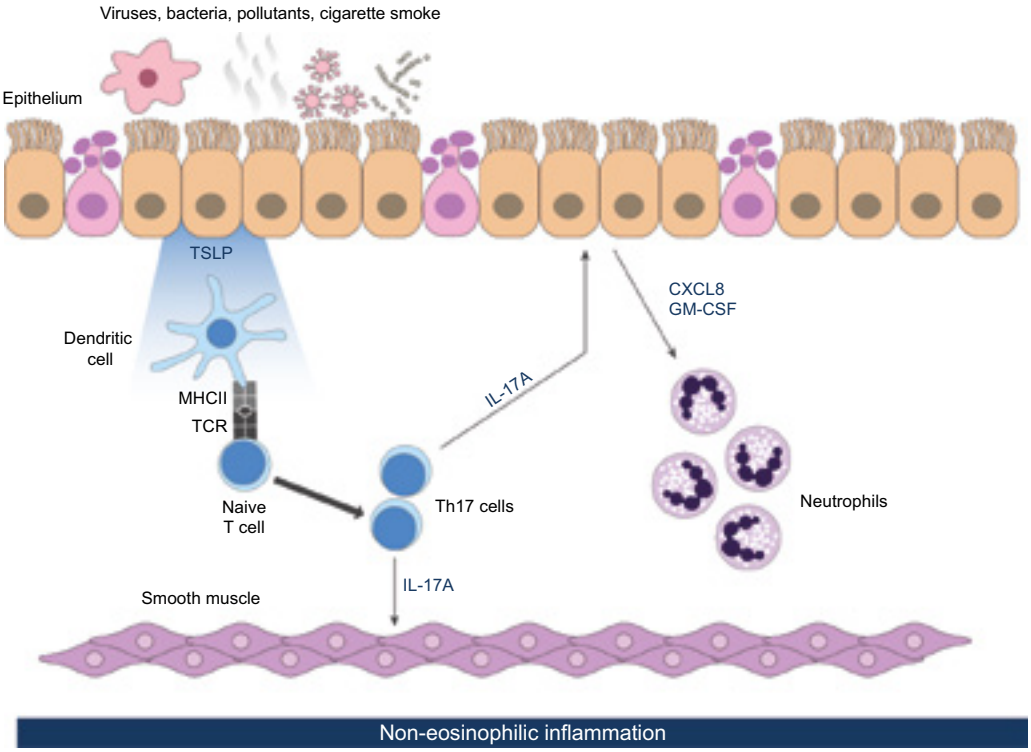

Figure 4. Immune mechanisms of TSLP in asthma relevant to non-eosinophilic inflammation. Exposure to environmental insults leads to airway neutrophilia. The mechanisms by which TSLP acts in this are not yet well understood but may involve Th17 cell differentiation through TSLP-promoted dendritic cell activation and subsequent effects on neutrophils via IL-17A production. IL-17A stimulates bronchial epithelial cells to produce neutrophilia-promoting cytokines such as CXCL8 (IL-8) and GM-CSF, and promotes airway remodeling by altering the function of airway smooth muscle cells. CXCL8, C-X-C motif chemokine ligand 8; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; MHCII, major histocompatibility complex class II; TCR, T cell receptor; Th, T helper; TSLP, thymic stromal lymphopoietin.

conditions through dendritic cell activation. Whether TSLP has a direct effect on Th17 activation and differentiation is unclear. TSLP also has the ability to enhance neutrophil killing of methicillin-resistant *Staphylococcus aureus* (MRSA) during *in vivo* skin infection, directly engaging the complement C5 system to modulate neutrophil reactive oxygen species production [115]. The researchers concluded that TSLP increases MRSA killing in a neutrophil- and complement-dependent manner, suggesting a link between TSLP and an innate immune response [115].

With respect to *in vivo* studies, Li *et al.* (2018) assessed the levels of alarmin cytokines in bronchoalveolar lavage fluid from individuals with asthma at different levels of severity and from healthy control individuals [35]. The concentrations of IL-33 and TSLP, but not IL-25, were significantly greater in those with asthma compared with controls, and these cytokine levels correlated inversely with lung function. Further, the concentration of TSLP alone correlated positively with neutrophil counts. Previous studies have shown that neutrophils are a source of TSLP in bronchial biopsy tissue [29,30,116], and this may explain the findings of Corren *et al.* (2017), who reported that anti-TSLP monoclonal antibody therapy reduced exacerbations in patients with severe asthma without blood eosinophilia. The authors proposed that TSLP may play a role in patients with low or absent T2 inflammation [47]. The prevalence of TSLP in other airway diseases, such as chronic obstructive pulmonary disease [30], further suggests that TSLP may be involved in other T2-independent inflammatory pathways.

5. Structural mechanisms of action of TSLP relevant to asthma

Further to its actions on specific immune cells, there is now substantial evidence that TSLP serves as a key mediator between immune cells and structural cells in the airway (Figures 1 and 5). Dysregulation of structural cells in asthma can result in characteristic alterations to the airway, collectively known as airway remodeling, which include thickening of the reticular basement membrane, goblet cell hyperplasia, subepithelial fibrosis and ASMC hyperplasia and/or hypertrophy [117].

5.1. TSLP and ASMCs

Numerous reports indicate that TSLP is an important modulator of ASMC activity. Human ASMCs express TSLPR [24], and stimulation with TSLP results in expression of IL-6, CCL11 and CXCL8, as well as migration through STAT3 signaling [118–122]. ASMCs are a significant source of TSLP [24,119,123], augmented in the presence of TNF-α and IL-1β via the p38 and MAPK signaling pathways [118,124]. Allakhverdi *et al.* (2009) showed that TNF-α and IL-1β can promote TSLP expression in ASMCs from healthy individuals,

and that supernatants of IgE/anti-IgE-activated mast cells induced TSLP release in ASMCs [93]. Cultures of supernatants of IL-1- and TNF-α-stimulated ASMCs triggered release of IL-5 and IL-13 by mast cells, which was attenuated by TSLP blockade [93]. Collectively, these findings indicate that TSLP can promote airway inflammation through a crosstalk between mast cells and airway structural cells, as well as communication between the airway epithelium and mast cells [18,24,30,93].

5.2. TSLP and fibroblasts

Studies using a human lung fibroblast cell line co-cultured with epithelial cells transfected with TSLP demonstrated significant production of collagen and alpha smooth muscle actin via a p38-MAPK- and STAT3-dependent pathway [125,126]. This indicates that TSLP released by lung epithelial cells in airway diseases such as asthma may promote airway remodeling through activation of fibroblasts. Furthermore, expression of TSLP in bronchial biopsy tissue has been shown to be localized to fibroblasts [116,123,127]. Specifically, TSLP has been shown to increase TGF-β1 and arginase 1 production by fibroblasts at the mRNA and protein levels [128]. It has been proposed that TSLP stimulation can induce fibroblast cellular senescence during airway remodeling in asthma and that inhibiting the signaling pathways of senescence overcomes TSLP-induced airway remodeling [129]. Mechanistic data from clinical studies with biologics targeting TSLP function will be required to corroborate these *in vitro* findings.

6. Clinical data with TSLP blockade

As described above, there is extensive evidence that TSLP plays a major role in the pathophysiology of asthma, based on its position at the top of the inflammatory cascade for both T2 and non-T2 inflammatory processes. Blockade of TSLP may therefore be effective for a broad population of patients with severe, uncontrolled asthma and thus has been actively pursued as a therapeutic treatment.

6.1. Drugs in development

There are two investigational medications in clinical development for the treatment of asthma that directly bind to TSLP. The first of the investigational medications to be tested in asthma is tezepelumab, a human monoclonal antibody that binds to TSLP and thus prevents binding and signaling through the TSLPR. Tezepelumab was initially tested as an intravenous formulation and is more recently being tested with subcutaneous delivery.

A second investigational anti-TSLP medication to be tested in asthma is CSJ117, a fully human neutralizing antibody antigen-binding fragment (Fab) that belongs to the IgG1/λ isotype subclass. CSJ117 has been developed as an inhaled formulation for targeted delivery to the lungs to bind to the TSLP

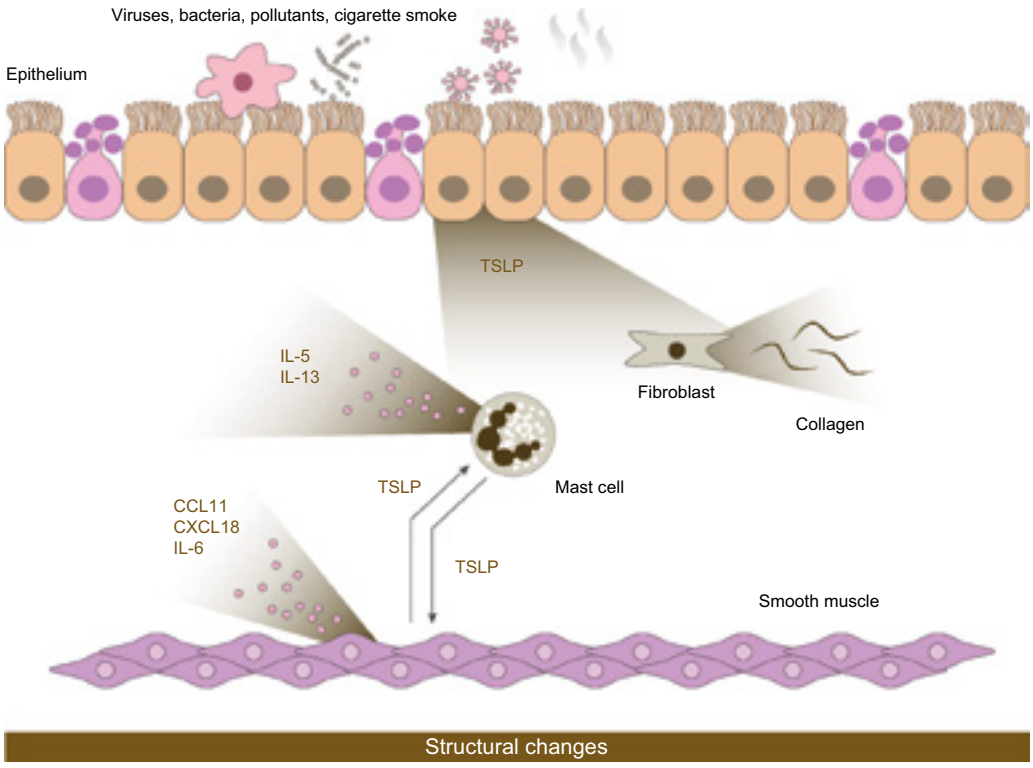

Figure 5. Structural mechanisms of TSLP relevant to multiple asthma inflammatory endotypes. These mechanisms include stimulating airway smooth muscle cell migration and mediating crosstalk between airway smooth muscle cells and mast cells, inducing both cell types to produce TSLP and inflammatory cytokines. TSLP also stimulates human lung fibroblast cells to produce collagen, promoting airway remodeling. CCL11, C-C motif chemokine ligand 11; CXCL8, C-X-C motif chemokine ligand 8; IL, interleukin; TSLP, thymic stromal lymphopoietin.

released by airway epithelial cells in response to common inhaled triggers [16,17]. This formulation has potentially greater convenience than the systemic delivery of tezepelumab, while directly targeting the lung epithelium via local distribution of drug may potentially reduce the chance of effects caused by impacting TSLP signaling outside the lung.

The TSLPR complex has been associated with a number of allergic inflammatory diseases in addition to asthma [130]. GSK2618960 is a humanized Fc-disabled IgG1 monoclonal antibody directed against the alpha component (IL-7Rα; CD127) of the TSLPR [131] and is currently in development for the treatment of autoimmune indications including multiple sclerosis [132]. Together with evidence of autoantibodies in airways of patients with severe eosinophilic asthma [133], blocking IL-7Rα may have utility for the treatment of asthma. Administered intravenously, this investigational medication has been well tolerated. By flow cytometry, GSK2618960 demonstrated greater than 95% receptor occupancy on $CD3^+$ T cells and effectively blocked IL-7 receptor signaling as measured by STAT5 phosphorylation following *ex vivo* exposure of whole blood to IL-7 stimulant [132].

6.2. Clinical studies of TSLP blockade

Two clinical trials of TSLP blockade in patients with asthma have been published, reporting favorable results of tezepelumab treatment. Results from a trial of CSJ117 are pending. Completed and ongoing clinical studies of TSLP blockade are summarized in Table 2.

The first trial completed in patients with asthma was a phase 1b, proof-of-concept study to evaluate the efficacy of tezepelumab in an allergen challenge model of allergic asthma. This randomized, parallel-group, double-blind, placebo-controlled study was conducted by the Clinical Investigators Collaboration in a Canadian population of adults with mild, allergic asthma [46]. Tezepelumab was administered intravenously at a dose of 700 mg and participants were dosed every 4 weeks for 3 months. In the tezepelumab group, blood eosinophil counts began to decline at 2 weeks post-dosing (the first time point measured) and reached normal levels by 4 weeks. Sputum eosinophils showed a significant improvement into the normal range (of < 2%) by the first time point measured, 6 weeks after the first dose. Remarkably, the level of fractional exhaled nitric oxide (FeNO) improved significantly by 1 week after the first dose. Inhaled allergen challenges were conducted on days 42 and 84 to induce eosinophilic inflammation in the airways; tezepelumab significantly inhibited the allergen-induced early and late asthmatic responses, as well as post-challenge measures of inflammation, including FeNO, and eosinophils in blood and sputum. It was noted that the systemic treatment was effective in regulating both circulating and local measures of inflammation.

The second completed trial in asthma (PATHWAY) was a large, phase 2, multicenter, randomized, parallel-group, double-blind, placebo-controlled study [47]. The trial evaluated the efficacy and safety of tezepelumab as an add-on therapy for patients with moderate-to-severe asthma and a history of exacerbations and uncontrolled disease, who were receiving inhaled corticosteroids and long-acting β_2-agonists with or without oral corticosteroids and additional asthma controllers. Three tezepelumab dose regimens were evaluated, low (70 mg every 4 weeks), medium (210 mg every 4 weeks) and high (280 mg every 2 weeks), administered subcutaneously for 1 year. The study reported significant reductions versus placebo in annualized exacerbation rates of 62%, 71% and 66% in the low-, medium- and high-dose tezepelumab groups, respectively, along with significant improvements in lung function and markers of inflammation (FeNO and blood eosinophils) in all active treatment groups. Of interest, these improvements were observed irrespective of patient phenotype and independent of baseline peripheral blood eosinophil counts, IgE levels and FeNO levels, indicating that tezepelumab provided similar efficacy in patients with T2-driven or non-T2-driven disease. Assessments of pro-inflammatory biomarkers and proteomics were also conducted. In the cohort receiving tezepelumab 210 mg every 4 weeks (the dose selected for phase 3 studies), serum IL-5 and IL-13 levels and numbers of blood eosinophils at 1 year decreased by at least 50% from baseline, along with 25% and 20% reductions in FeNO and total IgE, respectively [134]. Proteomics analyses revealed reductions in proteins associated with matrix remodeling (MMP-10 and periostin) demonstrating broad biological effects of TSLP blockade [135].

In addition to the studies of tezepelumab, a multinational proof-of-concept study of CSJ117 in the allergen challenge model with patients with mild, allergic asthma has been performed to evaluate safety, tolerability, pharmacokinetics and pharmacodynamics. Twenty-eight participants completed the study, which comprised daily inhalation of CSJ117 and allergen challenges conducted at 6 and 12 weeks. The study was completed in 2019 with results pending [136].

Further clinical trials to assess the efficacy, mechanisms and long-term safety of tezepelumab are underway. Two pivotal phase 3 studies (NAVIGATOR and SOURCE) are being conducted in patients with severe asthma who are receiving inhaled corticosteroids/long-acting β_2-agonists with or without maintenance oral corticosteroids and additional asthma controllers [137,138]. The primary outcomes are reductions in asthma exacerbation rate and daily oral corticosteroids, respectively. An additional bronchoscopy study (CASCADE) aims to improve understanding of the mechanisms of TSLP blockade by assessing the effects of tezepelumab on the number of inflammatory cells in endobronchial biopsies collected from adults with inadequately controlled, moderate-to-severe asthma [139]. Data regarding long-term safety and tolerability will be important and are currently being addressed in a tezepelumab extension trial (DESTINATION) [140]. Together, these studies will provide much-needed information regarding the benefits of blocking TSLP in asthma.

7. Conclusion

There is substantial evidence that TSLP plays a central role in epithelial-driven inflammation, beginning with the response to environmental insults and leading to multiple innate and

Table 2. Clinical studies of TSLP blockade: completed and ongoing studies in patients with asthma.

Study number	Drug	Estimated start and completion dates	Patient population	Phase	Primary outcome
NCT00972179	Tezepelumab (TSLP mAb)	Completed 2010	49 healthy volunteers	1	Safety of SC and IV doses
NCT00757042	Tezepelumab (TSLP mAb)	Completed 2011	78 healthy volunteers and individuals with moderate-to-severe atopic dermatitis	1	Safety of SC and IV doses
NCT01405963	Tezepelumab (TSLP mAb)	Completed 2013	31 adults with mild allergic asthma	1	Allergen-induced late asthmatic response
NCT01913028	Tezepelumab (TSLP mAb)	Completed 2014	24 adult healthy Japanese men	1	safety
Safety and tolerability of SC doses					
NCT02512900	Tezepelumab (TSLP mAb)	Completed 2016	21 adolescents with mild-to-moderate asthma	1	safety
Pharmacokinetic profile of SC doses					
NCT02054130 (PATHWAY)	Tezepelumab (TSLP mAb)	Completed 2017	584 adults with uncontrolled, moderate-to-severe asthma	2	Annualized asthma exacerbation rate
NCT02237196 (CATNIP)	Tezepelumab (TSLP mAb)	2015–2019	121 adults with moderate-to-severe allergic rhinitis	1/2	Allergen-induced total nasal symptom score
NCT02698501 (UPSTREAM)	Tezepelumab (TSLP mAb)	2016–2019	40 adults with asthma requiring ICS (± LABA)	2	Mannitol PD15
NCT03989544 (PATH-BRIDGE)	Tezepelumab (TSLP mAb)	2019–2019	315 healthy adults	1	Pharmacokinetics of SC administration via accessorized pre-filled syringe or autoinjector compared with vial and syringe
NCT03968978 (PATH-HOME)	Tezepelumab (TSLP mAb)	2019–2020	216 adults and adolescents with severe asthma	3	Successful SC administration via accessorized pre-filled syringe or autoinjector at home versus in the clinic
NCT03347279 (NAVIGATOR)	Tezepelumab (TSLP mAb)	2019–2020	1038 adults and adolescents with severe, uncontrolled asthma taking medium-to-high-dose ICS and at least one additional asthma controller with or without OCS	3	Annualized asthma exacerbation rate
NCT03406078 (SOURCE)	Tezepelumab (TSLP mAb)	2018–2020	150 adults with oral corticosteroid-dependent asthma (Americas, Europe)	3	Reduction in daily OCS dose
NCT03688074 (CASCADE)	Tezepelumab (TSLP mAb)	2018–2020	116 adults with inadequately controlled moderate-to-severe asthma, taking ICS and at least one additional asthma controller	2	Number of airway submucosal inflammatory cells/mm^2 of bronchoscopic biopsies
NCT03706079 (DESTINATION)	Tezepelumab (TSLP mAb)	2019–2022	966 adults and adolescents with severe, uncontrolled asthma	3	Exposure-adjusted incidences of adverse events and serious adverse events
NCT04048343 (NOZOMI)	Tezepelumab (TSLP mAb)	2019–2021	66 Japanese adults and adolescents with inadequately controlled severe asthma	3	Rate of adverse events
NCT03927157 (DIRECTION)	Tezepelumab (TSLP mAb)	2019–2023	396 Chinese adults with severe, uncontrolled asthma taking medium-to-high-dose ICS and at least one additional asthma controller with or without OCS	3	Annualized asthma exacerbation rate
NCT03138811	CSJ117 (TSLP mAb fragment)	2017–2019	28 adults with mild, stable, atopic asthma	1	Allergen-induced late asthmatic response

ICS, inhaled corticosteroids; IV, intravenous; LABA, long-acting β_2-agonist; mAb, monoclonal antibody; OCS, oral corticosteroids; SC, subcutaneous; TSLP, thymic stromal lymphopoietin.

adaptive inflammatory pathways. While it is well established that TSLP drives T2 inflammation following its release from the epithelium, there is growing evidence that TSLP also plays a role in non-T2 processes involving both immune and structural cells. Myriad effects of TSLP have been identified on a variety of cell types including ILC2s, hematopoietic progenitor cells, eosinophils, basophils, mast cells, monocytes/macrophages, dendritic cells, lymphocytes, neutrophils, smooth muscle cells and fibroblasts.

In patients with asthma, TSLP production appears to be dysregulated, being overexpressed compared with healthy individuals and correlating with asthma severity and airway obstruction. Elements of asthma pathophysiology, including airway hyperresponsiveness, mucus overproduction and airway remodeling, are at least partly driven by TSLP via its downstream, pro-inflammatory effects. The position of TSLP at the top of the inflammatory cascade makes it an attractive therapeutic target. Clinical trials of systemic TSLP blockade with tezepelumab in patients with asthma have yielded promising results, including significant reductions in exacerbation rates, improvements in lung function and reductions in multiple biomarkers of inflammation.

8. Expert opinion

Treatment options for asthma have rapidly expanded in recent years with the introduction of biologics developed for specific patient endotypes. The aim of these new medications is to control a specific inflammatory pathway that is dysregulated (i.e. IgE,

IL-5, IL-4/IL-13), and subsequently improve asthma control and prevent exacerbations. By activating a range of downstream inflammatory pathways, TSLP affects disease activity more broadly than a single downstream pathway. As TSLP expression in the airway is abnormally elevated in patients with asthma, TSLP blockade may be considered to have an immunomodulatory function, restoring homeostatic balance to the airways.

The results of clinical trials provide the strongest evidence of the importance of TSLP in driving asthma. Blocking TSLP is the first biological approach that has been shown to produce clinically meaningful reductions in blood eosinophils, circulating IgE, and FeNO. The airway epithelium is the site of a variety of asthma triggers, including viruses, allergens, bacteria and fungi, so targeting this compartment may be effective in treating asthma caused by multiple triggers. TSLP blockade has been shown to be a promising approach for treating both T2-driven and non-T2-driven (i.e. non-allergic, non-eosinophilic) inflammation in asthma when dosed for periods of up to 1 year. There are limited therapeutic options for patients with non-T2-driven inflammation, which is characterized by either neutrophilic or paucigranulocytic airway inflammation. Although the disease mechanisms of these asthma endotypes are not well understood, it is noteworthy that TSLP blockade has been shown to be effective in this population of patients. The potential glucocorticoid-sparing effects of TSLP blockade represents another important area of study.

As with other biologics, it will be important to investigate biomarkers to identify patients who best respond to anti-TSLP therapy. Blood eosinophils, serum IgE and FeNO have been used as biomarkers to guide treatment with anti-IL-5/IL-5Rα, anti-IgE and anti-IL-4/IL-13 monoclonal antibodies in severe asthma. While TSLP itself could hypothetically be used as a biomarker to identify patients with elevated levels, this has not been established largely owing to difficulties in accurately identifying and measuring low concentrations of this cytokine. Furthermore, in nasal polyp tissues, it has been shown that TSLP can be cleaved by endogenous proteases to generate bioactive peptides [141,142], which may contribute to lack of detection by anti-TSLP antibodies in *ex vivo* work and may also result in underestimation of actual TSLP production. The clinical relevance of systemic detection of TSLP is also unclear, as studies examining associations between TSLP expression and disease manifestations have examined airway levels of TSLP. Additionally, the episodic expression of TSLP protein in response to various triggers means that TSLP levels in circulation may not fully reflect the local tissue environment during an asthma exacerbation.

Attempts to quantify TSLP in patient samples are also complicated by the existence of two isoforms of the protein: the full-length protein, often called long-form TSLP (lfTSLP), and a form comprising about half the amino acid length (63-amino-acid acids), often called short-form TSLP (sfTSLP) [143–146]. It is not currently known if the anti-TSLP therapies in clinical development bind to the lfTSLP, sfTSLP, or both. While the role of lfTSLP has been well characterized, the function of sfTSLP remains uncertain. It is believed to be constitutively expressed in human tissues but not in rodents [147,148]. Furthermore, it does not bind to the TSLPR complex [147,149], suggesting a distinct biological function from lfTSLP. The relative ratio of lfTSLP to sfTSLP has not been established in patients with asthma, owing in part to a lack of available research reagents able to discern between the two forms of TSLP. At present, lfTSLP versus sfTSLP can only be distinguished at the mRNA level, by using specific primers. Such studies examining the two isoforms in human tissue have revealed that the long isoform of TSLP is pro-inflammatory and is expressed during inflammation [150], and that the TSLP isoform ratio may be altered during several inflammatory disorders [149]. Further research is needed to improve our understanding of the role of the two isoforms of TSLP, their regulation by SNPs and their expression under different pathological conditions.

Although TSLP is primarily expressed by epithelial cells at barrier surfaces (lung, gut, skin), TSLP can be also be produced by a range of immune cells and can thereby contribute to the pathology of asthma at sites distal to the airways, such as the bone marrow for hematopoiesis. As such, systemic dosing of anti-TSLP, blocking TSLP signaling throughout the body, may be an effective approach; however, it also has the potential to disrupt other homeostatic roles of TSLP [149,151]. The long-term safety and efficacy of anti-TSLP treatment therefore needs to be evaluated, ideally considering not only T2-driven and non-T2-driven inflammation, but also TSLP variants, gene polymorphisms and ethnically diverse populations. Furthermore, inhaled TSLP blockade, directly targeting TSLP produced in the airways, is an interesting alternative route being assessed. Future studies evaluating the effects of inhaled TSLP blockade may help us to understand the relative contribution of airway epithelial cell TSLP production to the pathology of asthma, and whether local delivery to the airways is effective and has relevance for the safety and tolerability of anti-TSLP therapy.

Acknowledgments

Medical writing support for this manuscript, limited to literature searches, editing, summarizing, and figure and table production, was provided by Richard Claes, PhD, of PharmaGenesis London, London, UK, funded by AstraZeneca and Amgen.

Funding

Medical writing support for this manuscript was funded by AstraZeneca and Amgen.

Declaration of interest

GM Gauvreau has received research funding from Amgen, AstraZeneca and Novartis for clinical trials testing anti-TSLP antibodies. R Sehmi has no conflicts of interest to declare regarding studies related to TSLP. CS Ambrose and JM Griffiths are employees of AstraZeneca and own stock and stock options in AstraZeneca. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial relationships or otherwise to disclose.

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torna all'abstract

Epithelial cell–derived cytokines: more than just signaling the alarm

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The epithelial cell–derived cytokines thymic stromal lymphopoietin (TSLP), IL-33, and IL-25 are central regulators of type 2 immunity, which drives a broad array of allergic responses. Often characterized as "alarmins" that are released by the barrier epithelium in response to external insults, these epithelial cell–derived cytokines were initially thought to act only early in allergic inflammation. Indeed, TSLP can condition dendritic cells to initiate type 2 responses, and IL-33 may influence susceptibility to asthma through its role in establishing the immune environment in the perinatal lungs. However, TSLP, IL-33, and IL-25 all regulate a broad spectrum of innate immune cell populations and are particularly potent in eliciting and activating type 2 innate lymphoid cells (ILC2s) that may act throughout allergic inflammation. Recent data suggest that a TSLP/ILC axis may mediate steroid resistance in asthma. Recent identification of memory Th2 cell subsets that are characterized by high receptor expression for TSLP, IL-33, and IL-25 further supports a role for these cytokines in allergic exacerbations. There is therefore growing interest in developing biologics that target TSLP, IL-33, and IL-25. This Review provides an overview of TSLP, IL-33, and IL-25 and the development of blocking antibodies that target these epithelial cell–derived cytokines.

The epithelial lining of the skin, gut, and lungs has long been known as a protective barrier against infection and physical or chemical injury. As the primary organ that senses the external environment, it is now clear that the barrier epithelium also functions as a key sensor and integrator of environmental cues. Allergic diseases encompass a wide breadth of pathological immune responses to otherwise innocuous antigens that are encountered at barrier sites of the body. These responses, called type 2 immune responses, also provide protection against helminth infections. In allergic diseases, type 2 inflammation can drive atopic dermatitis (AD) in the skin; food allergies and eosinophilic esophagitis (EoE) in the gastrointestinal tract; or asthma, allergic rhinitis, and chronic rhinosinusitis within the respiratory system. The prototypical type 2 response is characterized by induction of Th2 cells; B cell production of IgE; activation of specific innate cell populations such as type 2 innate lymphoid cells (ILC2s), eosinophils, mast cells, and basophils; and production of type 2 cytokines such as IL-4, IL-5, IL-9, and IL-13 by innate and adaptive immune cells. The itch response, mucus production, and bronchoconstriction may also be components of the type 2 allergic response.

Regulatory T cells (Tregs), which are important in maintaining immune homeostasis, also regulate type 2 immunity at barrier surfaces. Mice that lacked the CNS1 gene regulatory region at the *FOXP3* locus, which is required for peripheral induction of Tregs, spontaneously developed type 2 inflammation within the gastrointestinal tract and lungs (1). Mice whose Tregs lacked expression of the transcription factor RORα exhibited exaggerated type 2 skin inflammation in models of AD (2). This exaggerated inflammation in RORα-deficient Tregs may have been in part due to decreased expression of death receptor 3 (DR3; also known as TNF receptor superfamily member 25, or TNFRSF25) on Tregs. DR3 on Tregs can bind the ligand TL1A (also known as TNF superfamily member 15, or TNFSF15) and may sequester TL1A to restrain TL1A-driven inflammation by Th2 cells and ILC2s. Additional data also suggest that Tregs can regulate ILC2 function through ICOS-ICOSL interactions and production of IL-10 and TGF-β (3).

In epithelial regulation of allergic type 2 responses, three cytokines — thymic stromal lymphopoietin (TSLP), IL-33, and IL-25 — have emerged as critical mediators of type 2 inflammation. These cytokines alert the immune system to external insults and regulate tissue restoration and repair after injury. While our understanding of how these cytokines function initially focused on their roles early in type 2 responses, emerging data suggest that these three cytokines provide important tissue-specific signals to both innate and adaptive cell populations throughout type 2 inflammation. TSLP, IL-33, and IL-25 may therefore be important mediators of inflammation during allergic disease exacerbations and may prove to be key targets for therapeutic intervention even after disease is well established. This Review provides an overview of the regulation and function of TSLP, IL-33, and IL-25. We also discuss the current status of the development of treatments that target TSLP, IL-33, or IL-25.

TSLP

TSLP is a member of the IL-2 family of cytokines that was initially identified as a pre–B cell growth factor (4). Epithelial cells in the lungs, skin, and gastrointestinal tract are thought to be the primary source of TSLP during both homeostatic and inflammatory condi-

Conflict of interest: The authors have declared that no conflict of interest exists.

Reference information: *J Clin Invest*. 2019;129(4):1441–1451.
https://doi.org/10.1172/JCI124606.

tions, although dendritic cells (DCs), basophils, and mast cells can also express TSLP (5–9). TSLP expression and release from epithelial cells is increased in response to a broad array of stimuli, including mechanical injury, infection, inflammatory cytokines, and proteases such as trypsin and papain (6, 10, 11). Two main isoforms of TSLP have been described in mice, but the functional consequence of these variants is unknown. In humans, a short isoform appears to be expressed in basal conditions, whereas a longer isoform is induced by inflammatory stimuli (12). Cleavage of human TSLP by serine proteases may also regulate TSLP protein levels or function, although it is unclear whether a similar regulatory mechanism exists in mice (13, 14). TSLP genetic variants and high levels of TSLP expression have been linked to atopic diseases such as AD, asthma, allergic rhinoconjunctivitis, and EoE (15). TSLP overexpression has also been reported in Netherton syndrome, a genetic disease caused by mutations in SPINK5 that manifests in type 2 inflammation at multiple sites (16), and in some nonatopic pulmonary diseases such as chronic obstructive pulmonary disease (9).

TSLP is a distant paralog of IL-7 and shares a common receptor subunit, IL-7Rα, with IL-7. TSLP binds the TSLP receptor (TSLPR) that is coupled with IL-7Rα to activate downstream pathways (17); TSLP-mediated signaling has been studied primarily in DCs and T lymphocytes, in which signaling occurred primarily through JAK/STAT pathways (18–20). A number of non-hematopoietic cell populations have been shown to express TSLPR and to be responsive to TSLP. Although the implications in allergic inflammation are not known, the barrier epithelium can respond to TSLP, and TSLP mediated recovery from colonic inflammation in a mouse model of colitis by inducing intestinal epithelial production of secretory leukocyte peptidase inhibitor (SLPI) (21). A growing body of literature also suggests that TSLP can activate a subset of sensory neurons to drive the itch response in allergic diseases such as AD (22, 23).

TSLPR is broadly expressed within hematopoietic cell populations (24). The highest expression is seen on specific myeloid DC populations (25–27), which have been shown to be important TSLP-responsive populations in both humans and mice. TSLP-stimulated DCs upregulated the costimulatory molecules CD40, CD80, CD86, and OX40L (28, 29). When cocultured with TSLP-conditioned DCs, naive syngeneic T cells proliferated but did not differentiate; naive allogeneic T cells cocultured with TSLP-conditioned DCs acquired an inflammatory Th2-like phenotype with production of IL-4, IL-5, IL-13, and TNF-α but not IL-10 (30). TSLP-conditioned DCs could also support the maintenance of Th2 effector memory cells and promotion of IgA2 class switching in the intestines (31, 32). The actions of TSLP directly on T cells can also promote type 2 responses. TSLP signaling on naive T cells in the presence of TCR stimulation promoted proliferation and Th2 differentiation through induction of IL-4 gene transcription (33–35). Recent data demonstrated that TSLP could directly promote Th2 differentiation and type 2 cytokine expression from naive T cells in vitro, even in the absence of IL-4 (18). In vivo, in an OVA/alum immunization model using antigen-specific T cells, it was noted that T cells lacking TSLPR acquired an effector phenotype after immunization but were defective in the ability to generate Th2 memory (36). In a variety of models of allergic disease, TSLP can regulate induction of Th2 cells and Th9 cells, likely through its effects on DCs and T cells (28, 29, 33–35, 37–40). TSLP can also act directly on Tregs in the skin and has been implicated in regulating the generation of Tregs in the thymus and microbiota-driven expansion and maintenance of Helios-negative Tregs in the gut (41–43). The impact of TSLP regulation of Tregs in allergic inflammation remains unclear.

In addition to DCs, basophils and innate lymphoid cells (ILCs) have also emerged as important innate effector cell populations downstream of TSLP. In mouse models, a TSLP/basophil axis has been shown to be important in experimental EoE and food allergy (44–46), and TSLP drove basophil hematopoiesis independent of IL-3 (47). In some models, TSLP-driven allergic inflammation was mediated by ILCs (48, 49). Given the importance of respiratory virus infections in driving asthma exacerbations, it is interesting to note that respiratory viruses can induce TSLP expression, and type I interferons induced during the antiviral response can play a counterregulatory role by modulating ILC2 activity (50–52). Several recent publications have now suggested that a TSLP/ILC axis may play a pivotal role in steroid-resistant allergic airway inflammation. TSLP signaling induced expression of the antiapoptotic protein BCL-XL in ILC2s and prevented corticosteroid-induced apoptosis of ILC2s in vitro (53). In vivo, TSLP signaling was not required to drive inflammation following OVA/IL-33 administration, but lack of TSLP signaling greatly enhanced the ability of dexamethasone to suppress inflammation in this model of allergic lung inflammation (53). Data from human subjects suggest a similar and important role for a TSLP/ILC axis, since TSLP could also mediate resistance to corticosteroids in ILC2s from human PBMCs and bronchoalveolar lavage (BAL) fluid. Furthermore, TSLP levels in the BAL fluid from asthmatic patients were inversely correlated with dexamethasone-mediated inhibition of IL-5 production from BAL fluid ILC2s (54). Since steroid therapy is a cornerstone for many allergic and inflammatory diseases, further study of the TSLP/ILC axis is certainly warranted to determine whether similar mechanisms regulate inflammation at other tissue sites.

IL-33

IL-33 is an IL-1 family cytokine that may exert a broad spectrum of effects extending from early immune development to atopic disease exacerbations. IL-33 was initially named "nuclear factor in high endothelial venules" (NF-HEV) based on its high expression in the nucleus of HEVs (55). The link between IL-33 and type 2 immune responses was established when IL-33 was identified as the ligand for suppression of tumorigenicity 2 (ST2; sometimes referred to as IL-1RL1, T1, or IL-33R) (56), which had been characterized previously as an orphan receptor important in type 2 responses in the lungs (57, 58). Genetic studies have reproducibly demonstrated significant associations between *IL33* and *IL1RL1* genetic variants and asthma in humans (59–66). Genetic variants in *IL1RL1* are also associated with AD risk (67), and genetic variants in the *IL33* and *IL1RL1* loci are associated with EoE risk (68, 69).

Epithelial cells at barrier surfaces and endothelial cells, both of which express IL-33 constitutively in the nucleus, are thought to be the primary sources of IL-33 during homeostatic and inflammatory conditions (67, 70, 71). A variety of other hematopoietic and non-hematopoietic cell types have also been reported to express IL-33 under basal conditions or after treatment with inflammatory

stimuli (67, 70, 72–79). In specific contexts, cell types other than epithelial and endothelial cells may serve as important sources for IL-33. For example, mast cell–derived IL-33 may be important in experimental autoimmune encephalitis and in intestinal helminth infections (80, 81).

Like the activity of other IL-1 family cytokines, the activity of IL-33 is regulated both by its localization within the cell and by proteolytic cleavage. IL-33 contains an N-terminal chromatin-binding motif and a predicted nuclear localization sequence. Although some studies suggest a role for IL-33 in transcriptional regulation (82), the intranuclear localization of IL-33 in most cell types is thought to be important in sequestering this cytokine to prevent inappropriate release (83). Transgenic mice that expressed a form of IL-33 that lacked the nuclear localization signal died of systemic inflammation (84). Although IL-33 lacks a signal sequence required for conventional secretory pathways, it can be released as an "alarmin" in response to cellular injury or stress (85, 86). Full-length IL-33 appears to be biologically active, but proteolytic cleavage of IL-33 at various sites can modulate its activity. Mast cell and neutrophil proteases can cleave and further activate IL-33 (87–89). Certain allergens also contain proteases that can cleave and further activate IL-33 (90). In contrast, cleavage of IL-33 by caspase-1, -3, or -7 or oxidation of IL-33 results in inactivation (91, 92). Splice variants of IL-33 also exist, although how these different isoforms differ in activity and regulation is not fully understood (93–95).

IL-33 binds a heteromeric receptor consisting of ST2 and its coreceptor IL-1 receptor accessory protein (IL-1RAcP). Formation of the IL-33/ST2/IL-1RAcP complex results in recruitment of MyD88 and IL-1R–associated kinase (IRAK) to activate a variety of downstream signaling pathways (96). A soluble variant of ST2 (sST2) that lacks the transmembrane domain appears to function as a decoy receptor to negatively regulate IL-33/ST2 signaling (97, 98). ST2 is constitutively expressed on several immune cell types, including mast cells, ILC2s, Th2 cells, and a subset of Tregs, and can be induced on many other immune subsets; as a consequence, IL-33 can also directly activate and induce cytokine production from a broad number of cell types (79, 99–109). Recently, leukotrienes and IL-33 have been shown to act together in a signaling circuit that may be an important amplifier of inflammation in allergic disease exacerbations. Signaling through the leukotriene receptor CysLT2R on alveolar cells drove the production of IL-33, which acted on T cells to upregulate T cell expression of the leukotriene receptor CysLT1R (110, 111). Some non-hematopoietic cell types are also IL-33–responsive. ST2 expressed on human airway epithelium may mediate inflammatory cytokine production from the bronchial epithelium (112, 113); and, like TSLP, IL-33 has also been shown to mediate the itch response through activation of sensory neurons (114).

IL-33 is a particularly potent activator of ILC2s, which produce type 2 cytokines such as IL-13 and IL-5 and upregulate surface OX40L and PD-L1 in response to IL-33 (107, 115–118). In mice, systemic IL-33 also mobilized ILC2 precursors from the bone marrow (119). In a papain-driven model of allergic lung inflammation, IL-33–mediated activation of ILC2s was important in inducing Th2 cells in the draining lymph nodes and in promoting memory Th2 responses in the lungs (120, 121). Memory Th2 cells, which express ST2 at higher levels than effector Th2 cells, are another important effector cell type responsive to IL-33 (122, 123). In vivo, after house dust mite (HDM) exposure, IL-33 signaling on memory Th2 cells induced amphiregulin production, which then drove osteopontin production by eosinophils (123, 124). It is interesting to note that EGFR, the receptor for amphiregulin, can form a complex with ST2, and EGFR was required for IL-33–induced IL-13 production during helminth infections in mice (125). In helminth infection, an $ST2^+$ subset of memory Th2 cells was required to drive production of major basic protein (MBP) by eosinophils after infection (123, 124). While ILC2 and pathogenic memory Th2 cell populations both express high levels of ST2 and share transcriptional and epigenetic profiles (126), Th2 cells but not ILC2s express DUSP10, a phosphatase that can negatively regulate IL-33–mediated cytokine production (127). It is now also clear that IL-33 can promote the induction and function of Tregs in a variety of settings (103, 128–130). The implications of this role for IL-33 in suppressing inflammation in allergic disease are not fully understood, though IL-33 was shown to negatively regulate allergic inflammation by inducing Tregs via an IL-33/mast cell/IL-2 axis (131).

In the developing lungs in mice, IL-33 has been shown to have an important role in establishing the pulmonary immune environment that influences the risk and development of allergic lung inflammation later in life. These studies have shown direct links between perturbations in IL-33/ST2 signaling in the perinatal period and subsequent type 2 responses to allergen. Following a perinatal increase in IL-33 expression in the lungs, pulmonary ILC2 frequencies increased (132–134). ILC2s mediated eosinophil accumulation in the lungs during the neonatal period and were the primary source of IL-13 in the neonatal lungs that drove the phenotypic polarization of pulmonary macrophages. Tissue insults such as hyperoxia that occur during the perinatal period can increase IL-33 expression and mobilize ILC2s, leading to increased susceptibility to asthma later in life (135). IL-33 also drove a lung-specific transcriptional program in basophils in the developing lungs, since the gene expression profile of lung basophils from ST2-deficient neonatal mice was more similar to that of wild-type circulating basophils than to that of wild-type lung basophils (136). Thus, IL-33 signaling impacts all stages of allergy starting even from the establishment of the immune environment in the perinatal lungs.

IL-25

IL-25 (sometimes referred to as IL-17E) is a member of the IL-17 cytokine family, although the functions of IL-25 have been shown to be quite distinct from those of other IL-17 cytokine family members given IL-25's ability to amplify type 2 inflammation at multiple tissue sites (137–140). Blockade of IL-25 signaling can attenuate allergic inflammation in a variety of mouse models (141–143). Although initial reports described IL-25 as a Th2 cell–derived cytokine (137), epithelial cells, alveolar macrophages, mast cells, basophils, and eosinophils have now also been reported as potential sources of IL-25 (141, 144–148). IL-25 was constitutively expressed by epithelial cells of the skin and lungs in subjects with asthma or atopic disease, and expression of IL-25 was higher in the skin and lungs of subjects with asthma and atopic disease than in the skin and lungs of control subjects (147, 148). In subjects with chronic

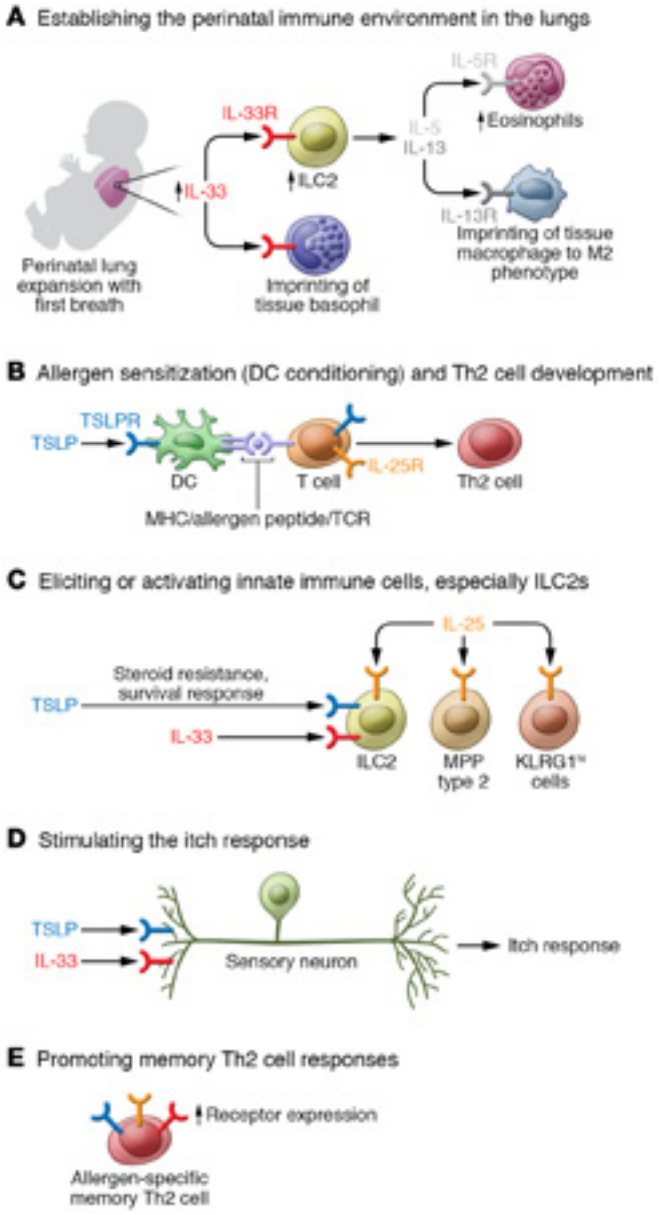

Figure 1. TSLP, IL-33, and IL-25 regulate a diversity of responses in type 2 immunity. (**A**) IL-33 release in the lungs at birth helps establish the pulmonary immune environment, which can influence asthma risk and development later in life. (**B**) TSLP acts directly on DCs to drive Th2 cell development; IL-25, along with IL-4, can also drive Th2 cell differentiation. (**C**) TSLP, IL-33, and IL-25 act on a broad array of innate immune cells and are particularly important in eliciting and activating ILC2s; IL-25 can also elicit MPP type 2 cells and IL-17^{+} KLRG1^{hi} cells. (**D**) TSLP and IL-33 can act on sensory neurons to stimulate the itch response. (**E**) TSLP, IL-33, and IL-25 can promote adaptive type 2 responses through subsets of memory Th2 cells that are characterized by high receptor expression for TSLP, IL-33, and IL-25.

rhinosinusitis, solitary chemosensory cells (SCCs) appeared to be the primary source of IL-25 within the sinonasal epithelium, and SCCs were expanded in nasal polyp tissue compared with adjacent turbinate epithelium (149). Within the lungs and intestines in mice, IL-25 expression was restricted to tuft cells, a rare type of chemosensory epithelial cell (150–152). IL-25 expression by tuft cells was further induced by succinate or helminth-derived products and mediated intestinal epithelial remodeling in response to colonizing protozoa, which protected against helminth infections (150, 153, 154). Few data exist on whether IL-25 expression and function are regulated by splicing or proteolytic cleavage; however, IL-25 has been reported to be a substrate for cleavage by matrix metalloproteinase 7 (MMP-7) (155).

IL-25 binds IL-17RB, and along with IL-17RA recruits the adapter protein Akt1 to activate downstream signaling pathways (138, 156–159). Cellular targets of IL-25 include T cells, ILC2s, specific myeloid populations, and invariant NKT cells, as well as non-hematopoietic cell populations such as fibroblasts, epithelial cells, endothelial cells, and mesenchymal cells (118, 137, 146, 147, 160–166). An IL-25–responsive, steroid-resistant myeloid population was a critical mediator of disease in a model of cockroach allergen–driven chronic allergic lung inflammation (167). In addition to acting on ILC2s, which are activated or elicited by TSLP and IL-33, IL-25 may also induce some functionally and phenotypically distinct ILC populations: multipotent progenitor (MPP) type 2 cells and IL-17–producing KLRG1^{hi} cells (162, 165). A subset of NKT cells has also been shown to produce type 2 cytokines in response to IL-25 and could drive airway hyperresponsiveness in an OVA/alum model of allergic airway inflammation (164). As with TSLP and IL-33, T cells also appear to be important target cells in IL-25–mediated inflammation. Ex vivo analyses of human peripheral blood demonstrated high expression of IL-17RB transcript and protein in memory Th2 cells that was greatly enhanced by coculture with TSLP-conditioned DCs (147). Augmentation of Th2 differentiation and function by IL-25 appeared to be dependent on IL-4, since naive T cells lacking IL-4 or antibody blockade of IL-4 abrogated the ability of IL-25 to induce Th2 differentiation in vitro (146). IL-25 does not appear to drive Th9 differentiation, but Th9 cells expressed IL-17RB and increased IL-9 production in response to IL-25 (168). Under homeostatic conditions, IL-25 can play an important role in limiting IL-17 expression within the gut. Intestinal commensal microbiota drove expression of IL-25 from the epithelium, which limited IL-23 expression and Th17 cell expansion in the large intestines and limited IL-22 production from RORγt^{+} ILCs (ILC3s) in the small intestines (169, 170). Although the implications in allergy are unclear, recent data also demonstrated an important role for IL-25 in driving keratinocyte proliferation and skin inflammation in an IL-17–dependent imiquimod-induced psoriasis model (171). Additional data linking IL-25 to Th17-type responses come from a hapten-mediated model of contact hypersensitivity (CHS). CHS mediated by transferred Th2 cells was comparable in wild-type and IL-25–deficient mice, yet transferred Th17 cells could drive inflammation in wild-type but not IL-25–deficient mice (172).

TSLP, IL-33, and IL-25: interplay and tissue-specific roles

Although TSLP, IL-33, and IL-25 can all promote type 2 inflammation through their effects on a broad array of cell populations (Figure 1 and Table 1), the downstream effector profiles can be distinct in response to these three cytokines (173), and cells may express different levels of receptors for TSLP, IL-33, or IL-25 in a tissue-dependent manner (174). Studies in mouse models have demonstrated

Table 1. Cellular sources and targets of TSLP, IL-33, and IL-25

	TSLP	IL-33	IL-25
Sources			
Non-hematopoietic	Epithelial cells	Epithelial cells	Epithelial cells (esp. tuft cells, solitary chemosensory cells)
	Stromal cells	Endothelial cells	
		Fibroblastic reticular cells, fibroblasts, fibroblast-like cells, myofibroblasts	
		Adipocytes	
		Smooth muscle cells	
		Glial cells	
		Hepatocytes	
Hematopoietic	Dendritic cells	Various myeloid cell types	Alveolar macrophages
	Mast cells	Mast cells	Mast cells
	Basophils	Platelets, megakaryocytes	Basophils
			Eosinophils
			Th2 cells
Targets			
Non-hematopoietic	Epithelial cells	Epithelial cells	Epithelial cells
	Sensory neurons	Endothelial cells	Endothelial cells
		Stromal cells, fibroblasts	Fibroblasts
		Sensory neurons	Mesenchymal cells
		Glial cells	
		Cardiomyocytes	
Hematopoietic	Type 2 ILCs (ILC2s)	Type 2 ILCs (ILC2s)	Type 2 ILCs
	$CD4^+$ T cells	$CD4^+$ T cells	(ILC2s, MPP type 2, IL-17^+KLRG1^{hi})
	(Th2, Th9, naive T cells)	(Th2, Th1, Th17)	$CD4^+$ T cells (Th2, Th9, naive T cells)
	Tregs	Tregs	iNKT cells
	$CD8^+$ T cells	$CD8^+$ T cells	Specific myeloid populations
	NKT cells	NK cells	
	B cells	iNKT cells	
	Mast cells	B cells	
	Basophils	Mast cells	
	Eosinophils	Basophils	
	Dendritic cells	Eosinophils	
	Monocytes, macrophages	Dendritic cells	
		Macrophages	
		Neutrophils	

differential requirements for TSLP, IL-33, and IL-25. In some models, the allergen dose or mouse genetic background can influence the requirements for these epithelial cell–derived cytokines. At low doses of HDM, blockade of either IL-33 or GM-CSF but not TSLP attenuated HDM-driven allergic lung inflammation, but inflammation driven by high-dose HDM was attenuated in the absence of TSLP signaling (175). C57BL/6 mice that lacked TSLP signaling had greatly attenuated skin inflammation after topical application of the vitamin D analog MC903 (48); yet, in BALB/c mice, IL-25 may play a more important role than TSLP, since MC903-driven inflammation was decreased to a greater extent in mice that lacked the IL-25 receptor IL-17RB than in mice that lacked either TSLPR or ST2 (117). Some studies suggest a tissue-specific role for TSLP, IL-33, and IL-25, as mice lacking TSLP, IL-33, and IL-25 signaling had impaired type 2 immune responses in tissue in helminth infection or after HDM challenge despite the fact that lymph node priming of adaptive type 2 immunity remained intact (126).

The recent identification of subsets of memory Th2 cells in humans and mice that are characterized by high expression of receptors for TSLP, IL-33, and IL-25 supports a role for these three cytokines in regulating adaptive immune responses in allergy. These Th2 subpopulations are enriched at affected sites in EoE and AD and constitute a higher frequency of circulating Th2 cells in subjects with seasonal allergies than in control subjects (176, 177). It is interesting to speculate whether the increased responsiveness of these memory Th2 cells to TSLP, IL-33, and IL-25 may be important in mediating the allergic phenotype, since the mere presence or absence of allergen-specific T cells does not appear to distinguish allergic from nonallergic subjects (178, 179). Additional research is required to assess whether each of these epithelial cell–derived cytokines is uniformly required across a spectrum of human allergic diseases or whether patterns of expression of these cytokines may distinguish distinct allergic endotypes or phenotypes.

Other epithelial cytokines

While TSLP, IL-33, and IL-25 have been highlighted as important epithelial cell–derived cytokines in allergy because of their potent ability to drive type 2 responses, it is important to note that numerous other cytokines produced by the barrier epithelium also have key roles in regulating allergic diseases. A growing body of literature implicates granulocyte-macrophage colony-stimulating factor (GM-CSF) in the regulation of allergic responses. GM-CSF was able to serve as an adjuvant to drive type 2 lung inflammation in response to low-dose HDM or OVA alone (180, 181), and blockade or loss of GM-CSF signaling attenuated allergic inflammation in a variety of mouse models (175, 182–185). Studies examining the role of GM-CSF in allergic lung inflammation suggest a primary role for GM-CSF during allergic sensitization (184). IL-1α may also be important during sensitization but not challenge since neutralization of IL-1α but not IL-1β during sensitization reduced type 2 inflammation in an HDM-driven asthma model, whereas inflammation was not affected by blockade of IL-1α or IL-1β during the challenge phase in this model (175). In fact, TLR4-induced IL-1α from bronchial epithelial cells provided an important autocrine signal for GM-CSF and IL-33 release, suggesting that IL-1α may be one of the earliest triggers of type 2 immunity in the lungs. Studies of AD also suggest that keratinocyte release of IL-1α can drive chronic skin inflammation (186).

Table 2. Clinical trials of drugs targeting TSLP or IL-33/ST2[A]

Study title	Clinical trial identifier	Stage	Drug	Condition/disease
Anti-TSLP clinical trials				
Safety Study of AMG 157 in Healthy Subjects	NCT00972179	Phase I	Tezepelumab	Healthy
A Phase 1 Study to Evaluate the Safety, Tolerability, Pharmacokinetics and Immunogenicity of MEDI9929 After Single Administration in Healthy Male Japanese Subjects	NCT01913028	Phase I	Tezepelumab	Healthy
A Study to Evaluate the Pharmacokinetics of MEDI9929 (AMG 157) in Adolescents with Mild to Moderate Asthma	NCT02512900	Phase I	Tezepelumab	Asthma
Double-blind, Multiple Dose Study in Subjects with Mild Atopic Asthma	NCT01405963	Phase I	Tezepelumab	Asthma
Safety Study of AMG 157 in Healthy Subjects and Subjects with Atopic Dermatitis	NCT00757042	Phase I	Tezepelumab	Healthy, atopic dermatitis
Anti-TSLP (AMG 157) Plus Antigen-Specific Immunotherapy for Induction of Tolerance in Individuals with Cat Allergy	NCT02237196	Phase I/phase II	Tezepelumab	Cat allergy/ hypersensitivity
Study to Evaluate the Efficacy and Safety of MEDI9929 (AMG 157) in Adult Subjects with Inadequately Controlled, Severe Asthma	NCT02054130	Phase II	Tezepelumab	Asthma (191)
Effects of Anti-TSLP in Patients with Asthma (UPSTREAM)	NCT02698501	Phase II	Tezepelumab	Asthma
Study to Evaluate Tezepelumab on Airway Inflammation in Adults with Uncontrolled Asthma (CASCADE)	NCT03688074	Phase II	Tezepelumab	Asthma
Phase 2a Study to Evaluate the Efficacy and Safety of MEDI9929 in Adults with Atopic Dermatitis (ALLEVIAD)	NCT02525094	Phase II	Tezepelumab	Atopic dermatitis (194)
Study to Evaluate Tezepelumab in Adults and Adolescents with Severe Uncontrolled Asthma (NAVIGATOR)	NCT03347279	Phase III	Tezepelumab	Asthma
Extension Study to Evaluate the Safety and Tolerability of Tezepelumab in Adults and Adolescents with Severe, Uncontrolled Asthma	NCT03706079	Phase III	Tezepelumab	Asthma
Study to Evaluate the Efficacy and Safety of Tezepelumab in Reducing Oral Corticosteroid Use in Adults with Oral Corticosteroid Dependent Asthma	NCT03406078	Phase III	Tezepelumab	Asthma
Anti–IL-33/ST2 clinical trials				
Proof of Concept Study to Investigate ANB020 Activity in Adult Patients with Severe Eosinophilic Asthma	NCT03469934	Phase II	Etokimab	Asthma
A Study Investigating the Efficacy, Safety, and PK Profile of ANB020 Administered to Adult Subjects with Moderate-to-Severe AD	NCT03533751	Phase II	Etokimab	Atopic dermatitis
Placebo-Controlled Study to Investigate ANB020 Activity in Adult Patients with Peanut Allergy	NCT02920021	Phase II	Etokimab	Peanut allergy
Etokimab in Adult Patients with Chronic Rhinosinusitis with Nasal Polyps (CRSwNP)	NCT03614923	Phase II	Etokimab	Chronic rhinosinusitis with nasal polyps
A First-in-Human, Double Blind, Single Dose Study in Healthy Subjects and Subjects With Mild Atopic Asthma	NCT01928368	Phase I	AMG 282	Asthma
A Study to Evaluate the Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of AMG 282 in Healthy Subjects and Subjects With Chronic Rhinosinusitis with Nasal Polyps	NCT02170337	Phase I	AMG 282	Chronic rhinosinusitis with nasal polyps
Safety and Tolerability of MEDI3506 in Healthy Subjects, in Subjects With COPD and Healthy Japanese Subjects	NCT03096795	Phase I	MEDI3506	Chronic obstructive pulmonary disease
Proof-of-Concept Study to Assess the Efficacy, Safety and Tolerability of SAR440340 (Anti-IL-33 mAb) in Patients With Moderate-to-Severe Chronic Obstructive Pulmonary Disease (COPD)	NCT03546907	Phase II	SAR440340	Chronic obstructive pulmonary disease
Efficacy and Safety Study of GSK3772847 in Subjects With Moderately Severe Asthma	NCT03207243	Phase II	GSK3772847	Asthma
Repeat Dose Study of GSK3772847 in Participants With Moderate to Severe Asthma With Allergic Fungal Airway Disease (AFAD)	NCT03393806	Phase II	GSK3772847	Asthma with allergic fungal airway disease
Anti-ST2 (MSTT1041A) in COPD (COPD-ST2OP)	NCT03615040	Phase II	MSTT1041A	Chronic obstructive pulmonary disease

[A]From ClinicalTrials.gov, accessed December 5, 2018. Includes trials that are completed or recruiting.

Studies have also highlighted a potential role for TGF-β, and, in particular, epithelial cell–derived TGF-β, in the pathogenesis of asthma. A critical role for TGF-β in mediating tolerance through Treg induction has been well established; thus, some studies have investigated TGF-β in allergy as a potential mode of therapy (187, 188). However, loss of TGF-β expression specifically from the bronchial epithelium reduced ILC2 accumulation and IL-13 production in the lungs following HDM administration (189). The association of SNPs in the promoter and coding regions of the *TGFB1* gene with asthma susceptibility and degree of atopy further supports an important role for TGF-β in allergic disease (190). Given the opposing roles that have been described for

TGF-β in suppressing or promoting allergic inflammation, further study is needed to better understand how TGF-β regulates type 2 immunity at barrier tissue sites.

Development of biologics against epithelial cytokines

Although biological therapies directed against IgE and some effector cytokines have been developed, studies in mouse models suggest that epithelial cell–derived cytokines such as TSLP, IL-33, and IL-25 may regulate allergic responses more broadly and through more diverse pathways than IgE or type 2 effector cytokines such as IL-4, IL-13, or IL-5. There is therefore growing interest in developing therapeutics to target TSLP, IL-33, and IL-25. Antibodies directed against IL-25 are under development but have not yet entered clinical trials. One antibody directed against TSLP and several antibodies that block IL-33/ST2 signaling are under development (Table 2). Therapeutics directed against TSLP and IL-33 have not yet been approved by the FDA, but several trials that have been conducted suggest that these antibodies may be both safe and effective in a variety of atopic diseases.

TSLP signaling blockade. Tezepelumab (AMG 157/MEDI9929) is a human IgG2 monoclonal antibody that binds human TSLP and prevents the binding of TSLP with TSLPR. Tezepelumab was effective in reducing rates of asthma exacerbations in patients with moderate to severe disease requiring long-acting β-agonists and medium to high doses of inhaled glucocorticoids (191). A study of responses to allergen challenge in patients with mild allergic asthma suggested that tezepelumab affects both early and late asthmatic responses to allergen exposure (192). Treatment with tezepelumab did not affect total IgE levels in patients with mild asthma but did decrease blood eosinophil counts compared with those of placebo-treated control subjects. Allergen-induced bronchoconstriction after allergen challenge was also attenuated in tezepelumab-treated patients compared with controls. Studies are currently ongoing to evaluate the efficacy of tezepelumab as adjunctive therapy to immunotherapy in inducing long-term tolerance to cat allergen (193). ALLEVIAD, a phase IIa study of safety and efficacy of tezepelumab in adults with AD, has also been completed, and although higher numbers of subjects treated with tezepelumab reached an improvement of 50% or more in the Eczema Area Severity Index (EASI-50), the study did not reach the level of significance in this primary endpoint (194). It will be particularly interesting to establish whether blockade of TSLP may be effective in steroid-resistant asthma given recent studies suggesting a central role for TSLP and ILCs in steroid resistance in mouse models of allergic inflammation.

IL-33 signaling blockade. Etokimab (ANB020) is a humanized anti–IL-33 IgG1 antibody generated by AnaptysBio that is being evaluated in a number of studies in the treatment of AD, eosinophilic asthma, peanut allergy, and chronic rhinosinusitis with nasal polyps (195). In vitro analyses demonstrated high-affinity binding to IL-33 and inhibition of IL-33 activity on primary human cells. Phase I and phase IIa trials of etokimab have been completed. Etokimab demonstrated a favorable safety profile, and a single dose of etokimab suppressed IL-33 function for 85 days based on ex vivo assays. In adult subjects with AD that was inadequately controlled with topical corticosteroids, single dosing was also able to achieve an improvement of 50% or more in the EASI-50 eczema grading index (196). Interim analyses of a phase IIa study of etokimab in adults with severe eosinophilic asthma demonstrated improvements in forced expiratory volume (FEV1) at day 2 in etokimab-treated patients over patients receiving placebo, and differences in FEV1 measurements between etokimab-treated and placebo-treated patients remained significant at day 64 (197). Improvements in lung function also correlated with reductions in blood eosinophil numbers. Positive responses have also been reported in interim analyses of a phase IIa trial of etokimab in adult peanut allergy patients with a clinical history of anaphylaxis (198).

Conclusions

Substantial progress has been made in understanding the mechanisms that underlie the development and progression of allergic diseases. The regulation of barrier tissue immune homeostasis by TSLP, IL-33, and IL-25 affects susceptibility to allergic disease development but also modulates the function of cell populations such as memory Th2 cells and ILCs that drive allergic disease exacerbations. Thus, biologics directed against these epithelial cell–derived cytokines may be effective across a broad spectrum of allergic diseases. Antibodies against IL-25 are still in the early stages of development. The anti-TSLP antibody tezepelumab and the anti–IL-33 antibody etokimab have now shown promising results in a variety of allergic diseases. Ongoing and future studies will help establish whether biologics targeting TSLP, IL-33, or IL-25 can offer safe, effective, and steroid-sparing treatments for allergy.

Acknowledgments

We thank Kathryn Pothoven for helpful discussions. Support for this work was provided by NIH grants R01AI068731, U19AI125378, and R01AI124220 to SFZ.

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HHS Public Access

Author manuscript

Clin Exp Allergy. Author manuscript; available in PMC 2016 April 01.

Published in final edited form as:

Clin Exp Allergy. 2015 April ; 45(4): 706–719. doi:10.1111/cea.12506.

Mechanisms of Airway Hyperresponsiveness in Asthma: The Past, Present and Yet to Come

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Abstract

Airway hyperresponsiveness (AHR) has long been considered a cardinal feature of asthma. The development of the measurement of AHR forty years ago initiated many important contributions to our understanding of asthma and other airway diseases. However, our understanding of AHR in asthma remains complicated by the multitude of potential underlying mechanisms which in reality are likely to have different contributions amongst individual patients. Therefore the present review will discuss the current state of understanding of the major mechanisms proposed to contribute to AHR and highlight the way in which AHR testing is beginning to highlight distinct abnormalities associated with clinically relevant patient populations. In doing so we aim to provide a foundation by which future research can begin to ascribe certain mechanisms to specific patterns of bronchoconstriction and subsequently match phenotypes of bronchoconstriction with clinical phenotypes. We believe that this approach is not only within our grasp but will lead to improved mechanistic understanding of asthma phenotypes and hopefully better inform the development of phenotype-targeted therapy.

1. Introduction

Airway hyperresponsiveness (AHR) is defined as the predisposition of the airways of patients to narrow excessively in response to stimuli that would produce little or no effect in healthy subjects (Figure 1). Cockcroft et al (1) are largely credited with popularising the non-specific test of AHR almost forty years ago; however, the abnormal responses of asthmatics to non-specific stimuli were first described by Tiffeneau and Beauvallet in 1945 (2) and later developed during the 1960s in both Europe (3) and the United States (4). AHR has long been considered a cardinal feature of asthma and its measurement has provided profound insights into the underlying pathophysiology of the disease. Our view of AHR has greatly matured, and because of recent findings it is important to re-assess the current knowledge of AHR particularly in our understanding of the underlying mechanisms. While we did not embark upon a systematical evaluation of all literature regarding mechanisms of

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AHR in asthma we have endeavoured to provide an extensive update on the most recent findings. We will first introduce a few key clinical studies to provide a foundation for discussing the way in which future research of the mechanisms of AHR may contribute significantly to clinical practice.

But let's start with a pair of case studies. Both authors have at one time responded positively to bronchial challenge. One (DC) had AHR to methacholine despite being concurrently negative to mannitol challenge. Following treatment, he is now in "AHR remission". The other (CI) responded positively in the past to exercise, methacholine and histamine when inhaled but negatively during systemic administration. Currently he does not respond to methacholine even up to high doses. Despite this small sample size, a few key concepts emerge. Firstly, AHR is a moving target, in that its severity and presence are dependent upon many factors, including the modality of agonist chosen and level of treatment. Secondly, AHR is likely due to a plethora of underlying mechanisms that will have greater or lesser contributions in individual patients. This heterogeneity is not always well appreciated. While the clinical utility of AHR has been extensively reviewed elsewhere (5–7) we believe that understanding the heterogeneity of the mechanisms underlying AHR is an often over-looked yet important piece of the puzzle. Therefore we propose that future research into the mechanisms of AHR should aim to can move our understanding from a "one size fits all" approach to ascribing specific mechanisms of AHR to distinct patient populations. In this way we believe that it will provide much needed insight into the ever-developing recognition of distinct clinical phenotypes of asthma.

2. Clinical Importance

Airway hyperresponsiveness is used as a tool in the diagnosis, classification of severity (8) and management (9, 10) of asthma. AHR is useful in those who report symptoms (9), particularly in those with normal baseline lung function as measured by spirometry (11). The presence of AHR is associated with increased decline in lung function (12), even in those with asymptomatic AHR (13), increased risk for the development of asthma (12) and increased likelihood of the persistence of wheeze from childhood to adulthood (14). Furthermore, the severity of AHR is associated with an increased risk of exacerbation (15), increased asthma severity as measured by symptoms (16) and an increased level of treatment required to control symptoms (1). While the clinical implications relating specifically to the loss of the maximal response plateau in asthma are unclear, an increased or absent plateau represents uninhibited airway narrowing or closure that has the potential for life-threatening exacerbations (17). Understanding the factors contributing to the presence and severity of AHR therefore provides an important component for improving asthma control and reducing disease progression.

Although AHR is considered a hallmark of asthma, it is important to recognise that the severity, and even presence, of AHR is not stable. AHR to non-specific stimuli, such as histamine and methacholine, is increased in some, but not all, subjects following allergen challenge (18). This increase in AHR occurs most frequently in those subjects with a late asthmatic response and its persistence can be short-lived or remain for up to several months from exposure (19). It is not surprising, then, that seasonal allergen exposure alters the

severity of AHR (20). In addition, anti-inflammatory therapy profoundly improves AHR and since its widespread introduction many patients on appropriate treatment regimens may not respond positively to bronchial challenge in the range associated with asthma. For example, in a population of poorly controlled, chronically undertreated asthmatics, Reddel *et al* (21) reported that 16 weeks of high dose inhaled corticosteroid (ICS) followed by dose titration led to a 4.0 doubling dose increase in $PD_{20}FEV_1$ (reduction in AHR). After 72 weeks, 40% of subjects had responses to methacholine challenge within the normal range. Consistent with this, in asthmatic subjects on regular controller therapy, the sensitivity of a positive methacholine challenge for the diagnosis of asthma is only 77% (22). This sensitivity is further reduced in Caucasian and non-atopic patients. Furthermore, the measurement of AHR is confounded by its moderate repeatability, with estimates of within-subject repeatability ranging from 1–3 doubling doses (reviewed in (23)). Variability of AHR is further increased in those with non-atopic disease and those over 50 years of age (24). It is important to acknowledge that the variability of AHR is not only due to variability in the underlying mechanisms but also due to the imprecision of the measurement itself. Although it is clear that a negative challenge does not exclude the presence of asthma, interpretation of a negative bronchial challenge must consider the presence or absence of current symptoms. In a patient with current symptoms a negative challenge may suggest that diagnoses other than asthma should be considered. However, a negative challenge in a period without symptoms does not preclude asthma and in consideration of history it may be more appropriate to label such a patient as "*currently* negative AHR".

3. Measurement of AHR: To what do we respond and how do we measure it?

Traditionally measurements of airway responsiveness have been presented using two different, yet qualitatively similarly, calculations. The provocative concentration (or dose) causing a 20% fall in FEV_1 ($PC_{20}FEV_1$) is calculated by interpolation from the dose causing ≥20% fall in FEV_1 and the penultimate dose on a semi-log scale. It is maybe not surprising that determining the actual dose delivered (PD_{20}) appears to be a more robust measure than simply using the concentration of agonist (PC_{20}) (25). The dose response slope (or DRS), also referred to as the response dose ratio (RDR), is calculated as the slope of the dose response curve plotted with a linear dose axis. The advantage of the DRS is that it provides a continuous measure of airway responsiveness allowing inclusion of subjects who do not reach a 20% fall in FEV_1. In a more philosophical sense, the DRS more accurately reflects that airway responsiveness is a continuous variable in which AHR merely describes those people at one extreme. As both calculations provide similar information studies providing either calculation will be subsequently mentioned without differentiation.

a. Methacholine vs Mannitol

There are two groups of stimuli utilised in the measurement of AHR; those which allegedly act directly on the airway smooth muscle to induce bronchoconstriction, such as methacholine and histamine, and those which indirectly cause bronchoconstriction through the release of upstream mediators. Indirect challenge tests include exercise, eucapnic voluntary hypernea (EVH), hypertonic saline, mannitol, adenosine 5'monophosphate (AMP)

and various allergens. Although extensive review of the clinical application (6) and mechanisms (5) of these various stimuli have been provided elsewhere, the increasing interest in mannitol is worth considering as an example of an indirect challenge. Airway eosinophilia, measured by both sputum eosinophils and exhaled NO (eNO), is more strongly associated with AHR to mannitol than to methacholine (26, 27). Although this suggests that mannitol may provide a marginally better reflection of airway eosinophilia, the clinical utility of this finding is unclear. In addition, mannitol is more variable than methacholine which probably reflects variability in underlying inflammation. Therefore mannitol might be useful in predicting those patients who respond best to anti-IL-13 or anti-IL-5 monoclonal antibody treatment given the projected high cost of these treatments. Compared to treatment guidelines based upon symptoms and lung function, treatment strategies targeting reductions in AHR to mannitol (STAMINA trial) (28) or methacholine (AMPUL trial) (9) both lead to improvements in the number of mild exacerbations. Mannitol appears more specific in detecting a diagnosis of asthma although it is less sensitive than methacholine (27, 29, 30). Interestingly, the biggest distinction may be in the effect of allergen exposure with increased responsiveness to methacholine but reduced responsiveness to mannitol three hours after allergen challenge (31). This highlights the distinct underlying mechanisms between the two tests and suggests that comparison of the two methods may be useful in identifying phenotypes of AHR, and the underlying mechanisms, in subpopulations of patients. Indeed, one recent study reported 15% of asthmatics in primary care had AHR only to methacholine while another 15% had AHR only to mannitol (32). Similar variability in response has been reported when comparing exercise, EVH and methacholine challenge (33). Further research is required to determine the clinical importance of these distinct phenotypes of AHR.

b. What lung function measurement to use?

Traditionally AHR has been measured as reductions in spirometric parameters, most particularly FEV_1. However, spirometry is highly effort dependent and therefore requires considerable subject co-operation which is impossible for children under five and difficult for the elderly and those with increased disease severity. In contrast, the forced oscillation technique (FOT) is a measure of the mechanics of the respiratory system which can be acquired without special breathing manoeuvers. The FOT imposes oscillations over tidal breathing with the subsequent changes in pressure and flow analysed to provide measures of respiratory system resistance (Rrs) or its inverse conductance (Grs), as measures of airway calibre, and reactance (Xrs), as a measure of elastance. Recent advancements have allowed the FOT to cross the divide into clinical practice. FOT is capable of detecting patients with AHR, as assessed by spirometry, during methacholine (34), mannitol (35) and carbachol challenges (36). Furthermore, the repeatability of Grs and Xrs is not different to that of FEV_1 (2.0 and 1.95 vs 1.67 doubling doses, respectively, (35)). However, it should be noted that the aforementioned comparative studies included deep inspirations (inherent in spirometry) which provide beneficial effects in non-asthmatic but not moderate to severe asthmatic subjects (37). More importantly, removing deep inspirations during FOT measurement may also alter responsiveness in asthmatics with mild AHR since reduced responses are observed in mild disease when challenge inhalation is performed with the deep inspiration method compared to the tidal breathing method (38). Therefore, measuring responses to bronchial challenge during only tidal breathing with FOT may reduce the

ability to discriminate borderline AHR from normal responsiveness. Furthermore, decades of research indicates the clinical utility of AHR measured by spirometry, leaving us with a scenario where the use of FOT during bronchial challenge may best be suited as an adjunct to spirometry. Comparison of spirometry and FOT responses to bronchial challenge, as well as comparisons between FOT variables, may provide important clues as to the underlying pathophysiology. Lastly, FOT measurements may be capable of detecting differences in the pattern of response between stimuli (39) which may further aid in assigning phenotypes of AHR to distinct clinical populations.

4. Mechanisms of Airway Hyperresponsiveness

Despite decades of research, there is still little consensus as to the mechanisms underlying AHR in asthma. This is most likely due to the numerous pathophysiological abnormalities associated with asthma and the likely reality that different mechanisms or a combination of these gives rise to AHR in different patient populations. The definition of asthma as an inflammatory airways disease characterised by exaggerated airway narrowing immediately brings attention to the role of airway inflammation and the airway smooth muscle (ASM) in the manifestation of AHR. In addition, the structural remodelling reported in many patients with asthma is also likely to contribute in some, but presently unclear, way to the severity of AHR. Lastly, there is currently renewed interest in airway closure as a cause of AHR rather than merely a consequence.

a. Genetics

The role of familial inheritance in asthma was formally acknowledged by Coca and Cooke in 1923 (40) and the heritability of AHR has since been reported to be approximately 30% (41). However, the mechanisms linking genetics and AHR remain to be defined. Levitt and Mitzner (42) showed the large genetic contribution to AHR in mice by demonstrating that airway responsiveness to acetylcholine was controlled by a single autosomal recessive gene. Genome-wide association studies have revealed a substantial number of genes associated with susceptibility to asthma (recently reviewed in (43)), with genes corresponding to inflammatory pathways, airway epithelial function and ASM function likely contributing to AHR (mechanisms discussed below). Indeed, β_2-adrenergic receptor genotype appears to partially determine the improvements in AHR to methacholine following salmeterol/ICS therapy (44). Although not substantiated, this may represent distinct genotype-dependent mechanisms of AHR rather than differences in general treatment efficacy since improvements in baseline lung function, eNO and bronchodilator responsiveness were not affected by genotype. Similarly, it has been reported that allergen exposure in mice induces epigenetic changes in the transforming growth factor-β signaling pathway which are associated with development of AHR (45). Despite considerable advancement of our understanding of genetics in asthma future research is required to determine if and how genetic/epigenetic alterations are causally linked to the development and severity of AHR in human asthma.

b. Airway Inflammation

Asthma is a disease associated with chronic inflammation and the influx of inflammatory proteins likely contributes to AHR. Although allergic asthma has long been known to be associated with increased eosinophils in the airways, recent research suggests that subsets of asthmatic patients have elevated neutrophils with or without increased eosinophils (46). In asthmatic subjects there is a positive correlation between the severity of AHR and the number of eosinophils and metachromic cells in sputum (47, 48), as well as the number of mast cells in the airways (49). Furthermore, the level of exhaled nitric oxide (eNO), considered a biomarker for eosinophilic inflammation (50), correlates with the severity of AHR to methacholine in asthmatic subjects (51, 52). Interestingly, the link between eNO and AHR appears driven by airway narrowing, but not airway closure (53). In a small study of mild asthmatics, Brusasco et al (54) found no relationship between baseline AHR and inflammatory cells in bronchoalveolar lavage. However, after allergen challenge a strong correlation was reported between the increase in AHR and increase in eosinophils, further supporting the role of eosinophils in AHR. In contrast, there is little evidence that airway neutrophilia contributes to the severity of AHR in asthma. Indeed, Porsbjerg *et al* (48) were unable to show any correlation between neutrophils or neutrophil mediators and AHR. However, sputum neutrophils were correlated with an increased contribution of airway closure to the overall level of bronchoconstriction. Taken together, these findings suggest that eosinophillic airway inflammation may contribute to the severity of AHR whereas airway neutrophilia may be associated (causally or coincidentally) with an alteration in the type of bronchoconstriction towards predominance of airway closure.

c. Airway Smooth Muscle

Bronchoconstriction is due, at least in part, to constriction of the airway smooth muscle (ASM) surrounding the airway. Therefore it is not surprising that increased contractility of the ASM has long been touted as a principal cause of AHR. Abnormal ASM function could be due to intrinsic abnormalities of the ASM itself or to the effects of the asthmatic environment in which it resides.

i. Intrinsic factors—Despite considerable research it is still unclear as to whether asthmatic ASM is intrinsically hyper-contractile, and if so, what factors are mechanistically involved. Recent gene expression profiling of ASM revealed four novel genes that not only differentiated asthmatic and non-asthmatic patients, but were related to the severity of AHR (55). Furthermore, the expression of contractile proteins α-smooth muscle actin and desmin in ASM from asthmatics correlates with the severity of AHR (56) suggesting a role of intrinsic ASM dysfunction. Some *in vitro* studies have reported increased force generation of ASM from asthmatic patients (57, 58), while others have reported no difference when compared to healthy controls (59–61). However, increased airway narrowing could be due to an increase in the shortening velocity of ASM despite normal force generation. This would theoretically occur because a muscle that shortens quickly would produce greater airway narrowing during expiration before the dilatory effect of the proceeding inspiration (62). Indeed, *in vivo* findings support an effect of ASM shortening velocity on the magnitude of ASM shortening (63).

ASM contraction involves the formation of actin-myosin cross-bridges with the rate of formation dependent upon the activity of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). An increase in the activity of either MLCK or MLCP would lead to increased shortening velocity of ASM. Indeed, both an increased expression of MLCK and increased shortening velocity of ASM have been reported in asthma (64). Furthermore, the fast myosin heavy chain isoform is also increased in patients with asthma which murine models suggest would also increase cross-bridge cycling and AHR (65). Functionally, this may not be important as increased shortening velocity in asthma is not a consistent finding (61). Asthmatic ASM is also more sensitive to oxidative stress with the extent of oxidative damage within the ASM bundle correlated with the severity of AHR (66). This relationship is in part mediated by increased NOX4 expression since siRNA knockdown of NOX4 attenuates *in vitro* ASM contractility. In contrast to dysregulation of the molecular pathways controlling ASM contraction, subcellular structure of the ASM appears similar between asthmatic and non-asthmatic subjects (67).

ii. Extrinsic factors—The asthmatic airway resides in a pro-inflammatory environment which likely contributes to ASM dysfunction independent of any intrinsic abnormalities. Pro-inflammatory cytokines such as IL-4, IL-13 and tumour necrosis factor-α (TNFα) increase ASM responsiveness *in vitro*, possibly via effects on calcium signaling (68). Proteases, such as matrix metalloproteinase-1 (MMP-1), are increased within ASM bundles of asthmatics and also regulate *in vitro* ASM contractility (69) and structural integrity. Additionally, the number of mast cells within the ASM correlates with the severity of AHR in asthma (70). Although the mechanisms are not yet clear, mast cell mediators such as histamine, leukotriene D_4 (71) and prostaglandin D_2 (72) may contribute to increased basal ASM tone. Bossé and colleagues (73) reported that ovine tracheal ASM adapts to increased basal tone so that subsequent ASM shortening is synergistically amplified. Computational modelling suggested that this synergistic effect on ASM shortening, termed force adaptation, would translate to an increase in airway narrowing as high as 48% and increase in airflow resistance up to 274% for a prototypic ninth generation airway (74). Although force adaptation has recently been demonstrated in mice *in vivo* (75), it is unknown whether force adaptation occurs in humans *in vivo* and to what extent, if any, that would contribute to AHR measured by spirometry.

Alternatively, the inflammatory milieu may induce the transition of asthmatic ASM from a contractile to a “synthetic” phenotype (reviewed in (76)). This synthetic ASM phenotype is characterised by reduced contractile-associated proteins but increased proliferation and chemokine secretion. *In vitro* stimulation of human ASM with TNFα or IL-1β induces the secretion of chemokines such as regulated on activation, normal T cells expressed and secreted (RANTES), interleukin-6 (77, 78) and IL-8 (79, 80). This proliferative/secretory phenotype is associated with reduced expression of contractile proteins such smooth muscle myosin heavy chain, smooth muscle α-actin, myosin light chain kinase (81). It is presently unclear whether the transition of ASM to the synthetic phenotype confers protection against, or further contributes to, AHR.

Damage to the airway epithelium, which provides an initial barrier for inhaled spasmogens, also likely contributes to AHR. Disruption of the airway epithelium would increase the

amount of stimulus interacting with the ASM and thus potentiate bronchoconstriction. In addition, epithelial damage or dysregulation likely reduces the ability of the epithelium to maintain relaxation of ASM via release of epithelial-derived relaxing factor(s) (82). For example, intratracheal administration of cationic proteins reduces both the barrier effect and control of ASM relaxation by the airway epithelium, and results in AHR in animal models (83). Additionally, damage to the epithelium may also directly contribute to airway narrowing. Recent murine *in vitro* findings suggest that rupture of small airway epithelial cells induce intracellular [Ca^{2+}] waves and subsequent contraction in neighboring ASM (84). The contribution of damage of the airway epithelium is most likely to be highly relevant to AHR following exposure of noxious inhalants, such as in occupational asthma.

d. Structural airway remodelling

The lung of the asthmatic exhibits a gamut of structural pathologies that are collectively termed airway remodelling. Remodelling here merely means that the structure is no longer normal with the implication that the change is permanent. These changes include subepitheial fibrosis (85), ASM hypertrophy/hyperplasia (86), angiogensis (87) and changes in extracellular matrix composition (88). AHR correlates with airway wall thickening (89, 90) , reticular basement membrane thickness (91) and components of the extracellular matrix (56, 92), although others have been unable to replicate these findings (70).

Thickening of the airway wall could contribute to excessive bronchoconstriction in two ways. Airway resistance is inversely related to airway radius such that an increase in the submucosal area would amplify the reduction in airway calibre for any given degree of ASM shortening. Although an attractively simple explanation, it remains unclear whether reduced airway calibre is causally associated with increased severity of AHR. On one hand, several studies report a correlation between baseline airway calibre and AHR, measured by FEV_1/FVC (53, 93) and $FEF_{25–75}$/FVC (94, 95). On the other hand, *improvements* in airway calibre appear dissociated from improvements in AHR. Salome et al (96) administered fenoterol prior to histamine challenge and reported that although baseline FEV_1 and AHR both improved, AHR returned to control levels much more rapidly than FEV_1. This disconnect was later strengthened by Britton et al (97) who reported that ipratropium did not alter AHR despite increasing baseline FEV_1 and sGaw. However, determining whether *reductions* in airway calibre are similarly disassociated from AHR is confounded by an inability to reduce airway calibre without confounding effects, such as those related to transpulmonary pressure or ASM tone.

An increase in the thickness of the adventitial layer has the potential to uncouple the ASM layer from the surrounding parenchyma. Under this condition, the ASM is essentially untethered from the lung parenchyma, reducing the load against which the ASM shortens. This would allow for increased ASM shortening. Increased ASM mass, due to either hypertrophy or hyperplasia, is thought to increase the total force generated by ASM and thus exaggerate airway narrowing without any alteration in ASM contractile function (98). Indeed, increased ASM area in explanted bronchial segments from asthmatics correlates with increased *in vitro* airway narrowing (99). However, airway remodelling may protect against AHR. Should the remodelling processes increase airway wall stiffness, it would in

fact oppose, and therefore limit, airway narrowing during bronchoconstriction (100). On the other hand, some features of airway remodeling may be a consequence, rather than a cause, of AHR since bronchoconstriction itself is sufficient to induce subepithelial fibrosis and mucous metaplasia without affecting AHR (101).

e. Airway Closure

It is not well recognized that bronchoconstriction is also associated with increased airway closure. Moreover, it is important to determine whether increased airway closure is merely the consequence of exaggerated airway narrowing (102) or whether asthma is associated with a predisposition to airway closure. Increased airway closure assessed by bronchial challenge is associated with increased disease severity (103), oral steroid use (104) and a history of exacerbations requiring intubation (103). Irvin and Bates (105) reviewed the literature that supports the notion that bronchoncostriction in asthma is not due to central airway narrow as commonly assumed, but rather due to peripheral airway closure. Consistent with this hypothesis, AHR in allergically sensitised mice can be fully attributed to an increased susceptibility to small airway closure (106, 107). The importance of airway closure to human asthma was validated by the finding that the extent of airway closure during methacholine challenge was a significant determinant of the severity of AHR, independent of airway narrowing (53). However, unlike in the allergically sensitised mouse, there is great variability in the contribution of airway closure to bronchoconstriction in human asthma. As shown in Figure 2A and B, asthmatic patients can respond to bronchial challenge through predominantly airway narrowing or airway closure. It is important to highlight that these examples are the extremes of a continuum with the majority of subjects falling in between. Hence, the severity of AHR in specific phenotypes of asthmatic patients is likely due in large part to airway closure. However, the clinical features or underlying mechanisms of patients who respond predominantly due to airway closure require further investigation.

There are several mechanisms by which asthma pathophysiology may lead to increased airway closure during bronchoconstriction. Firstly, mucous plugging would obviously induce airway closure so it is not surprising that pharmacologically blocking the release of mucous protects against AHR in allergically inflamed mice (108). Secondly, increased airway closure may be due to surfactant dysfunction caused by inflammation (109, 110). Similarly, fibrin is known to inactivate surfactant (111), accumulate in the airways of asthmatics and is associated with AHR (107). A role of surfactant dysfunction in AHR is consistent with the protective effect of inhaled surfactant against allergen-induced bronchoconstriction (112). On the other hand, recent computational and physiological evidence suggests that increased baseline ventilation heterogeneity may promote increased airway closure during bronchoconstriction. Venegas et al (113) developed a highly advanced lung model that takes account of the effects of the parenchymal tethering forces, the intra- and extra-luminal pressures and ASM forces. The model predicted that uniform ASM contraction with the addition of small, random heterogeneities in airway calibre would lead to the abrupt development of airway closure when ASM contraction reached a critical level of instability. The validity of the model predictions have been strengthened by subsequent findings that the severity of AHR in asthma strongly correlates with the degree of baseline

ventilation heterogeneity (52, 114, 115). This has recently been extended by the report that baseline ventilation heterogeneity correlates with the increase in airway closure during methacholine challenge (116). Importantly, the association between baseline ventilation heterogeneity and AHR remained following three months of ICS treatment suggesting that it is independent of (steroid-responsive) airway inflammation (52). Further research is needed to ascertain the causes of the baseline ventilation heterogeneity and whether they can be targeted to treat AHR, which may provide more effective treatment strategies for asthma.

5. Can AHR contribute to our understanding of asthma phenotypes?

Asthma is not a single disease but a combination of many pathophysiological features culminating in the clinical presentation of asthma symptoms. This underlies the importance of personalised medicine, in which the foundation has been built on improved phenotyping of asthmatic patients utilising a variety of clinical, inflammatory and physiological features (117, 118). However, these approaches are yet to include charateristics of AHR, such as differences between modalities or the pattern of bronchostriction. Below we highlight three phenotypes associated with worse asthma control and discuss the current understanding of AHR in each group (Table 1).

a. Asthma in the elderly

Asthma control worsens with age (119) with one recent study reporting that 25% of asthma patients over 65 years experienced at least one severe exacerbation in the preceding year (120). Hardaker et al (114) recently compared the physiological determinants of AHR in young and elderly asthmatics. In those below 55 years, the severity of AHR was predicted by increased eosinophilic airway inflammation (exhaled NO) and baseline ventilation heterogeneity in conducting airways. In contrast, AHR in the elderly was associated with baseline gas trapping and ventilation heterogeneity in acinar airways. This suggests that AHR in the elderly is associated with more peripheral disease. This is consistent with previous reports that AHR in the elderly is associated with increased airway closure during methacholine challenge (121). This may be due to the increase in neutrophilia with age, since increased airway closure during bronchoconstriction in elderly asthmatics correlates with sputum neutrophil levels (48). Alternatively the distinct AHR of the elderly may be due to ural change of the loss of elastic recoil due to emphysema-like changes associated with aging (122). Bronchial challenge with AMP has been suggested to induce a more peripheral response than that due to methacholine (123) and therefore age may have greater effects on AHR to AMP than to methacholine challenge. However, it is unclear whether the more peripheral disease is due to the additive effects of age on asthma or a synergistic effect of disease duration.

b. Asthma in the obese

Cross-sectional studies report an increased prevalence of asthma in the obese (124), while obesity and weight gain appear to precede the development of asthma (125). Obesity appears to worsen asthma control (126) while weight loss leads to an improvement in asthma symptoms (127). There is growing recognition that obese asthmatics comprise two distinct clinical populations; those with high IgE and early-onset asthma (allergic), and those

with late-onset disease and low serum IgE (non-allergic). Following weight loss, only the non-allergic obese asthmatics had an improvement in methacholine responsiveness suggesting that obesity negatively impacts AHR only in those with non-allergic disease (128). This effect of obesity on AHR is due to increased collapsibility of peripheral airways that predisposes to increased airway closure during methacholine challenge (129, 130). This is illustrated in Figure 2C, in which the extent of airway closure, adjusted for the level of airway narrowing, is substantially greater in non-allergic obese asthmatics compared to non-obese asthmatics (ie significantly steeper slope). Interestingly, following weight loss, the response to methacholine appears identical to that of non-asthmatic obese subjects (ie position and slope of regression). These data suggest that allergic obese asthmatics may have asthma that is complicated by obesity, whereas non-allergic obese asthmatics have asthma secondary to obesity. Since the severity of AMP appears closely associated with atopy and IgE levels (131, 132), it is possible that AMP challenge may be better able to differentiate these two phenotypes of obese asthma.

c. Asthma in smokers

Asthmatics who smoke report worse asthma control (133) and smoking history is associated with the severity of AHR (134). Airway closure during AMP challenge is increased in asthmatic smokers compared to non-smokers, but not during methacholine challenge (135). This is consistent with a more peripheral response during AMP than methacholine challenge (123). Interestingly, improvements in AHR to AMP following smoking cessation occur earlier than improvements in AHR to methacholine (136). This may reflect a greater sensitivity of AMP to smoking-related pathophysiology. On the other hand, methacholine challenge may better reflect underlying structural changes than AMP (131) such that differences between the two stimuli may reflect distinct mechanisms underlying the severity of AHR in smoking asthmatics. Further research will determine whether these differences can be used to detect early smoking-related disease or determine those smokers who may respond to asthma guideline therapy and those unlikely to benefit.

6. Looking through the crystal ball: the future of AHR testing

We must ensure that our view of the measurement of AHR does not remain as a “one size fits all” approach. On one hand we have many different stimuli for bronchial challenge testing and on the other, an extensive list of potential mechanisms underlying AHR. Currently we do not completely understand whether specific mechanisms play a greater role in AHR assessed by one challenge test over another. Similarly, we do not know whether differences between challenge modalities provide a better assessment of the various clinical asthma phenotypes. Physiologists have long known that FEV_1 is a polyvalent measure of lung function that provides little information about the precise pattern of bronchoconstriction. Understanding the pattern of bronchoconstriction in an individual patient, whether through comparison of spirometric variables or from measurements such as the FOT and inert gas washout, may allow us to ascribe certain phenotypes of bronchoconstriction to specific clinical phenotypes. This, too, would help elucidate the underlying mechanisms and may contribute to more targeted therapies. As discussed above, a combination of these two approaches has already been applied to asthma in the obese,

elderly and those who smoke. While only in its infancy, the evidence to date suggests that phenotyping AHR may help to uncover the pathophysiology contributing to poor asthma control in numerous distinct subsets of patients with asthma.

7. Conclusion

The development of the measurement of AHR forty years ago sparked many important contributions to our understanding of asthma and other airway diseases. However, it is time to re-evaluate our assumptions of AHR in light of the current population of asthmatic patients. We must look towards the future, embracing the technological advancements which provide potentially complimentary techniques to measure the response to bronchial challenge. These complementary measurements may lead us to better partition global bronchoconstriction into its components of airway narrowing and airway closure as well as proximal and distal airway effects. This enhancement has to the potential of allowing us to assign certain mechanisms to specific patterns of bronchoconstriction, opening the door for matching phenotypes of bronchoconstriction with clinical phenotypes. In doing so we are likely to gain improved mechanistic understanding of asthma phenotypes, and help better focus as well as better assess the development of phenotype-targeted therapy.

Acknowledgments

Grant support: DGC is a recipient of a CJ Martin Fellowship from the National Health and Medical Research Council of Australia (1053790). CGI is supported by the American Lung Association and the National Institutes of Health (P30 GM103532).

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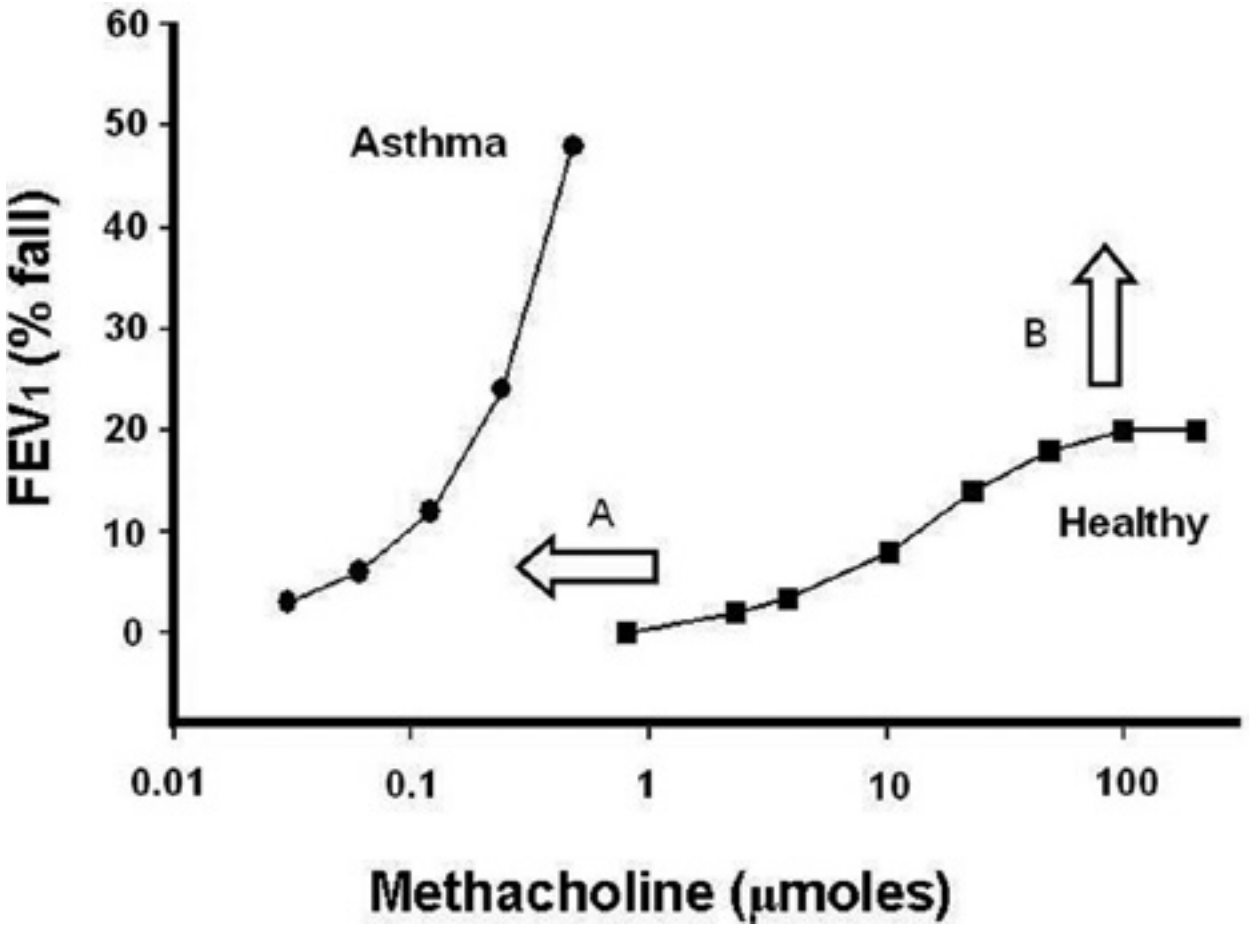

Figure 1. Representative dose response curves (DRC) to methacholine in a healthy and a severely asthmatic subject

Airway hyperresponsiveness is characterised by both an increased sensitivity, seen as the leftward shift in the DRC of the asthmatic patient (A), and excessive bronchoconstriction, resulting in the loss/increase in the maximal response plateau (B).

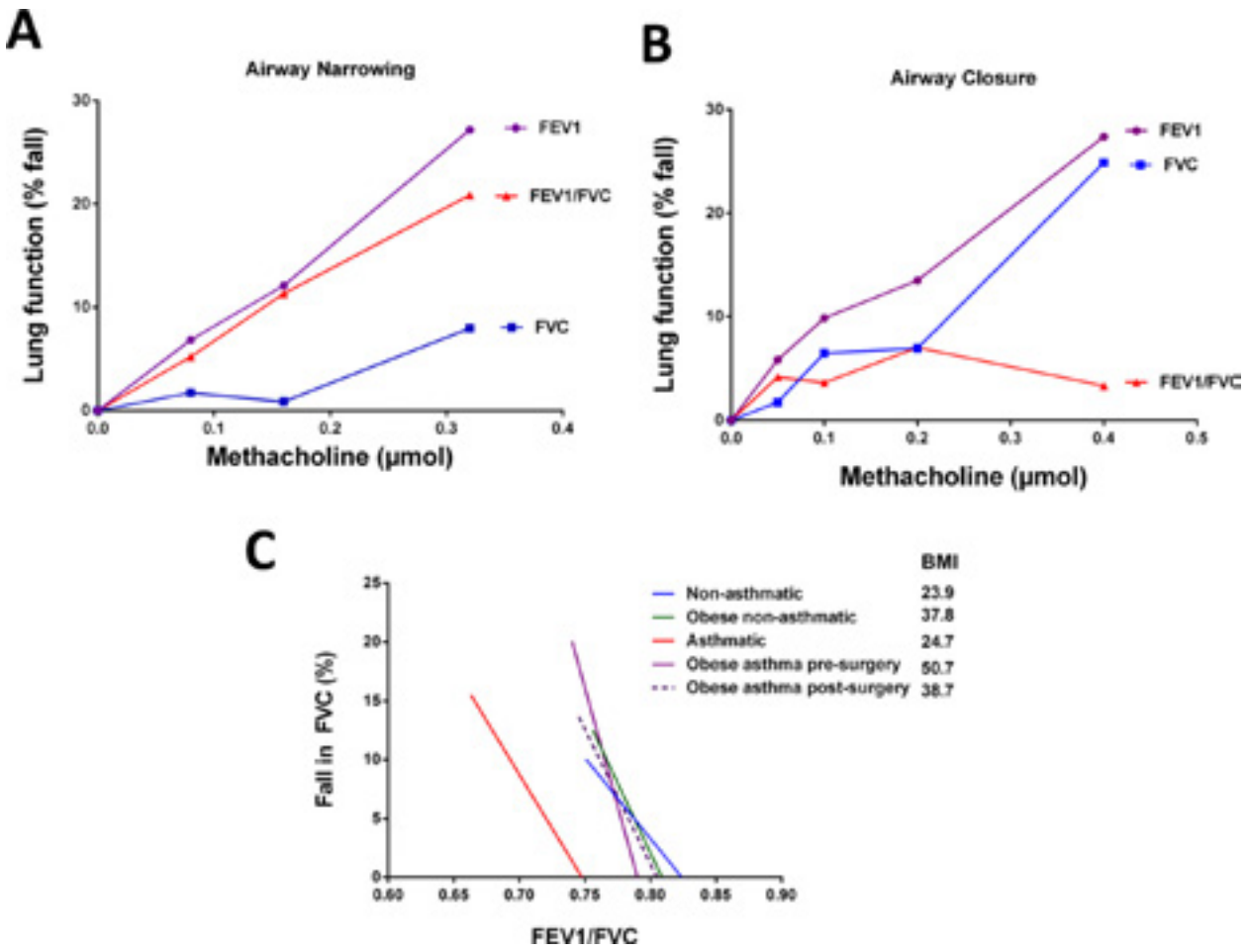

Figure 2. The contribution of airway narrowing and airway closure to the fall in FEV_1 during bronchial challenge

FEV_1 is reduced by airway narrowing because a narrowed airway loses some capacity to transmit flow. However, FEV_1 is also determined by the number of parallel airways contributing to flow and is thus reduced by functional airway closure (both true airway closure and severe airway narrowing). By contrast, FVC is determined by the volume of air in communication with the mouth and is reduced by functional airway closure but not by airway narrowing. Air narrowing, per se, is thus reflected in the ratio FEV_1/FVC. There is substantial variation in the contribution of airway narrowing and airway closure to the fall in FEV_1 amongst patients with asthma. Shown are dose response curves for an asthmatic subject with predominantly airway narrowing (A: 24 years old, baseline FEV_1 122%pred, $PC_{20}FEV_1$ 0.23μmol) and one with predominantly airway closure (B: 24 years old, baseline FEV_1 78%pred, $PC_{20}FEV_1$ 0.28μmol). These examples represent extremes of a continuum of responses, with the majority of subjects falling in between. The extent of airway narrowing is expected to contribute to airway closure so to determine excessive airway closure we have analysed the relationship between %fall FVC and FEV_1/FVC (C). A steeper slope represents greater airway closure for a given level of airway narrowing. Absolute

FEV_1/FVC, rather than % fall FEV_1/FVC, maintains the contribution of baseline airway calibre. Representative regression lines were calculated from the mean baseline FEV_1/FVC, mean fall in FEV_1/FVC and mean % fall FVC for lean non-asthmatics (blue), lean asthmatics (red), obese non-asthmatics (green), non-allergic obese asthmatics prior to bariatric surgery (purple) and the same subjects 12 months following bariatric surgery (dashed purple). Data were adapted from two of our previous studies (53, 130). Important to note is the increased slope in asthmatics compared to non-asthmatics, and in all obese groups compared to the two lean groups. Following weight loss, the slope of the obese non-allergic asthmatics decreased suggesting reduced predisposition to airway closure. Interestingly, the position and slope of obese non-allergic asthmatics post-surgery is almost identical to obese non-asthmatics suggesting that the effect of obesity on airway closure is dependent upon the level of adiposity in non-allergic subjects.

Table 1

Current understanding of AHR in asthma phenotypes associated with worse control

Asthma Phenotype	Severity of AHR	Pattern of AHR (Closure vs narrowing)	Modality	Associated pathophysiology
Elderly	Increased	Closure	Methacholine	↑ Neutrophils ↓ Elastic recoil
Obese				
• non-allergic	Increased	Closure	Methacholine	↓ FRC volume ↑ airway compliance ↓ surfactant (?)
• allergic	Unaltered	Closure	Methacholine	↓ FRC volume
Smoking	Increased	Closure	AMP	Inflammation/ Acutely reversible
	Increased	~ Equal	Methacholine	Structural

FRC = functional residual capacity

Published in final edited form as:
Adv Pharmacol. 2013 ; 66: 129–155. doi:10.1016/B978-0-12-404717-4.00004-4.

The biology of thymic stromal lymphopoietin (TSLP)

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Abstract

Originally shown to promote the growth and activation of B cells, thymic stromal lymphopoietin (TSLP) is now known to have wide-ranging impacts on both hematopoietic and non-hematopoietic cell lineages, including dendritic cells (DCs), basophils, eosinophils, mast cells, $CD4^+$, $CD8^+$ and natural killer (NK) T cells, B cells and epithelial cells. While TSLP's role in the promotion of TH2 responses has been extensively studied in the context of lung- and skin-specific allergic disorders, it is becoming increasingly clear that TSLP may impact multiple disease states within multiple organ systems, including the blockade of TH1/TH17 responses and the promotion of cancer and autoimmunity. This review will highlight recent advances in the understanding of TSLP signal transduction, as well as the role of TSLP in allergy, autoimmunity and cancer. Importantly, these insights into TSLP's multifaceted roles could potentially allow for novel therapeutic manipulations of these disorders.

Keywords

allergy; atopy; cancer; cytokines; TSLP

I. Introduction

Thymic stromal lymphopoietin (TSLP) is a member of the IL-2 cytokine family, and a distant paralog of IL-7 (Leonard, 2002). Murine TSLP was discovered in thymic stromal cell line supernatants that supported B cell development (Friend et al., 1994). Like IL-7, TSLP can stimulate thymocytes and promote B cell lymphopoiesis. Accordingly, TSLP was initially studied as a B cell growth factor (Levin et al., 1999). A human homolog was subsequently identified, and further characterization of the cytokine revealed a four-helix bundle structure containing six conserved cysteine residues and multiple potential sites for N-linked carbohydrate addition. As discussed later, in spite of only 43% amino acid identity, human and murine TSLP share a significant degree of functional homology (Reche et al.,

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VII. Conflict of Interest Statement The authors have no conflicts of interest concerning the work in this review.

2001; Sims et al., 2000). During allergic inflammation, the primary producers of TSLP are epithelial cells, keratinocytes and stromal cells, although recent data have demonstrated that both dendritic cells (DCs) and mast cells are capable of TSLP production (Soumelis et al., 2002; Watanabe et al., 2004; Ying et al., 2005; Kashyap, Rochman, Spolski, Samsel, & Leonard, 2011; Moon, Choi, & Kim, 2011). Several groups identified a receptor capable of binding TSLP with low affinity (TSLPR subunit), which shares 24% identity to the common γ receptor chain (γ_c) (Pandey et al., 2000; Park et al., 2000). Upon further analyses, the functional receptor (TSLPR) was shown to include both the TSLPR subunit and the IL-7Rα chain in humans and mice (Quentmeier et al., 2001; Park et al., 2000). The functional TSLPR is expressed by a variety of hematopoietic cell populations, such as T cells, B cells, NK cells, monocytes, basophils, eosinophils and DCs, as well as some non-hematopoietic cell lineages such as epithelial cells (Ziegler, 2010; Reardon et al., 2011). While classified as a hematopoietin receptor based on structural homology, the TSLPR subunit contains notable differences from canonical hematopoietin receptors. The TSLPR subunit contains the conserved box1 sequence, which regulates Janus protein tyrosine kinase (JAK) binding in other cytokine receptors, but lacks the conserved box2, and contains only one tyrosine (Y) residue four amino acids from its carboxy terminus (Park et al., 2000). Additionally, it contains a modified WSXWS motif and multiple potential *N*-linked glycosylation sites (Tonozuka et al., 2001).

II. TSLP signaling

As a member of the hematopoietin receptor family, it was originally hypothesized that the TSLPR would utilize JAKs to activate STAT proteins downstream of the TSLPR. Indeed, TSLP stimulation of multiple cell lines leads to STAT5 phosphorylation. However, initial experiments in these cell lines showed that TSLPR signaling occurred in the absence of JAK activation, and dominant-negative forms of JAK-1 and -2 did not affect TSLP-mediated STAT5 activation (Isaksen et al., 1999; Levin et al., 1999). Several alternatives were implicated in TSLPR signaling, such as Src kinases and phosphinositol 3 kinase (Isaksen et al., 2002). However, two recent papers have demonstrated robust and sustained activation of JAK-1 and -2 following TSLP signaling in primary human dendritic cells and primary human and mouse $CD4^+$ T cells (Arima et al., 2010; Rochman et al., 2010). Surprisingly, unlike IL-7Rα and γ_c in IL-7 signaling, which utilize JAK-1 and -3, the TSLPR subunit bound and utilized JAK-2 in concert with IL-7Rα-associated JAK-1. These latest findings resolve a long-standing question about the mode of TSLP signaling, and show that TSLP-induced JAK activation precedes the activation of STAT proteins. In human peripheral blood-derived CD11c+ DCs TSLP stimulation activated STAT 1,3,4,5, and 6, as well as JAKs 1 and 2(Arima et al., 2010). Similar results have been seen using mouse DCs, with the exception that no phosphorylation of Stat6 was seen (B.D. Bell, M. Kitajima and S.F. Ziegler, manuscript submitted). These data suggest that TSLP is capable of activating multiple STAT proteins. Whether TSLP utilizes similar signaling pathways in other cell lineages and how each STAT molecule contributes has yet to be elucidated.

III. TSLP-Responsive Cells

A plethora of cell types have been shown to be capable of responding to TSLP in vivo and in vitro. These include DCs, CD4 and CD8 T cells, B cells, mast cells, basophils, eosinophils, and NKT cells. This long list of responding cell types suggests the important role of this cytokine in orchestrating the initial response to an epithelial insult. While the normal function of TSLP is likely the maintenance of Th2-type homeostasis at barrier surfaces(Ziegler & Artis, 2010), as will be discussed below, dysregulated TSLP expression can result in the development of type 2 inflammatory responses leading to allergic disease.

A. Dendritic Cells

It has now become apparent that a major TSLP-responsive cellular subset in both humans and mice are myeloid-derived dendritic cells (mDCs)(Reche et al., 2001; Zhou et al., 2005). Co-culture of TSLP-activated DCs with naïve syngeneic CD4+ T cells led to T cell proliferation but no differentiation, suggesting a role for TSLP in CD4+ T cell homeostasis(Watanabe et al., 2004). However, when TSLP-stimulated DCs primed CD4+ T cells in an antigen-specific manner (e.g., in an allogeneic culture), the resulting T cells display characteristic features of Th2 differentiated cells (production of IL-4, IL-5, IL-13, and TNFα), with the exception that IL-10 production was not evident(Soumelis et al., 2002). These data suggest that TSLP-activated DCs primed for inflammatory Th2 cell differentiation. Interestingly, TSLP, in the absence of IL-12, induced OX40L expression on DCs, and OX40-OX40L interactions were critical for the ability of the DCs to drive Th2 cell differentiation(Ito et al., 2005). Consistent with a role in regulating Th2 cytokine responses, TSLP-activated DCs were also capable of supporting the maintenance and further polarization of CRTH2+ Th2 effector memory cells(Wang et al., 2006). In contrast, autologous TSLP-activated DCs supported the expansion and functions of $CRTH2^{+}$ $CD4^{+}$ TH2 memory cells (Wang et al., 2006), but led to T cell proliferation and elaboration of high levels of IL-2, but not IL-4, IL-5 or IL-13, when co-cultured with naïve T cells (Watanabe et al., 2004).

TSLP-conditioned DCs also augmented intestinal epithelial cell-mediated IgA2 class switching through the induction of APRIL (He et al., 2007). Finally, some in vitro studies have suggested a role for TSLP in the generation of tolerogenic DCs that can drive the differentiation of regulatory T cells (Tregs) (Watanabe et al., 2005; Besin et al., 2008; Iliev et al., 2009), although other studies have indicated that TSLP may hinder the production and/or maintenance of FOXP3+ Tregs in vivo in certain disease processes (Lei, Zhang, Yao, Kaplan, & Zhou, 2011; Duan et al., 2010).

B. T lymphocytes

Early work from the Leonard lab showed that TSLPR-deficient mice had normal lymphocyte numbers, but that γ_c/TSLPR double deficient mice had a more pronounced defect that γ_c-deficient mice along(Al Shami et al., 2004). The also showed that TSLP could drive the expansion of T and B cells when injected into γ_c-deficient mice, showing that TSLP can effect lymphoid homeostasis. Subsequent studies showed that TSLP can also act directly on CD4+T cells, and in the presence of TCR stimulation, promoted proliferation and

TH2 differentiation of naïve $CD4^+$ T cells through induction of IL-4 gene transcription (Omori & Ziegler, 2007; Rochman, Watanabe, Arima, Liu, & Leonard, 2007). IL-4 further upregulated TSLPR on $CD4^+$ T cells, resulting in a positive feedback loop. Although IL-4 maintained TSLPR expression on both *in vitro* differentiated TH2 and TH17 cells, higher TSLPR levels were present on TH2 than on TH1 and TH17 cells, which correlated with the ability of TSLP to drive the proliferation and survival of activated TH2 cells (Kitajima, Lee, Nakayama, & Ziegler, 2011). Naïve mouse $CD8^+$ T cells also express TSLPR, though TSLPR expression is low to absent on naïve human $CD8^+$ T cells; however, following activation, TSLPR expression is upregulated on both mouse and human $CD8^+$ T cells (Rochman & Leonard, 2008; Akamatsu et al., 2008). In both $CD4^+$ and $CD8^+$ T cells, TSLP stimulation upregulated the survival protein Bcl-2 in a STAT5-dependent manner (Rochman & Leonard, 2008; Rochman et al., 2010; Kitajima et al., 2011).

C. B lymphocytes

The initial studies describing TSLP demonstrated that TSLP can support B cell lymphopoiesis (Friend et al., 1994; Levin et al., 1999). In *in vitro* studies, pro-B cells derived from fetal liver, but not bone marrow, responded to TSLP, although pre-B cells from both origins could proliferate in response to TSLP (Vosshenrich, Cumano, Muller, Di Santo, & Vieira, 2003). The role of TSLP in normal B cell development or during inflammatory responses remains undefined. However, it is clear that aberrant TSLP signaling can have a significant impact on B cells, as has been demonstrated by the association of TSLPR mutations with a subtype of B cell leukemia(Chapiro et al., 2010; Roll & Reuther, 2010; Tasian & Loh, 2011). In addition, elevated systemic TSLP has been shown to lead to aberrant B cell development and function, with both direct effects on early B cell development and indirect effects leading to autoimmune hemolytic anemia(Astrakhan et al., 2007; Iseki et al., 2012).

D. Innate immune cells

Multiple innate immune cells express the TSLPR and respond to TSLP. For example, TSLP can enhance cytokine production from mast cells, NKT cells and eosinophils (Nagata, Kamijuku, Taniguchi, Ziegler, & Seino, 2007; Allakhverdi et al., 2007; Wong, Hu, Cheung, & Lam, 2009). In addition, TSLP has very recently been shown to induce eosinophil extracellular traps (EETs), extrusions of mitochondrial DNA toxic granule molecules released in response to infection(Morshed, Yousefi, Stöckle, Simon, & Simon, 2012). Finally, TSLP has also been shown to be important for the development and function of a subset of basophils(Siracusa et al., 2011). This subset is IL-3-independent, and is recruited to site of type-2 inflammation where is it speculated that they play a role in promoting Th2-type responses(Siracusa et al., 2011; Sokol et al., 2009; Siracusa, Wojno, & Artis, 2012). Thus TSLP not only can directly promote type 2 responses through CD4 T cell differentiation, it can also influence responses through the recruitment and activation of innate immune cells capable of producing cytokines involved in type 2 inflammation.

IV. TSLP-associated diseases

The variety of TSLP-responsive cell types demonstrates that TSLP can impact type 2 inflammation through a myriad of different pathways. In addition, numerous studies in both humans and mice now implicate TSLP in a growing number of different disorders beyond allergic inflammation, including infection, cancer and autoimmunity. The following sections describe the disorders associated with TSLP and what is known about the mechanisms through which TSLP may act.

A. Skin disorders

Atopic dermatitis (AD) is a chronic inflammatory skin disease that affects an estimated 10 to 20 percent of infants and young children in the United States (Leung, Boguniewicz, Howell, Nomura, & Hamid, 2004; Boguniewicz & Leung, 2011). Interestingly, there is suggestive evidence of linkage between single nucleotide polymorphisms (SNPs) in the TSLP gene and AD(Gao et al., 2010). In addition, while TSLP protein was undetectable in non-lesional skin in AD patients, TSLP was highly expressed in acute and chronic AD lesions (Soumelis et al., 2002). TSLP was also over-expressed in the skin of individuals with Netherton syndrome (NS), a severe skin disease characterized by atopic dermatitis-like lesions as well as other allergic manifestations that result from mutations in the serine peptidase inhibitor Kazal-type 5 (SPINK5) gene, which encodes the serine protease inhibitor lymphoepithelial Kazal-type–related inhibitor (LEKTI) (Briot et al., 2009).

In mice, over-expression of TSLP specifically in the skin was sufficient to induce a disease phenotype characterized by all of the hallmark features of AD (Yoo et al., 2005). In the steady-state, TSLP expression in the skin appears to be negatively regulated by retinoid X receptors (RXR), since keratinocyte-specific ablation of the retinoid X receptor isotypes RXRα and RXRβ resulted in upregulation of TSLP and development of AD-like skin inflammation (Li et al., 2005). RXRs heterodimerize with many nuclear receptor partners, including the vitamin D receptor and peroxisome proliferator-activated receptors. Administration of vitamin D or its analogs upregulated TSLP and resulted in the development of dermatitis (Li et al., 2006; Li et al., 2009), suggesting that vitamin D administration may result in RXR derepression and recruitment of co-activators to promote transcription. Keratinocyte-specific deletion of Notch signaling, which causes severe epidermal differentiation defects, also resulted in high systemic levels of TSLP. However, TSLP expression in this model may be due to responses to the resulting skin barrier defect rather than directly from the loss of keratinocyte-specific Notch signaling itself, since wild-type and mutant keratinocytes produced similar amounts of TSLP in *in vitro* cultures (Demehri et al., 2008). In SPINK5 knockout (SPINK5 −/−) mice, which reproduce many of the key features of NS, the absence of LEKTI resulted in unrestrained activity of the serine protease kallikrein 5, which directly activated proteinase-activated receptor 2 (PAR-2) and induced nuclear factor κB (NF-κB)–mediated overexpression of TSLP without contribution of the adaptive immune system (Briot et al., 2009; Kouzaki, O'Grady, Lawrence, & Kita, 2009). Interestingly, in SPINK5/PAR-2 double knockout mice, TSLP expression was greatly diminished, although inflammation still occurred (Briot et al., 2010). Whether the cytokine milieu differs in the absence of TSLP remains to be determined.

TSLP may influence both the initiation and progression of allergic skin inflammation, but the relative contribution to these stages and the cellular requirements may differ depending on the context. Langerhans cell (LC) migration and activation was seen in human AD lesions *in situ* (Soumelis et al., 2002). Furthermore, TSLP has been shown to increase the number and maturation status of migratory LCs in human skin explants cultures and to condition LCs to prime co-cultured naïve $CD4^+$ T cells to adopt an inflammatory TH2 phenotype (Ebner et al., 2007). However, mouse models of AD implicate additional cell types in the initiation and promotion of AD by TSLP. A recent study by Oh *et al*. implicated TSLP in mediating skin fibrosis downstream of IL-13, in part through the stimulation of fibrocyte collagen production (Oh et al., 2011). In a model of allergic skin inflammation using epicutaneous (EC) sensitization to ovalbumin (OVA) on tape-stripped skin, TSLP acted directly on T cells during the challenge phase to potentiate TH2 cytokine production (He et al., 2008). T cells and eosinophils were also required for TSLP-mediated dermal inflammation induced through intradermal delivery of recombinant TSLP protein (Jessup et al., 2008). In contrast, TSLP was involved in both sensitization and challenge phases of FITC-mediated contact hypersensitivity, since ear swelling was minimal if blockade of TSLP occurred prior to sensitization, but was only modestly reduced when TSLP blockade occurred after sensitization but prior to challenge (Larson et al., 2010; Boehme et al., 2009). While DC migration was intact in the absence of TSLP in EC sensitization, loss of TSLP signaling in the FITC CHS model was associated with reduced migration and activation of skin-derived antigen-bearing DCs. In addition, TSLP-responsive $CD4^+$ T cells were not required to induce a TH2 response in the CHS model (R.P. Larson and S.F. Ziegler, unpublished observations). In the setting of chronic high TSLP expression, skin inflammation also occurred in the absence of T cells (Yoo et al., 2005), possibly due to the ongoing stimulation of innate immune cells by TSLP.

TSLP has also been implicated in the phenomenon referred to as the atopic march, which describes the increased likelihood of individuals with AD of developing allergic rhinitis (AR) and asthma later in life (Bieber, 2008). Several models of induced TSLP expression in mouse keratinocytes result in subsequent allergic airway inflammation following intranasal challenge, suggesting that TSLP may be an important factor contributing to this progression from AD to AR and asthma (Zhang et al., 2009; Demehri, Morimoto, Holtzman, & Kopan, 2009; Leyva-Castillo, Hener, Jiang, & Li, 2012; Jiang et al., 2012). While many of these methods used to induce TSLP expression result in artificially high systemic levels of TSLP that are not seen in AD patients, we have found that intradermal administration of TSLP triggers progression from atopic dermatitis to asthma in the absence of systemic TSLP(Han et al., 2012). In this study, TSLP was the airway response to antigen challenge was shown to be TSLP-independent. These models, as well as approaches that allow for more specific expression or deletion of TSLP, will be helpful in identifying the cellular targets of TSLP and the mechanisms involved in the progression from AD to AR and asthma.

B. Respiratory Diseases

The initial report demonstrating high TSLP expression in AD and potentiation of inflammatory TH2 responses by TSLP also suggested a potential role for TSLP in allergic airway disease(Soumelis et al., 2002). This hypothesis was supported by the demonstration

that TSLP mRNA was present in human lung fibroblasts and bronchial epithelial and smooth muscle cells (Soumelis et al., 2002), and that aberrant levels of TSLP were associated with certain human respiratory disorders (Ying et al., 2005; Zhang et al., 2007; Ying et al., 2008; Kamekura et al., 2009; Semlali, Jacques, Koussih, Gounni, & Chakir, 2010; Kimura et al., 2011; Shikotra et al., 2011; Xu et al., 2010). Lung epithelium and submucosa samples from asthmatics and chronic obstructive pulmonary disease (COPD) patients contained a greater number of TSLP mRNA positive cells, and bronchoalveolar lavage (BAL) samples from these patients had higher concentration of TSLP protein compared to healthy controls (Ying et al., 2005; Ying et al., 2008; Semlali et al., 2010; Shikotra et al., 2011). Although the level of TSLP expression can be variable in asthmatic patients, it has been shown to correlate directly with TH2 cytokine and chemokine expression and inversely with lung function (Shikotra et al., 2011; Ying et al., 2008). Increased expression of TSLP in the nasal epithelium has also been found in biopsies from allergic rhinitis patients and was associated with TH2 cytokine production and eosinophilic infiltration in epithelial-associated tissue (Mou et al., 2009; Kamekura et al., 2009; Kimura et al., 2011; Xu et al., 2010). Genetic studies also support a critical role for TSLP in allergic airway disease. Several SNPs at the TSLP genomic locus found across multiple ethnic backgrounds were associated with increased asthma susceptibility or protection (Harada et al., 2009; Hunninghake et al., 2010; Bunyavanich et al., 2011; Harada et al., 2010; Torgerson et al., 2011; Shamim et al., 2007). One such SNP present in the genomic TSLP locus creates a novel AP-1 transcription factor binding site that could potentially lead to increased TSLP transcription (Harada et al., 2009).

A role for TSLP in human asthma has been well supported by a variety of mouse models, such as the surfactant protein c (SPC)-TSLP mouse, in which TSLP is constitutively expressed by the lung epithelium under control of the SPC promoter (Zhou et al., 2005). With increasing age, these mice developed a progressive asthma-like disease characterized by lung infiltration of eosinophils and TH2 $CD4^+$ T cells, airway remodeling and airway hyperreactivity. Disease in these mice was largely dependent on IL-4, IL-13, $CD4^+$ T cells and antigen (Headley et al., 2009; Zhou et al., 2008). $CD4^+$ T cells and antigen were also required in an acute asthma model using intranasal administration of TSLP in conjunction with antigen (Seshasayee et al., 2007; Headley et al., 2009). In addition to driving allergic inflammation in the lung following direct TSLP administration, TSLP played a crucial role in the well-established ovalbumin (OVA)/alum allergic airway inflammation model. In this model, TSLP protein was found in the BAL and lung after intranasal OVA challenge, and disease symptoms were curtailed in the absence of TSLPR or when TSLP activity was blocked by antibody or recombinant TSLPR protein (Zhou et al., 2005; Al Shami, Spolski, Kelly, Keane-Myers, & Leonard, 2005; Shi et al., 2008; Li et al., 2010; Zhang, Huang, Hu, Song, & Shi, 2011). In an OVA-driven mouse model of allergic rhinitis, blocking TSLP also inhibited disease development (Miyata et al., 2008).

Most data currently point to a primary role for TSLP in the sensitization/priming stage of allergic airway disease. TSLP produced by activated human-derived lung cells stimulated human DCs to prime $CD4^+$ TH2 cell development and mast cell production of TH2-associated cytokines (Soumelis et al., 2002; Allakhverdi et al., 2007; Bleck, Tse, Gordon, Ahsan, & Reibman, 2010). Furthermore, multiple studies have shown that TSLP-mediated

DC activation was responsible for the disease phenotype observed in mouse models of asthma (Zhou et al., 2005; Seshasayee et al., 2007; Shi et al., 2008; Li et al., 2010; Zhang et al., 2011). TSLP-induced DC expression of costimulatory molecules, in particular OX40L, and DC production of TH2 chemokines, such as CCL17 and CCL21, are likely the predominant mechanisms of action (Zhou et al., 2005; Seshasayee et al., 2007). However, TSLP may also influence the challenge stage of allergic airway disease by supporting TH2 $CD4^+$ T cell cytokine production (Shi et al., 2008; Miyata et al., 2008; Li et al., 2010; Zhang et al., 2011; Al Shami et al., 2005; He et al., 2008). As mentioned above, TSLP may also influence the regulatory T cell compartment. Several reports have shown the ability of TSLP to promote the development of thymic regulatory T cells (Tregs) *in vitro* (Mazzucchelli et al., 2008; Hanabuchi et al., 2010); however, *in vivo*, its role is less clear. In allergic airway disease, TSLP inhibited IL-10 mediated Treg function and the formation of inducible Tregs to exogenous antigen (Nguyen, Vanichsarn, & Nadeau, 2010). Importantly, the BAL fluid from asthmatics inhibited pulmonary Treg function in a TSLP-dependent manner (Nguyen et al., 2010). In the OVA allergen model, TSLP was shown to interfere with tolerance by inhibiting the generation of allergen-specific Tregs(Lei et al., 2011). In the same model, nucleotide-binding oligomerization domain-containing protein 2 (Nod2), and to a lesser extent Nod1 stimulation blocked tolerance to OVA intranasal challenge in a TSLP- and OX40L-dependent manner (Duan et al., 2010). In this model, loss of TSLP signaling correlated with increased antigen-specific $FOXP3^+$ T cells following Nod2 stimulation.

A variety of stimuli, such as IL-4, IL-13, TNF-α, IL-1, bacterial peptidoglycan, lipoteichoic acid, double-stranded RNA (dsRNA), respiratory viruses, air pollutants and allergens have been shown to induce TSLP expression by lung-derived parenchymal cells and immune cells (Soumelis et al., 2002; Allakhverdi et al., 2007; Lee & Ziegler, 2007; Zhang et al., 2007; Bleck et al., 2010; Kouzaki et al., 2009; Smelter et al., 2010; Kashyap et al., 2011; Kato & Schleimer, 2007). In particular, stimulation of Nod1 and Nod2 in non-hematopoietic cells were potent inducers of TH2 immunity via TSLP (Magalhaes et al., 2011). These stimuli likely all drive NF-κB-dependent expression of TSLP, as was shown to occur in human lung epithelial cells (Lee & Ziegler, 2007). Furthermore, TSLP transcription was negatively regulated by 9-cis-retinoic acid via retinoid X receptors in lung cells (Lee, Headley, Iseki, Ikuta, & Ziegler, 2008). Exposure to certain infectious agents or repeated environmental irritants may prime production of TSLP, leading to TH2-mediated human disease. For example, even in the absence of known lung disease, lung samples from smokers contained increased TSLP levels as compared to nonsmokers (Ying et al., 2008). In addition, lung epithelial cells from asthmatics produced more TSLP in response to dsRNA (viral analog) stimulation in culture (Uller et al., 2010; Brandelius et al., 2011), which may explain, at least in part, why patients with asthma tend to suffer more airway dysfunction after respiratory infections compared to healthy individuals (Jackson & Johnston, 2010). This aberrant TSLP production in response to lung insults may thus influence both the susceptibility of certain individuals to develop allergic respiratory diseases such as asthma, as well as the clinical complications that arise after environmental insults to the lungs of these individuals.

Collectively, these data illustrate that aberrant lung expression of TSLP is associated with human allergic airway disease and can mimic asthma-like disease in mice. According to genetic studies and *in vitro* analyses, lung samples from individuals with asthma or COPD produce more TSLP in response to lung insult as compared to samples from healthy individuals. Clinical trials targeting TSLP in these conditions are currently underway. According to mouse asthma models, TSLP appears to influence the sensitization stage of allergic airway responses, but a more in depth examination of TSLP's influence on the allergic effector response is required. Where and when TSLP acts during allergic airway disease will likely explain any trial results and dictate future therapeutic design.

C. Intestinal Inflammation

TSLP is constitutively expressed in both the mouse and human gastrointestinal tract, but can be further induced by a variety of cytokines, microbes and microbial products (Rimoldi et al., 2005; Zaph et al., 2007; Taylor et al., 2009; He et al., 2007; Tanaka et al., 2010; Zeuthen, Fink, & Frokiaer, 2008; Humphreys, Xu, Hepworth, Liew, & Grencis, 2008). Mice carrying gene deletions specifically affecting the gut mucosa provide additional clues into the regulation of TSLP expression within the gut. TSLP mRNA levels were significantly decreased in mice with intestinal epithelial-specific deletion of Dicer (Biton et al., 2011), an enzyme involved in microRNA biosynthesis, or IκB kinase-β (Zaph et al., 2007). Both of these knockout mice showed increased susceptibility to infection with the mouse whipworm *Trichuris muris*. TSLP expression was also decreased in mice carrying a missense mutation in the *Muc2* mucin gene that resulted in an epithelial defect and spontaneous colitis (Eri et al., 2011). In *in vitro* analyses of TSLP intestinal function, human colonic or gastric epithelial-derived TSLP has been implicated in conditioning DCs to drive development of inflammatory TH2 cells (Kido et al., 2010), regulatory T cells (Iliev, Mileti, Matteoli, Chieppa, & Rescigno, 2009) or T cell-independent IgA(2) class switching (He et al., 2007). While supernatants from both human and mouse intestinal epithelial cells (IECs) can condition DCs to drive Treg differentiation, the requirements for TSLP may differ in humans and mice, since the presence of TSLP was required in mouse but not human IEC supernatants to drive a tolerigenic DC phenotype (Iliev et al., 2009; Iliev et al., 2009). Additional studies are just beginning to define whether and under what conditions TSLP may function in these pathways *in vivo*.

As is seen in atopic diseases of the skin and lung, aberrant expression of TSLP was also seen in allergic diseases of the gut. Polymorphisms in TSLP and the TSLPR were associated with the food allergy-related disorder eosinophilic esophagitis (EoE), and this association persisted when comparing EoE patients with allergic individuals without EoE (Rothenberg et al., 2010; Sherrill et al., 2010). Additionally, TSLP mRNA expression was higher in the esophagus of pediatric patients with EoE compared to controls, and was decreased in homozygotes of the protective GG minor allele for the rs3806932 SNP. Some studies suggest, however, that TSLP not only plays an important role in the promotion of TH2 responses, but is also a key player in maintaining intestinal homeostasis and modulation of TH1/TH17 inflammation. In contrast to the increased TSLP expression seen in EoE, decreased TSLP expression was seen in non-inflamed colonic tissue in Crohn's disease (CD) and ulcerative colitis (UC), the two types of inflammatory bowel disease (IBD) (Noble et al.,

2010; Noble et al., 2008; Rimoldi et al., 2005; Iliev et al., 2009). However, studies of UC have indicated that in inflamed tissue, TSLP expression is upregulated compared with non-inflamed tissue from either UC patients or controls (Noble et al., 2008; Tanaka et al., 2010).

Mouse models of TH2- and TH1-type inflammation also suggest important roles for TSLP in TH2-mediated immunity, maintenance of homeostasis and modulation of TH1/TH17 responses within the gut. TSLP was required to induce diarrheal disease in a mouse model of food allergy (Blazquez, Mayer, & Berin, 2010) and protective TH2 responses to infection with *Trichuris muris* (Zaph et al., 2007). However, TSLP was not required for oral tolerance to OVA, or for anaphylaxis and IL-4, IL-13 and IgE production following intragastric OVA/cholera toxin sensitization and challenge (Blazquez et al., 2010). Additionally, other helminths such as *Heligmosomoides polygyrus*, *Nippostrongylus brasiliensis* and *Schistosoma mansoni* still induced TH2 responses in TSLPR knockout mice, although in some cases, these responses were modified or slightly attenuated (Massacand et al., 2009; Ramalingam et al., 2009). Thus, while TSLP may promote TH2 responses in the gut, it is not absolutely required for TH2-type inflammation. In contrast to *T. muris*, both *H. polygyrus* and *N. brasiliensis* produce excretory/secretory (ES) products that acted on DCs to attenuate IL-12/23p40 production. Of note, protective TH2 responses can be induced in *T. muris* infections in the absence of TSLP following the blockade of either IFN-γ or IL-12/23p40 (Taylor et al., 2009; Massacand et al., 2009), suggesting that TSLP may play a prominent role in attenuating TH1 and TH17 responses.

Studies using mouse models of colitis have demonstrated important effects of TSLP in modulating the disease phenotype in intestinal inflammation, although there have been some conflicting results. In a chemical colitis model using dextran sulfate sodium (DSS), Taylor *et al*. showed that mice lacking the TSLPR developed more acute weight loss and increased colonic inflammation that correlated with higher levels of IFN-γ and IL-17A within the mesenteric lymph nodes (Taylor et al., 2009). In contrast, Reardon *et al*. reported comparable disease onset and severity in the DSS colitis model between mice that lack TSLP signaling versus controls. However, while wild-type mice recovered after DSS withdrawal, mice lacking either TSLP or its receptor had progressive disease and weight loss (Reardon et al., 2011). Reardon *et al.* showed that secretory leukocyte peptidase inhibitor (SLPI) was induced in DSS colitis in wild-type mice and that this induction was lost in TSLP knockout (TSLP KO) mice. Neutrophil elastase (NE) is a target of SLPI, and functions to degrade a number of substrates, including progranulin, a protein important in wound healing. Consistent with a role for TSLP in the inhibition of NE, TSLP KO mice displayed increased NE activity after treatment with DSS, and inhibition of NE reduced mortality in TSLP KO mice in this colitis model. While methodological differences may account for some of the discrepancies between these studies, a growing body of evidence demonstrates that differences in microbiota among various facilities can have profound effects on the development and function of the intestinal as well as systemic immune system (Gill & Finlay, 2011). Thus, further exploration of how the gut microbiota affects TSLP expression and function may be warranted.

These studies support a role for TSLP in the promotion of TH2 responses in the gastrointestinal system, but also provide important evidence that TSLP plays a key role in

the maintenance of immune homeostasis within the gut. Not only does TSLP function to attenuate TH1/TH17 responses, but also acts directly on the intestinal epithelium to support wound healing in colitis. Whether TSLP also contributes to wound healing and blockade of TH1/TH17 responses at other sites remains to be determined.

D. Cancer

A series of recent studies have implicated TSLP in the growth and metastasis of breast and pancreatic cancer, especially those which display an increased infiltration of TH2 cells (De Monte et al., 2011; Olkhanud et al., 2011; Pedroza-Gonzalez et al., 2011). Breast and pancreatic cancer cells and cancer-associated fibroblasts have been shown to produce TSLP in response to tumor-derived inflammatory cytokines and possibly other unidentified stimuli (De Monte et al., 2011; Olkhanud et al., 2011; Pedroza-Gonzalez et al., 2011). Furthermore, treatment of DCs with supernatants from these cells induced theTH2-attracting chemokines CCL17 and CCL22, as well as upregulation of DC costimulatory molecules CD80, CD86, OX40L and TSLPR, in a TSLP-dependent manner. Additionally, these primed DCs were able to promote TH2-polarizition of $CD4^+$ T cells *in vitro*. In support of these *in vitro* data, activated DCs and CCL17 and CCL22 were detected in the tumor and draining lymph nodes, but not non-draining lymph nodes of human patients (De Monte et al., 2011). Importantly, a decreased ratio of TH1/TH2 cells in human pancreatic cancer cases was associated with disease progression and was an independent prognostic marker of reduced survival (De Monte et al., 2011). While breast cancer cells with intact TSLP expression were able to induce tumor growth and metastasis in mice, shRNA knockdown of TSLP in these cells resulted in clones with minimal growth or metastasis (Olkhanud et al., 2011). Tumor progression and metastasis of an injected breast cancer or melanoma cell line was also decreased in TSLPR-deficient mice compared to wild-type mice (Olkhanud et al., 2011).

Previous work has shown that TH2 cytokines promote disease progression through increased survival of cancer cells, M2 macrophage differentiation, and fibrosis (collagen degradation and synthesis) (Wynn, 2004; Aspord et al., 2007; Mantovani, Romero, Palucka, & Marincola, 2008; Joyce & Pollard, 2009). TSLP may be linked to these phenomena in some human cancers, possibly based on its ability to drive TH2 differentiation and M2 macrophage differentiation ((Ziegler, 2010) and Han, H. and Ziegler, S.F., manuscript submitted). Alternatively, TSLP may promote tumor progression by controlling Treg migration. CCL22 production in human breast cancer is involved in the influx of tumor Tregs that may then alter the immunoregulatory environment (Gobert et al., 2009; Ménétrier-Caux, Gobert, & Caux, 2009). Further investigation is needed to identify the important sources and targets of TSLP within the tumor environment.

In addition to the association of TSLP with certain solid tumors, the TSLPR has been shown to be over-expressed in 5 to 10 percent of childhood B cell progenitor acute lymphoblastic leukemia (ALL) cases and approximately 60 percent of acute lymphoblastic leukemia cases in children with Down's Syndrome (Roll & Reuther, 2010; Tasian & Loh, 2011; Mullighan et al., 2009; Russell et al., 2009; Ensor et al., 2011). Approximately 15 percent of adult and high-risk pediatric B-ALL that lack characteristic rearrangements demonstrated TSLPR over-expression (Yoda et al., 2010). In addition, some cases of activating TSLPR mutations

were found(Chapiro et al., 2010). In almost all cases, TSLPR over-expression was associated with intra-chromosomal deletion or rearrangement of the TSLPR/CRLF2 locus with the immunoglobulin heavy chain (IGH) locus, placing TSLPR/CRLF2 under alternate transcriptional control downstream of the P2YR8 promoter (Russell et al., 2009; Mullighan et al., 2009; Yoda et al., 2010). These rearrangements were highly correlated with the presence of JAK2 mutations and were associated with a poor prognosis (Roll & Reuther, 2010; Mullighan et al., 2009; Russell et al., 2009; Cario et al., 2010; Harvey et al., 2010; Yoda et al., 2010; Ensor et al., 2011). In murine Ba/F3 cells, expression of TSLPR and JAK2 mutant alleles promoted growth factor-independent growth (Mullighan et al., 2009; Yoda et al., 2010). Mice with systemic over-expression of TSLP may provide a model for understanding the signaling mechanisms involved. In particular, loss of keratinocyte-specific Notch signaling resulted in high systemic levels of TSLP which correlated with a rapid expansion of pre-B cells in the early postnatal period that contributed to early mortality in these animals (Demehri et al., 2008). Interestingly, over-expression of TSLP early in the postnatal period was sufficient to drive a B cell lymphoproliferative disorder, but administration or induction of TSLP after postnatal day 14 was not, although other studies have shown expansion of B cell compartments following TSLP expression in adult mice (Astrakhan et al., 2007).

The association of TSLP and TSLP signaling pathways with hematologic malignancies as well as solid tumors implicates TSLP/TSLPR in numerous regulatory pathways that support cell growth and survival in cancer. In B-ALL, activation of signaling pathways downstream of TSLP directly promotes the growth and survival of malignant cells, whereas in breast and pancreatic cancer, TSLP likely contributes to multiple components of the tumor environment that affect growth and metastasis as well as immune evasion. Several reports suggest that TSLP/TSLPR may be useful as a prognostic marker and may present a novel target for therapeutic intervention in cancer.

E. Other Autoimmune Diseases and Issues of Tolerance

Mouse models with constitutive or inducible over-expression of TSLP have demonstrated that TSLP can be associated with autoimmune phenomena. TSLP over-expression in these mice was associated with the development of cryoglobulinemic glomerulonephritis due to increased production and kidney deposition of systemic polyclonal IgM and IgG via a monocyte/macrophage dependent mechanism (Taneda et al., 2001; Astrakhan et al., 2007). In addition, these mice developed red blood cell-specific auto-antibodies and autoimmune hemolytic anemia in a $CD4^+$ T cell and IL-4-dependent manner (Iseki et al., 2012). Whether TSLP is involved in human mixed cryoglobulinemia or autoimmune hemolytic anemia is unknown.

As discussed earlier, TSLP expression was decreased in IBD, a disorder that is thought to arise due to inappropriate immune activation against normally harmless microflora. Additionally, loss of TSLP signaling in a mouse model of autoimmune gastritis resulted in more severe disease (Nishiura et al., 2012). Although the impact of TSLP on colitis in mice appears more complex (Taylor et al., 2009; Reardon et al., 2011), this supports a model in which loss of TLSP, which can block TH1/TH17 responses, leads to increased

inflammation. However, data from humans and mouse models suggest that TSLP may actively promote inflammation in TH1/TH17-associated autoimmune diseases such as rheumatoid arthritis (RA) and multiple sclerosis (MS). In a proteoglycan-induced arthritis mouse model of RA, TSLPR-deficient mice had reduced immunopathology associated with decreased levels of production of IL-17, IL-1β, and IL-6, but increased IFNγ and IL-10 (Hartgring et al., 2011). Furthermore, blocking TSLP in a collagen-induced arthritis model ameliorated disease, while administering recombinant TSLP protein exacerbated disease (Koyama et al., 2007; Hartgring et al., 2011). Increased synovial concentrations of TSLP, as well as TNFα, have also been seen in synovial fluid from RA patients compared to samples from patients with osteoarthritis. In *in vitro* studies, TSLP-primed human myeloid DCs induced proliferation of self-reactive $CD4^+$ T cells capable of TH1 or TH2 differentiation, and TSLP priming of DCs, in conjunction with TLR3 ligand, supported TH17 differentiation (Watanabe et al., 2004; Tanaka et al., 2009; Koyama et al., 2007). Thus, although the role of TSLP in RA is largely undefined, these data provide intriguing evidence of its possible involvement.

SNPs in the IL-7Rα gene locus have been associated with multiple sclerosis (MS) and altered Treg numbers or function (Gregory et al., 2007; Lundmark et al., 2007). While TSLPR pairs with IL-7Rα and TSLP can affect Treg development, neither disease has yet been directly linked to TSLP. However, administration of TSLP or TSLP-treated bone marrow-derived DCs into nonobese diabetic mice prevented the development of diabetes in these mice (Besin et al., 2008), suggesting a possible role for TSLP in disease therapy. Although the mechanisms involved in protection from diabetes have not been determined, protection was associated with an increased number of Tregs. One final link that has been made between TSLP and immune tolerance is in maternal-fetal tolerance during pregnancy(Li & Guo, 2009). TSLP was produced and secreted by first semester trophoblasts, and tissue from normal pregnancies demonstrated a TH2 bias and higher levels of TSLP expression than samples from miscarriages (Guo et al., 2010; Pu et al., 2012; Wu, Guo, Jin, Liang, & Li, 2010). Thus, while TSLP expression and a TH2 bias may lead to disease progression in cancer, TSLP may contribute to tolerance at the maternal-fetal interface.

V. Conclusion

Much progress has been made in the understanding of TSLP biology and its role during TH2-type inflammation. Multiple cell lineages express the functional TSLPR that helps drive the immune response. More recent data has illustrated that TSLP is also involved in numerous disorders beyond just allergy, and may play a role in maintaining homeostasis in diseases such as IBD or in disease progression in cancer and autoimmunity. In order to utilize the knowledge gained about TSLP's biological effects, a better understanding of cell-specific signaling pathways must be delineated. Of utmost importance is deciphering whether TSLP invokes similar signaling pathways within different cells. Knowledge of the key targets and sources of TSLP in different disease states will also be important in furthering our comprehension of the pathophysiology of TSLP-associated disorders. Tools that can address these questions, such as approaches that use conditional deletion of the

TSLPR and cytokine, will be important in the continued investigation of the role of TSLP during both atopic and non-atopic conditions.

Acknowledgments

Support was provided by the Crohn's and Colitis Foundation of America (F.R.) and NIH grants 5T32AI007411-19 (B.D.B. and T.A.S.) and AI068731, AR056113, 5AR055695, HL098067, HL102708, AR059058 (S.F.Z.).

Abbreviations

TSLP	thymic stromal lymphopoietin
TSLPR	thymic stromal lymphopoietin receptor
IL-7Rα	interleukin-7 receptor alpha
JAK	Janus kinase
STAT	Signal Transducers and Activators of Transcription
γ_c	common γ receptor chain
AD	atopic dermatitis
NK	natural killer
SNP	single nucleotide polymorphism
NS	Netherton's syndrome
SPINK5	serine peptidase inhibitor Kazal-type 5
LEKTI	lymphoepithelial Kazal-type–related inhibitor
RXR	retinoid X receptor
PAR-2	protease-activated receptor 2
LC	Langerhans cell
EC	epicutaneous
FITC	fluorescein isothiocyanate
CHS	contact hypersensitivity
AR	allergic rhinitis
COPD	chronic obstructive pulmonary disease
BAL	bronchoalveolar lavage
SPC	surfactant protein C
FOXP3	forkhead box P3
EoE	eosinophilic esophagitis
CD	Crohn's disease
UC	ulcerative colitis

IBD	inflammatory bowel disease
DSS	dextran sulfate sodium
SLPI	secretory leukocyte peptidase inhibitor
NE	neutrophil elastase
ALL	acute lymphoblastic leukemia
B-ALL	B cell ALL
CRLF2	cytokine receptor-like factor 2
TNF	tumor necrosis factor
OX40L	OX40 ligand (CD134)
TLR	toll-like receptor
APRIL	a proliferation inducing ligand
IEC	intestinal epithelial cell

VIII. Reference List

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CHEST

Original Research

ASTHMA

Mast Cell-Airway Smooth Muscle Crosstalk

The Role of Thymic Stromal Lymphopoietin

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Background: **The mast cell localization to airway smooth muscle (ASM) bundle in asthma is important in the development of disordered airway physiology. Thymic stromal lymphopoietin (TSLP) is expressed by airway structural cells. Whether it has a role in the crosstalk between these cells is uncertain. We sought to define TSLP expression in bronchial tissue across the spectrum of asthma severity and to investigate the TSLP and TSLP receptor (TSLPR) expression and function by primary ASM and mast cells alone and in coculture.**

Methods: **TSLP expression was assessed in bronchial tissue from 18 subjects with mild to moderate asthma, 12 with severe disease, and nine healthy control subjects. TSLP and TSLPR expression in primary mast cells and ASM was assessed by immunofluorescence, flow cytometry, and enzyme-linked immunosorbent assay, and its function was assessed by calcium imaging. The role of TSLP in mast cell and ASM proliferation, survival, differentiation, synthetic function, and contraction was examined.**

Results: **TSLP expression was increased in the ASM bundle in mild-moderate disease. TSLP and TSLPR were expressed by mast cells and ASM and were functional. Mast cell activation by TSLP increased the production of a broad range of chemokines and cytokines, but did not affect mast cell or ASM proliferation, survival, or contraction.**

Conclusions: **TSLP expression by the bronchial epithelium and ASM was upregulated in asthma. TSLP promoted mast cell synthetic function, but did not contribute to other functional consequences of mast cell-ASM crosstalk.** ***CHEST 2012; 142(1):76–85***

Abbreviations: ASM = airway smooth muscle; CFSE = carboxyfluorescein succinimidyl ester; DAPI = 4′,6-diamidino-2-phenylindole; DMSO = dimethyl sulfoxide; FBS = fetal bovine serum; GINA = Global Initiative for Asthma; GMFI = geometric mean fluorescence intensity; HLMC = human lung mast cell; HMC-1 = human mastocytoma cell line; IQR = interquartile range; ITS = insulin transferrin sodium selenite; mRNA = messenger RNA; MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium; rh-TSLP = recombinant human thymic stromal lymphopoietin; Th2 = T-helper cell type 2; TNF = tumor necrosis factor; TSLP = thymic stromal lymphopoietin; TSLPR = thymic stromal lymphopoietin receptor

Asthma affects 5% to 10% of adults, and 10% of those with asthma have severe disease.[1] These patients consume over 50% of the health-care resources.[2] Asthma is characterized by variable airflow obstruction; airway hyperresponsiveness and mast cell-airway smooth muscle (ASM) interactions are important in the development of disordered airway physiology.[3,4]

Thymic stromal lymphopoietin (TSLP) is implicated in both the innate and adaptive immune response.[5] T-helper cell type 2 (Th2) polarization of the inflammatory response is an important component of the asthma paradigm.[5,6] A major effector axis resulting in this polarization is the recognition of allergen presented by dendritic cells in local lymph nodes to the $CD4^+$ T cell. The differentiation of naive T cells or reactivation of memory T cells is dependent upon costimulatory molecules, such as OX40, and their cognate ligand OX40L,[5-10] which is increased in the

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bronchial submucosa in asthma[9] and upregulated by TSLP.[5,8] TSLP messenger RNA (mRNA) is upregulated in the bronchial epithelium and submucosa in asthma in response to allergen, viruses, and environmental stimuli.[11] TSLP is expressed by mast

cells[12,13] and ASM[14,15] supporting the view that TSLP may have a role beyond Th2 polarization and may be important in mast cell-ASM interactions.

We hypothesized that expression of TSLP and TSLP receptor (TSLPR) is increased in asthma and that this axis plays a role in mast cell-ASM crosstalk. To test our hypothesis, we investigated TSLP expression in bronchial tissue across the spectrum of asthma severity compared with healthy control subjects, and defined TSLP and TSLPR expression and function by ASM and mast cells.

Materials and Methods

Subjects

Subjects were recruited from Leicester, England. Asthmatic subjects had a consistent history and objective evidence of asthma.[16] Asthma severity was defined by Global Initiative for Asthma (GINA) treatment steps (mild-moderate GINA 1-3, severe GINA 4-5).[17] Subjects underwent clinical characterization including sputum induction[18] and video-assisted fiber-optic bronchoscopic examination.[19] The study was approved by the Leicestershire Ethics Committee, approval number 4977, and all patients gave their written informed consent.

Cell Isolation and Culture

Pure ASM bundles were isolated from bronchoscopic samples (n = 14 asthma, n = 8 nonasthma) and from lung resection (n = 1). ASM was cultured and characterized as previously described.[16,20] The human lung mast cells (HLMCs) were isolated and cultured[16,20] from nonasthmatic lung (n = 10). The human mastocytoma cell line (HMC-1) was a generous gift from J. Butterfield, MD, (Mayo Clinic).

TSLP/TSLPR Expression

Sequential 2-μm sections were cut from glycomethacrylate-embedded bronchial biopsies and stained using a sheep polyclonal antihuman TSLP antibody (R&D Systems), monoclonal antibody mast cell tryptase (clone no. AA1; Dako), and appropriate isotype control sheep IgG (R&D Systems) and mouse IgG1 (Dako), respectively. The number of positively stained nucleated cells was enumerated per mm^2 of the lamina propria by a blinded observer. TSLP expression by the ASM or epithelium was also assessed using (1) a semiquantitative intensity score of no staining = 0, low = 1, moderate = 2, and high = 3 and (2) thresholding of red hue using Image J software (ImageJ 1.40g/java 1.6.0_05; NIH Image). The red/green/blue image was converted to hue/saturation/brightness stack. Hue image was thresholded to include pixels with non-red hue 30-210 (scale of 0-255) for all images, and the percentage area covered by red hue pixels was calculated by deducting non-red pixels from the total.[21] Tryptase was colocalized with TSLP within the ASM using sequentially cut sections and a minimum area of 0.1 mm^2 was considered assessable as described previously.[19]

TSLP and TSLPR expression was assessed in ASM, HLMC, and HMC-1 cells by flow cytometry and immunofluorescence. Isotype control subjects were used where appropriate (Dako). TSLP protein release was measured in ASM, HLMC, HMC-1, and sputum by enzyme-linked immunosorbent assay (R&D Systems Inc). Recombinant TSLP recovery was unaffected by the mucolytic dithiothreitol. TSLP and TSLPR mRNA levels were examined in ASM cells using the Human Genome U133A probe array (GeneChip; Affymetrix).[22]

Functional Assays

Changes in intracellular calcium $[Ca^{2+}]_i$ concentrations in ASM cells in response to recombinant human TSLP (rh-TSLP) were measured by fluo-3/Fura Red acetoxymethyl ester ratios (Invitrogen) using flow cytometry. ASM cells were primed with rh-TSLP and collagen gel contraction assessed. Gel surface area was measured using ImageJ by a blinded observer. The concentration of a panel of cytokines and chemokines were measured in ASM and HMC-1 cells stimulated with TSLP by electrochemiluminescence detection and pattern arrays (Mesoscale Discovery).

Proliferation and Apoptosis

ASM proliferation was assessed by cell counts and the CellTiter 96 Aqueous One Solution with the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H (MTS) (Promega) according to the manufacturer's instructions. Morphologic features of apoptosis (nuclear condensation and fragmentation) were assessed by DAPI (4′,6-diamidino-2-phenylindole) staining.

Coculture ASM and HLMC

HLMC proliferation was assessed by carboxyfluorescein succinimidyl ester (CFSE) (CellTrace Proliferation Kit; Invitrogen) in HLMC cocultured with ASM cells. Changes in ASM phenotype were studied in the presence of HLMC lysate and stained with α-smooth muscle actin (Sigma-Aldrich).

Statistical Analysis

Statistical analysis was performed using PRISM, version 4 (GraphPad Software). Parametric data were presented as mean (SEM) and nonparametric data as median (interquartile range [IQR]). Parametric data were analyzed with paired and unpaired *t* tests or one-way analysis of variance and the Tukey posthoc test for intergroup comparison as appropriate. Nonparametric data were analyzed using Mann-Whitney or Kruskal-Wallis tests and the Dunn test for posthoc comparison as appropriate. Correlations between parametric data were assessed by Pearson correlation and nonparametric data by Spearman rank correlation. A P value $< .05$ was considered significant.

Manuscript received July 15, 2011; revision accepted October 12, 2011.
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Funding/Support: This work was supported by GlaxoSmithKline. Dr Brightling was supported by a Wellcome Senior Clinical Fellowship [082265].
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DOI: 10.1378/chest.11-1782

Results

TSLP Expression in the ASM Bundle in Asthma

The clinical characteristics of the subjects assessed by immunohistochemistry are shown in Table 1. TSLP staining was apparent in the epithelium, ASM, and cells within the lamina propria (Fig 1A).

TSLP expression was significantly increased in the ASM and lamina propria in mild to moderate asthma and in the epithelium across all severities (Figs 1B-D, Table 1). There was a good correlation between TSLP expression assessed by the semiquantitative score and by the percentage of hue thresholding ($r = 0.94$, $P < .001$). The intensity of TSLP expression (percentage of red hue) in the ASM and epithelium were correlated ($r = 0.77$, $P < .0001$) and both were related to the number of epithelial cells in the sputum ($r = 0.58$, $P < .01$ and $r = 0.64$, $P < .001$, respectively). $TSLP^+$ cells/mm^2 in the lamina propria was also correlated with TSLP intensity (percentage of red hue) in the ASM, epithelium ($r = 0.49$, $P = .002$ and $r = 0.46$, $P = .003$, respectively) and sputum epithelial cell counts ($r = 0.55$, $P < .01$). There were no correlations with other sputum cell counts or with lung function. The median (IQR) proportion of $TSLP^+$ cells in the lamina propria that were mast cells was 40% (77). All the inflammatory cells in the ASM that colocalized to TSLP were mast cells. Sputum TSLP was measured in 12 patients with asthma and four healthy control subjects and was below limit of detection in all except for two asthmatics.

TSLP Expression by Primary Cells

There was no difference in expression between ASM cells derived from patients with asthma and from normal control subjects in all studies (data not shown). Therefore, normal and asthmatic ASM data were combined throughout. TSLP expression was identified in ASM and human mast cells by immunofluorescence (Figs 2A, 2B) and flow cytometry (Figs 2C-F). The expression of TSLP in unstimulated ASM cells compared with isotype control was geometric mean fluorescence intensity (mean ΔGMFI) fold (95% CI) 2 (1.6-2.6); $P < .001$, n = 8, which was not affected by stimulation with IL-1β, tumor necrosis

Table 1—*Clinical Characteristics of Asthmatic and Healthy Control Subjects*

	Normal	Mild-Moderate Asthma GINA 1-3 (1 = 11, 2 = 1, 3 = 6)	Severe Asthma GINA 4-5 (4 = 7, 5 = 5)
No.	9	18	12
Age, y[a]	45 (27)	53 (29)	51 (14)
Male (female) sex	5 (4)	7 (11)	4 (8)
Never/current/ex-smokers	8/1/0	14/4/0	10/0/2
Atopy, %	44	67	75
Inhaled corticosteroids,[a] μg/d beclomethasone equivalent	0	0 (0-500)	1,800 (1,600-2,000)
Oral corticosteroid,[a] mg/d	0	0	0 (0-10)
PC_{20} FEV_1,[b] mg/mL	> 16	0.5 (0.2-1.3)[d]	0.8 (0.3-2.3)[d]
FEV_1% predicted[c]	94 (3)	82 (7)	77 (7)[d]
Pre-BD FEV_1/FVC,[c] %	82 (2)	72 (2)[d]	67 (5)[d]
BD,[c] %	0 (0)	11 (4)[d]	9 (2)[d]
Sputum cell counts			
TCC[c]	0.9 (0.1)	2.4 (0.5)	5.9 (1.4)[d]
Eosinophil,[a] %	0.4 (0.8)	1.0 (5.2)	4.6 (18.0)[d]
Neutrophil,[c] %	56 (12)	48 (7)	65 (7)
Macrophage,[c] %	37 (12)	39 (7)	18 (6)[d]
Lymphocyte,[a] %	1 (5)	1 (2)	0.3 (3.1)
Epithelial cells,[a] %	1 (12)	4 (11)	1 (2)
TSLP expression in bronchial biopsy			
Epithelium,[a] SQS	0 (0.3)	0.75 (1.9)[d]	0.75 (1.3)[d]
Epithelium,[a] % red hue	0 (4)	11 (19)[d]	11 (12)[d]
Lamina propria,[a] cells/mm^2	2.2 (4.1)	16.3 (59.5)[d]	15.8 (17.7)
Airway smooth muscle,[a] SQS	0.25 (0.4)	0.88 (0.7)[e]	0.75 (0.9)
Airway smooth muscle,[a] % red hue	2 (4)	10 (10)[d]	9 (9)

BD = bronchodilator; GINA = Global Initiative for Asthma; IQR = interquartile range; PC = provocation concentration; SQS = semiquantitative score; TCC = total cell count.

[a]Median (IQR).

[b]Geometric mean (95% CI).

[c]Mean (SE).

[d]$P < .05$ compared with control subjects.

[e]$P < .001$ compared with control subjects.

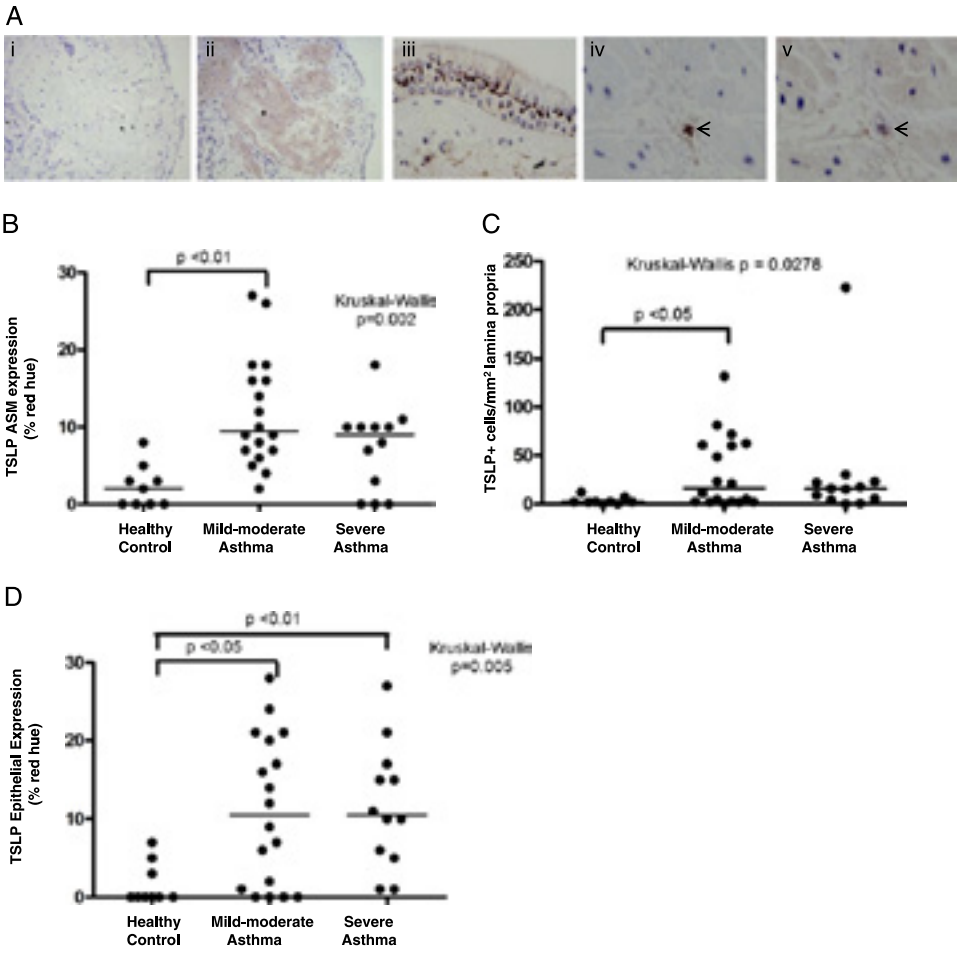

FIGURE 1. Mast cells in the ASM bundle express TSLP. A, Representative photomicrographs of a bronchial biopsy specimen from asthmatic subjects (original magnification, ×100) showing negative isotype control (i), TSLP staining in ASM bundles (ii), and epithelium (magnification ×400, iii). Sequential sections of the ASM bundle highlighting the same cells across sections showing mast cell tryptase-positive staining (iv) and TSLP (v). The short arrows illustrate the cells that are both tryptase$^+$ and TSLP$^+$ within the ASM bundle. B, Dot plot showing ASM TSLP expression determined by % red hue. C, TSLP-positive cells per square millimeter of lamina propria in subjects with and without asthma. D, TSLP expression in the epithelium by % red hue. Horizontal bar represents median. ASM = airway smooth muscle; TSLP = thymic stromal lymphopoietin.

factor-α (TNF-α), or IL-4 (Fig 2E). TSLP expression was also increased in unstimulated HMC-1 cells ΔGMFI fold (95% CI) 1.3 (1.07-1.5), $P = .01$, n = 5 and HLMC 2.8 (0.6-5), $P = .03$, n = 3, compared with isotype control (Fig 2F). TSLP was measurable in unstimulated ASM cells (305 ± 63 pg/10^6 cells, n = 11), HMC-1 cell supernatants (19 ± 1 pg/10^6 cells, n = 4), HLMC supernatants (82 ± 14 pg/10^6 cells, n = 3), and HLMC cell lysates (334.8 ± 54 pg/10^6 cells, n = 3), but was not affected by stimulation (Fig 2G). Resting HLMC released significantly more TSLP in cell supernatant compared with the resting HMC-1 cell mean difference ([95% CI] 59 [23-153] pg/10^6 cells, $P = .004$) (Fig 2G). Quantified by gene array analysis, TSLP mRNA was present in all ASM donors (1.47% ± 0.32%) of GAPDH mRNA (n = 11).

TSLPR Expression by Primary Cells

TSLPR expression was expressed in ASM and mast cells both by immunofluorescence (Figs 3A, 3B) and flow cytometry (Figs 3C, 3D). The expression of surface TSLPR in unstimulated ASM cells compared with isotype control was mean ΔGMFI fold (95% CI) 1.5 (1.1-1.8), $P = .01$, n = 7, which was not affected by stimulation with IL-1β, TNF-α, or IL-4 (Fig 3C), for unstimulated HMC-1 cells 1.4 (0.1-2), $P = .03$, n = 7, and for HLMC 1.8 (0.9-1.4); n = 4. TSLPR intracellular expression was detected in ASM and HMC-1 cells and was unaffected by stimulation (Fig 3D). Functional responses of TSLPR were studied; cells were loaded with fluo-3 and Fura Red and stimulated with rh-TSLP at 100-200 ng/mL to trigger Ca^{2+} flux through the membrane linked receptor (TSLPR) or

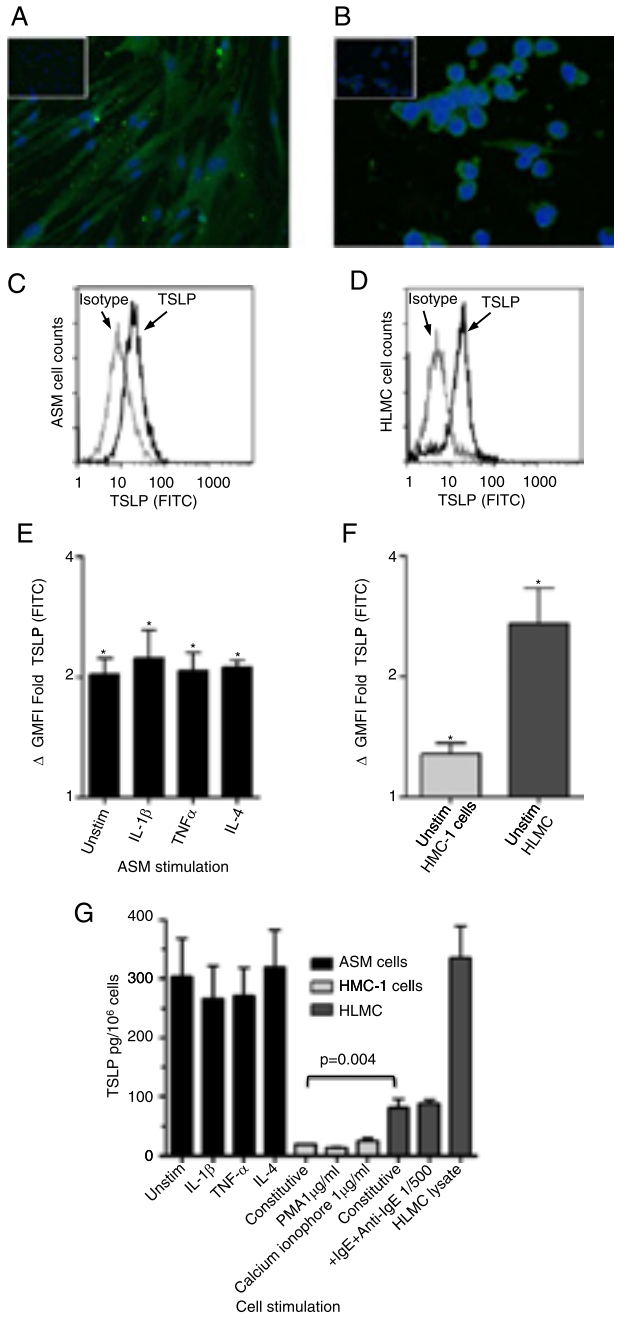

FIGURE 2. TSLP expressed by ex vivo ASM and mast cells. A, B, TSLP expression was confirmed in (A) ASM and (B) HMC-1 cells by immunofluorescence (nuclei stained blue, TSLP stained green, isotype shown as insert, magnification ×400, n = 3). C, D, The example fluorescent histograms for (C) ASM and (D) HLMC cells represent populations of TSLP (black line) plotted with the corresponding isotype control (gray line). E, The expression of intracellular TSLP was investigated by flow cytometry on unstimulated and stimulated ASM cells with 10 ng/mL proinflammatory cytokines IL-1β, TNFα, and IL-4 over 20 h (n = 8; $*P < .05$ compared with isotype control). F, Expression was also seen in unstimulated mast cells (n = 3-5; $*P < .05$ compared with isotype control). G, TSLP protein release was measured by enzyme-linked immunosorbent assay (ELISA) in ASM (n = 8-11, unstimulated and stimulated for 20 h), HMC-1 (n = 4), and HLMC (n = 3-5) supernatants (and lysate for HLMC). HMC-1 cell protein release was studied in cell supernatants following PMA stimulation with 1 μg/mL or calcium ionophore 1 μg/mL over 24 h and HLMC IgE sensitized (2.4 μg/mL) and then activated with anti-IgE (1:500) for 1 h. All data presented as mean ± SEM. Statistical differences were assessed using the *t* tests, and *P* values are as shown FITC = fluorescein isothiocyanate; GMFI = geometric mean fluorescence intensity; HLMC = human lung mast cell; HMC-1 = human mastocytoma cell line; PMA = phorbol myristate acetate; TNF = tumor necrosis factor; Unstim = unstimulated. See Figure 1 legend for expansion of other abbreviations.

calcium ionophore which induces a rapid release of Ca^{2+} from intracellular stores (Fig 3E). The response of HMC-1 cells and ASM cells stimulated with rh-TSLP showed a similar pattern with an increase in GMFI (fluo 3/Fura Red ratio, Fig 3E) for ASM cells stimulated with 100 ng/mL ($P = .03$, n = 5) and 200 ng/mL ($P = .04$, n = 8) rh-TSLP (Fig 3E) compared with baseline.

Neutralization of TSLP in Primary Cells

ASM assessed by the MTS assay demonstrated a significant increase in cell proliferation/metabolic activity after 96 h for cells cultured in either 10% fetal bovine serum (FBS) ($P = .004$) or ITS media compared with ASM at 0 h ($P = .04$) (Figs 4A, 4B). Recombinant TSLP (12.5-100 ng/mL) had no effect on the MTS assay in the presence of FBS and ITS media (data not shown). Neutralizing TSLP also had no effect on ASM metabolic activity both in FBS and ITS media (Figs 4A, 4B), and in contrast to the staurosporine positive control in FBS and ITS media ($P < .0001$, n = 6).

The percentage of ASM nuclei showing nuclear condensation and fragmentation characteristic of apoptosis, detected by DAPI staining, was unaffected by incubation with 100 ng/mL TSLP for 96 h (untreated, 6.3% ± 1.4% vs 100 ng/mL TSLP 9.6 ± 2.7, n = 6, $P = .2$). In the presence of staurosporine (1 μM, 96 h), a positive control ASM cells showed nuclear morphology characteristic of cells undergoing apoptosis compared with dimethyl sulfoxide (DMSO) control ($P < .0001$, n = 6) (Fig 4C). ASM cells primed for 48 h with rh-TSLP (10 ng/mL) embedded within collagen gels did not result in altered gel contraction compared with unprimed ASM over 7 days (n = 4) (Fig 4D).

ASM-Mast Cell Coculture

To track mast cells cocultured with ASM over 7 days, mast cells were labeled with fluorescent marker CFSE, a stable dye that is not passed between cells upon adhesion. Using flow cytometry, CFSE-labeled mast cells were gated (Fig 5A) and CFSE GMFI analyzed compared with cells cocultured with isotype and anti-TSLP (Fig 5B). Cocultured HLMC survived and proliferated with ASM cells alone and in the presence of anti-TSLP determined by CFSE (Fig 5B) and cell counts (Fig 5C).

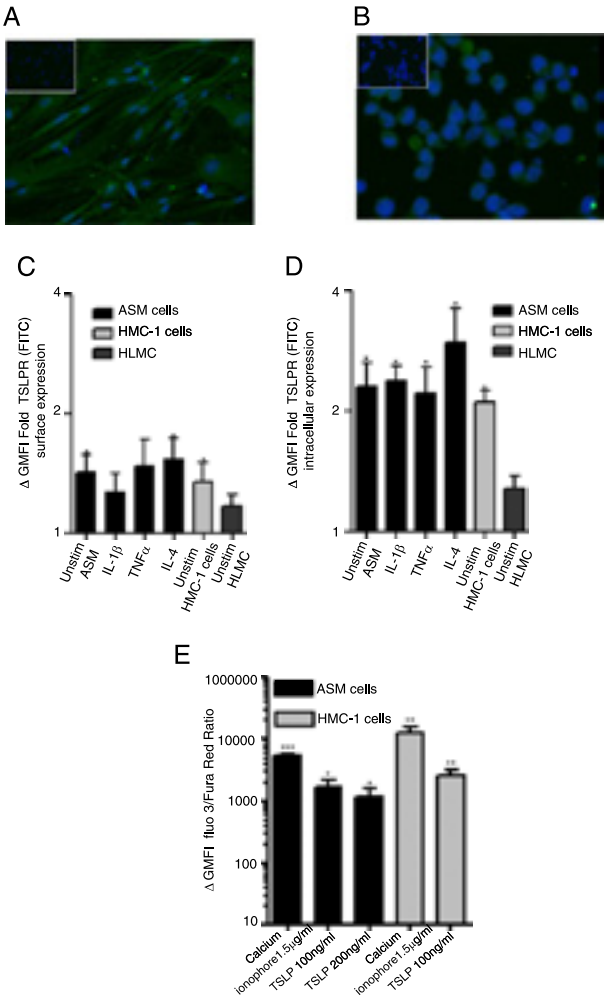

FIGURE 3. TSLPR expressed by ex vivo ASM and mast cells. A, B, TSLPR expression was confirmed in (A) ASM and (B) HMC-1 cells by immunofluorescence (nuclei stained blue, TSLPR stained green, isotype shown as insert, magnification ×400, n = 3). C, D, The expression of (C) surface and (D) intracellular TSLPR was investigated by flow cytometry on unstimulated ASM, mast cells, and stimulated ASM cells with 10 ng/mL proinflammatory cytokines IL-1β, TNFα, and IL-4 over 20 h (n = 3-7, $*P < .05$ compared with isotype control). TSLPR activation was studied by calcium flux assays in human ASM and HMC-1 cells. Cells were loaded with fluo-3 and Fura Red and baseline calcium levels were recorded for 60 s followed by the addition of either 100-200 ng/mL recombinant human TSLP (rh-TSLP) or 1.5 μg/mL calcium ionophore, (positive control) over a further 180 s (n = 5-8). E, The ΔGMFI was determined by the difference between the total stimulated GMFI minus the matched baseline GMFI for each cell type ($*P < .05$, $**P < .01$, $***P < .001$ compared with baseline GMFI). All data presented as mean ± SEM. Statistical differences were assessed using the *t* tests. TSLPR = thymic stromal lymphopoietin receptor. See Figure 1 and 2 legends for expansion of other abbreviations.

ASM cells cocultured with HLMC lysate (1:4 ratio of mast cells:ASM) showed a significant increase in metabolic activity compared with ASM alone over 7 days compared with baseline (n = 4) (Fig 5D). TSLP neutralization had no effect on ASM metabolic activity mediated by HLMC lysates (Fig 5D). ASM cells cultured with HLMC lysate showed increased α-smooth muscle actin GMFI compared with ASM cells, but was also unaffected by anti-TSLP (Figs 5E, 5F).

Chemokine and Cytokine Release in Human Cultured Cells

HMC-1 cell release of most chemokines and cytokines was significantly upregulated following TSLP (1 ng/mL) activation for 24 h (Table 2). ASM release of cytokines and chemokines was not upregulated by TSLP activation over 24 h (1-10 ng/mL, data not shown).

Discussion

We report for the first time, to our knowledge, that TSLP expression by ASM is increased in mild to moderate asthma, and that mast cells within the ASM bundle express TSLP. We confirm that the bronchial epithelium is an important source of TSLP. Primary ASM and mast cells also express TSLP and TSLPR constitutively. We confirmed that mast cells cocultured with ASM cells survive and proliferate, but that this was not affected by TSLP. Additionally, ASM contraction and synthetic capacity was not modulated by TSLP. In contrast, TSLP potently activated mast cells to release an array of cytokines and chemokines, suggesting that ASM-derived TSLP may play a role in mast cell activation.

TSLP is both necessary and sufficient for the development of Th2 cytokine-associated inflammation of the airways in rodents. Mice expressing a TSLP transgene in the airway epithelium develop a spontaneous, progressive inflammatory disease with all the characteristics of human asthma,[23] whereas direct intranasal delivery of TSLP (in the presence of antigen) leads to rapid onset of severe disease.[24] In human disease, genetic analysis has shown an association of polymorphisms in *TSLP* with asthma and airway hyperresponsiveness, IgE concentrations, and eosinophilia.[25-27] In addition, asthmatics have higher concentrations of TSLP in their lungs.[13] TSLP is expressed mainly by epithelial cells at barrier surfaces.[28] Factors known to be involved in either the development of asthma or the exacerbation of existing disease can induce TSLP expression in airway epithelial cells such as inflammatory cytokines and respiratory viruses.[28,29] We confirm here that TSLP expression was upregulated by the bronchial epithelium in asthma, independent of disease severity. Interestingly, the intensity of TSLP expression was related to the number of epithelial cells in the sputum supernatant, suggesting that TSLP expression was associated with epithelial damage. Other cells express TSLP including mast cells[12,13] and ASM.[14,15] TSLP expression was upregulated in the ASM bundle in chronic obstructive pulmonary disease[14] and increases with

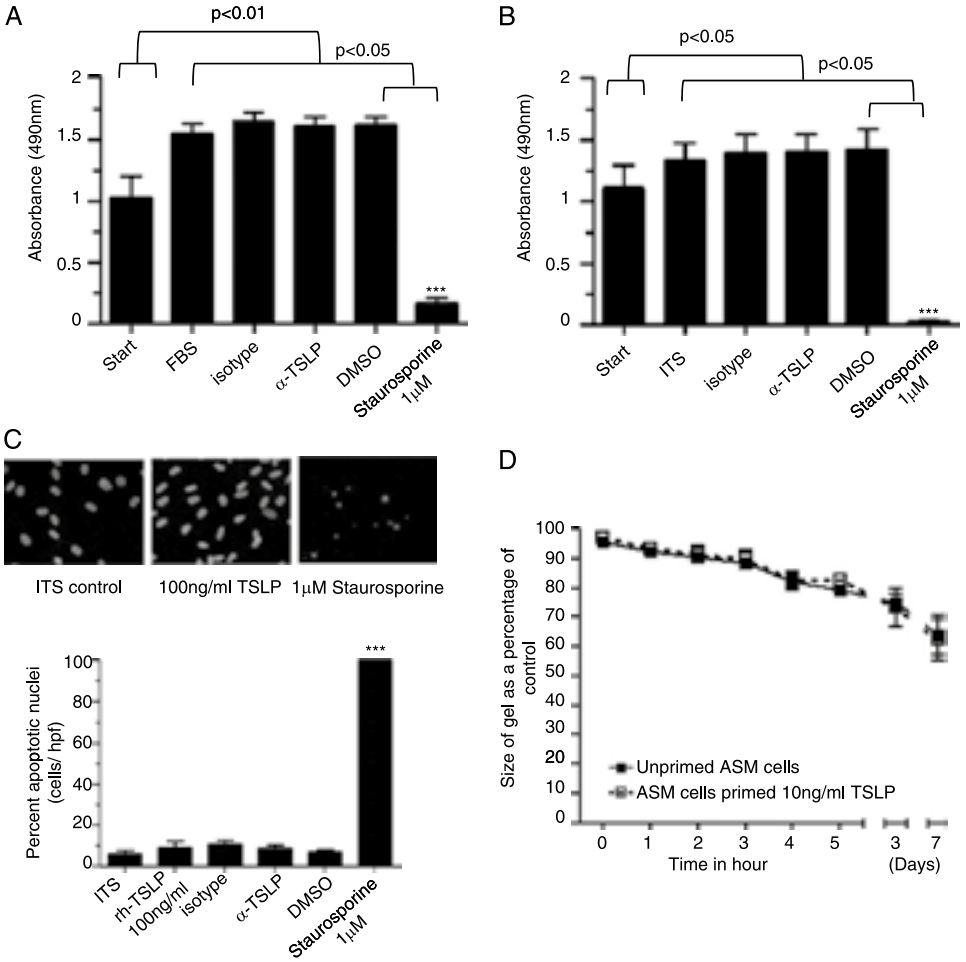

FIGURE 4. Neutralization of TSLP in ex vivo human cells. A, B, ASM cell metabolic activity or proliferation in the presence of (A) 10% FBS media and (B) serum-free ITS media was assessed over 96 h in the presence of isotype control, α-TSLP 1 μg/mL, DMSO, and 1μM staurosporine (positive control, n = 6). C, Representative micrographs of ASM cells showing DAPI (4′,6-diamidino-2-phenylindole) staining of cells in the presence of ITS media alone,100 ng/mL rh-TSLP, and 1μM staurosporine over 96 h. The percentage of apoptotic nuclei of ASM cells identified by nuclear morphology over 96 h of ASM cells alone in ITS, presence of rh-TSLP 100 ng/mL, α-TSLP, isotype, 1μM staurosporine, and DMSO control (n = 6). Comparisons were made between DMSO control vs staurosporin treated cells, ***$P < .001$. ASM cells were primed with TSLP (10 ng/mL) over 48 h and impregnated in the collagen gel and left in the gel without stimulation for 7 days (n = 4) to assess collagen gel contraction (D). All data presented as mean ± SEM. Statistical differences were assessed using the *t* tests and *P* values are as shown. DMSO = dimethyl sulfoxide; FBS = fetal bovine serum; ITS = insulin transferrin sodium selenite. See Figure 1-3 legends for expansion of other abbreviations.

exposure to cigarette smoke.[30] Here, we extend these observations to demonstrate for the first time that the number of cells in the lamina propria and the intensity of TSLP staining in the ASM bundle are also upregulated in mild to moderate, but not severe, asthma. Whether the relatively attenuated TSLP expression in severe disease represents the response to high-dose corticosteroid therapy or a feature of disease severity warrants further investigation.

Mast cell-ASM crosstalk has been implicated in the development of disordered airway physiology in asthma. Indeed, the number of mast cells within the ASM bundle is related to the degree of airway hyperresponsiveness.[19] Coculture of primary mast cells with ASM promotes mast cell activation,[31] differentiation,[32] survival and proliferation,[31,32] and ASM contractility.[33] We considered here whether the TSLP/TSLPR axis plays a role in these interactions. We confirmed that mast cells and ASM express both TSLPR and its ligand and that the receptor is functional using calcium imaging. Interestingly, activation of ASM TSLPR did not affect the function of these cells in terms of proliferation, survival, contraction, or synthetic response. Similarly, TSLP did not affect mast cell survival or proliferation. In contrast TSLP had a marked effect upon mast cell synthesis of chemokines and cytokines. This is consistent with previous work using cord[11] or peripheral blood-derived differentiated mast

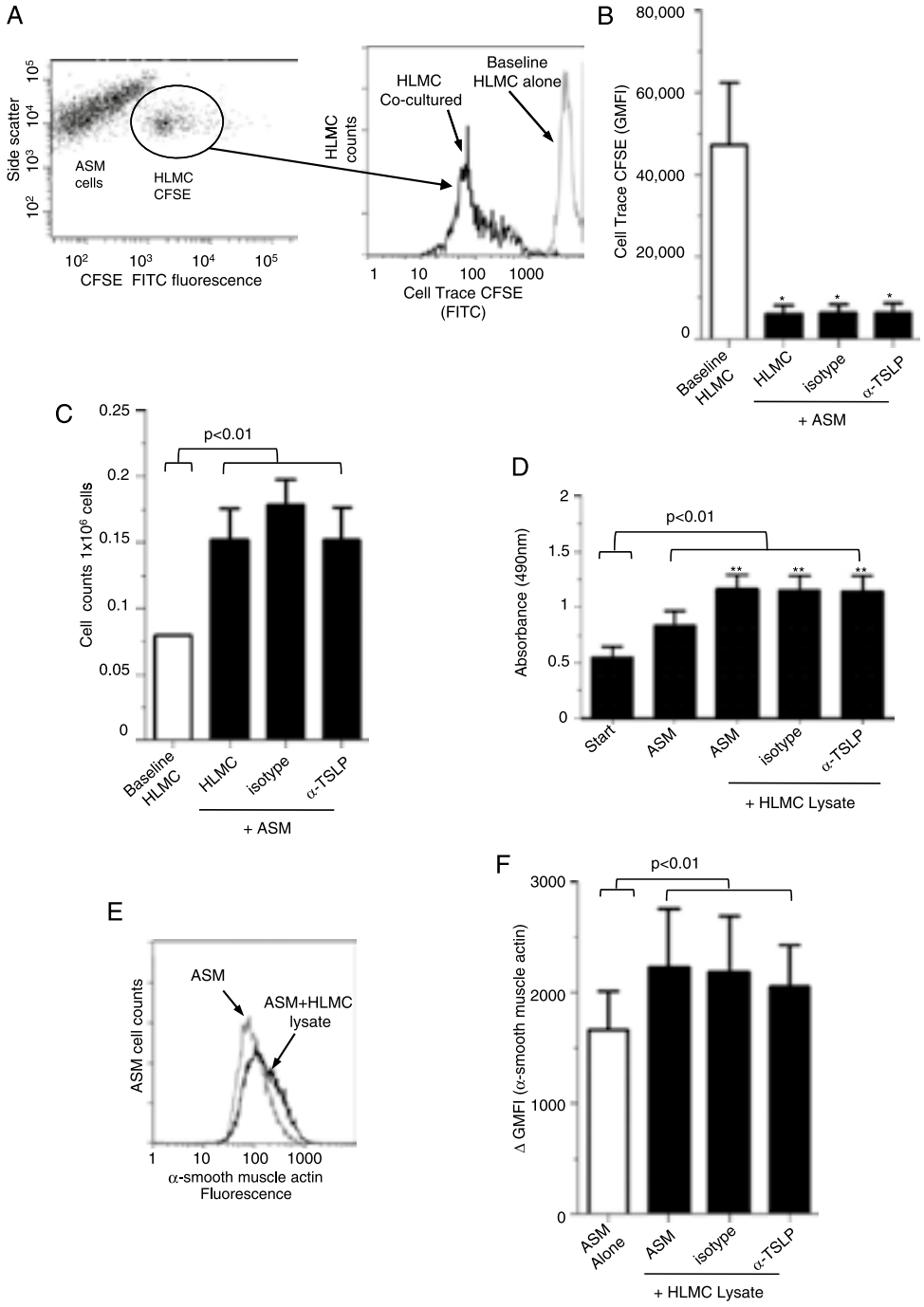

FIGURE 5. Mast cell coculture and lysate. A, Representative dot plot for ASM and HLMC (prelabeled with CFSE-FITC) cocultured for 7 days. After 7 days, coculture-labeled CFSE HLMC were gated and analyzed for CFSE GMFI intensity compared with HLMC baseline. Flow cytometric histogram illustrating CFSE fluorescence at baseline for HLMC alone and then cocultured for 7 days in the presence of ASM cells. B, HLMC CFSE proliferation was assessed over 7 days for HLMC cocultured with ASM with and without isotype control and α-TSLP (1 μg/mL) n = 6). Comparisons were made between HLMC GMFI at baseline compared with HLMC cocultured over 7 days. C, The number of HLMC present over 7 days in coculture ± isotype, α-TSLP 1 μg/mL was assessed and comparisons were made between baseline HLMC counts vs cocultured HLMC. D, ASM cell metabolic activity/proliferation was assessed over 7 days in the presence of ASM + HLMC lysate, ± isotype, α-TSLP 1 μg/mL (n = 4). E, Example fluorescent histogram of ASM cells stained with α-smooth muscle actin with ASM cells alone (gray) or ASM coculture with HLMC lysate (ratio-1 HLMC lysate:4 ASM, black line). F, The ΔGMFI of α-smooth muscle actin in ASM cells with and without neutralizing TSLP over 7 days (n = 4). All data presented as mean ± SEM. Statistical differences were assessed using the *t* tests and *P* values are as shown $^{*}P < .05$, $^{**}P < .01$. CFSE = carboxyfluorescein succinimidyl ester. See Figure 1-4 legends for expansion of other abbreviations.

Table 2—*Mean (SEM) Chemokine and Cytokine Release by HMC-1 (pg/10⁶ Cells) With and Without TSLP Stimulation (1 ng/mL) Over 24 h*

Chemokine/Cytokine	Unstimulated	Stimulated	*P* Value
CCL11	585 (110)	2,994 (60)	< .001
CCL4	374 (9)	11,362 (267)	< .001
CCL26	411 (121)	2,618 (256)	.003
CCL17	966 (52)	2,146 (123)	.003
CXCL10	798 (30)	2,380 (112)	< .001
CXCL8	63 (15)	4,970 (128)	< .001
CCL2	5,616 (173)	16,308 (242)	< .001
CCL22	922 (97)	2,074 (66)	< .001
CCL13	419 (143)	1,553 (9)	.004
CCL5	4.5 (6)	219 (14)	< .001
IFN-γ	125 (52)	105 (9)	.74
IL-1β	0	4.8 (2)	.121
IL-2	102 (2)	292 (39)	.016
IL-4	36 (18)	25 (14)	.662
IL-5	0	132 (8)	< .001
IL-10	80 (4)	354 (6)	< .001
IL-12p70	84 (22.84)	96 (35)	.79
IL-13	36 (36)	325 (19)	.005
TNF-α	81 (4)	236 (34)	.0138

HMC-1 = human mastocytoma cell line; IFN = interferon; TNF = tumor necrosis factor; TSLP = thymic stromal lymphopoietin.

cell progenitors.[34] Interestingly, we have previously reported that mast cells within the ASM-bundle are activated with increased expression of important Th2 cytokines including IL-13.[35] One possible explanation for this upregulated IL-13 expression by mast cells is that ASM-derived TSLP in the asthmatic airway may promote mast cell activation. Indeed, we confirmed that TSLP modulated mast cell production of IL-13 and in coculture, this is in part dependent upon ASM-derived TSLP.[15] Therefore, TSLP may present a novel target for asthma that may exert effects beyond epithelial repair and Th2 polarization to include the potential to modulate mast cell-ASM cell interactions.

Possible criticisms of this study are that the in vivo findings are cross-sectional and the mechanistic studies are ex vivo. We are, therefore, unable to determine whether modulating TSLP expression by mast cells or ASM within the asthmatic airway would lead to important clinical outcomes. We were unable to demonstrate a relationship between airflow obstruction and TSLP expression questioning the role of this axis in airway dysfunction. Interestingly, we did not find differences in TSLP expression in vitro in primary cells from health and disease, suggesting that in vivo either upregulation of TSLP in asthma or downregulation in health is a consequence of differences in the microenvironment.

In conclusion, we report here that both mast cells and ASM express TSLP and TSLPR. Mast cell-ASM interactions are important in the pathogenesis of asthma and coculture of these cells leads to reciprocal activation and differentiation. We found that TSLP potently activates mast cells, but we were unable to demonstrate further roles for TSLP in mast cell-ASM crosstalk. The mechanisms leading to mast cell activation within the ASM bundle and the consequences of this activation are likely to be manifold and, thus, the relative importance of the TSLP axis in these interactions requires further investigation. The results from specific TSLP therapy in humans are eagerly awaited.

Acknowledgments

Author contributions: Dr Brightling guarantees the integrity of the work.

Dr Kaur: contributed to study design and culture of all ex vivo cell experiments, studied TSLP and TSLPR expression and function, conducted the statistical analysis, ran the gene array system, contributed to the writing of the manuscript, and approved the final decision for submission.

Ms Doe: conducted all immunohistochemistry, contributed to the writing of the manuscript, and approved the final decision for submission.

Dr Woodman: ran the electrochemiluminescence arrays (Mesoscale Discovery), contributed to the writing of the manuscript, and approved the final decision for submission.

Ms Wan: ran the electrochemiluminescence arrays (Mesoscale Discovery), contributed to the writing of the manuscript, and approved the final decision for submission.

Ms Sutcliffe: acquired the statistical analysis, ran the gene array system, contributed to the writing of the manuscript, and approved the final decision for submission.

Dr Hollins: conducted the collagen gel contraction assay, contributed to the writing of the manuscript, and approved the final decision for submission.

Dr Brightling: was involved in the recruitment of volunteers and isolation of tissue, supervised all experimental design, contributed to the writing of the manuscript, and approved the final decision for submission.

Financial/nonfinancial disclosures: The authors have reported to *CHEST* the following conflicts of interest: Dr Brightling has received consultancy fees and research funding from AstraZeneca, MedImmune LLC, GlaxoSmithKline, Chiesi Ltd, Hoffmann-La Roche Inc, and Novartis AG. The remaining authors have reported that no potential conflicts of interest exist with any companies/organizations whose products or services may be discussed in this article.

Role of sponsors: The sponsor had no role in the design of the study, the collection and analysis of the data, or in the preparation of the manuscript.
Other contributions: We thank B. Hargadon, RGN, and S. McKenna, RGN, for assistance in clinical characterization of subjects that underwent bronchoscopy. We also thank Roberta Milone, BSc, and Rick Williamson, PhD (GlaxoSmithKline, Stevenage, England) for providing access to a Mesoscale Discovery Electrochemiluminescence reader and assay plates and for their advice, and to Neeta Kulkarni, MD, for assistance on hue thresholding.

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torna all'abstract

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The influence of TSLP on the allergic response

MR Comeau[1] and SF Ziegler[2]

Exposure to allergens first occurs at body surfaces in direct contact with the environment such as the skin, airways, and gastrointestinal tract, and compelling evidence suggests that allergic inflammatory responses are profoundly influenced by the products of epithelial cells located at these sites. One such product is thymic stromal lymphopoietin (TSLP), which is capable of affecting multiple cell lineages involved in allergic reactions. In this review we discuss recent work that has provided insight into the role TSLP plays in both aberrant and protective allergic inflammatory responses, as well as regulation, associations with disease, sources, and functions of this important cytokine.

INTRODUCTION

The increasing prevalence of diseases involving an allergic component is of global concern, accounting for a significant portion of annual healthcare expenditures worldwide.[1,2] The most prevalent forms of allergic disease are allergic rhinitis, asthma, atopic dermatitis (AD), and food allergies. In general, allergy is characterized by an overreaction of the immune system to normally harmless foreign protein substances, known as allergens, following exposure through various routes such as inhalation, direct contact, injection, or ingestion. The symptoms of allergic reactions present themselves systemically and vary widely in their severity, with the organs and tissues involved dependent upon the point of contact with the allergen.[3] Although some allergic conditions develop through IgE-independent pathways,[2,4] allergy is primarily viewed as an IgE-mediated response that progresses from an immediate hypersensitivity reaction due to products of mast cell and basophil degranulation, to a late-phase reaction characterized by leukocyte infiltration and swelling.[5,6]

The primary immune cell lineages involved in the initiation and progression of allergic inflammation include dendritic cells (DCs), mast cells, basophils, eosinophils, and type-2 helper T (Th2) cells. The responses of these principal players in allergic reactions are influenced by the local environments in which they reside. Immune cells localized within the epithelium at mucosal surfaces are at the site of primary exposure to pathogens and allergens. Consistent with this, recent evidence has demonstrated the importance of epithelial cross-talk with immune cells in developing innate and adaptive immune responses. In addition to providing a barrier to the external environment, epithelial cells express a variety of cell-surface and secreted factors in order to appropriately control immune responses.[7,8] Among the various secreted factors epithelial cells are capable of producing, cytokines have emerged as having a broad influence on the development of allergic inflammation and many are targets of novel therapeutics currently in development. While several epithelial cell-derived cytokines are capable of influencing immune responses, one of the most thoroughly studied in the context of allergic inflammation is thymic stromal lymphopoietin (TSLP). The focus of this review will primarily be to discuss the role of TSLP in the cascade of events leading to allergic inflammation, the association of TSLP and gene variants with allergic disease, and to highlight recent progress that has been made in the identification of novel cell sources, functions, species-specific differences, and regulation of this potent cytokine.

TSLP: AN EPITHELIAL CELL-DERIVED CYTOKINE ASSOCIATED WITH HUMAN ALLERGIC DISEASE

TSLP is a member of the hematopoietic cytokine family that includes interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-13, IL-15, and IL-21. The initial studies to elucidate the biological activities of TSLP were focused on lymphopoiesis and similar to IL-7, TSLP was found to support mouse B-cell expansion both *in vitro* and *in vivo*.[9–12] Using a variety of methods several groups identified a TSLP-binding protein in mouse, referred to as TSLP receptor (TSLPR), and showed that it was a low-affinity receptor for TSLP. Sequence analysis showed that TSLPR was similar to the common cytokine-receptor γ-chain (γ_c).[13–15] Further analysis of

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Received 25 September 2009; accepted 16 November 2009; published online 16 December 2009. doi:10.1038/mi.2009.134

the TSLP-receptor complex showed that the interleukin 7 receptor alpha (IL-7Rα) chain was a component of the high-affinity receptor, further linking these four-helix bundle cytokines. Later, *in silico* methods were used to isolate clones of human TSLP and TSLPR.[16–18] The human proteins were found to be quite divergent from those in mouse at the sequence level, but, as described below, functionally they behave in a similar manner. Epithelial cells were found to be the principle source of TSLP,[19] while several hematopoietic lineage cells were found to express both TSLPR and IL-7Rα, including DCs, monocytes, and T-cells.[17]

Studies of TSLP in humans demonstrated a potential role in Th2 inflammatory responses. The first supporting data along these lines were provided by a series of elegant experiments demonstrating several key findings: Primary human $CD11c^+$ myeloid DCs were found to coexpress the IL-7Rα and TSLPR chains, and respond to TSLP stimulation by producing the CCR4-binding, Th2 T-cell-attracting chemokines CCL17 and CCL22. Unlike other common DC-activating factors, TSLP-treated DCs are capable of priming naïve $CD4^+$ helper T-cells to differentiate into proinflammatory Th2 T cells producing IL-4, IL-5, IL-13, and tumor necrosis factor-α (TNF-α), but lower levels of interferon-γ and IL-10.[19] Additionally, TSLP was shown to be a potent DC survival and maturation factor inducing upregulation of surface HLA-DR, CD40, CD80, CD83, and CD86.[17] Uniquely, TSLP induces OX40-L on DCs in the absence of IL-12, and the interaction between OX40 and OX40-L was identified as the molecular signal TSLP uses to prime naïve T-cells for Th2 differentiation.[20,21] TSLP-treated DCs were also found to interact with $CRTH2^+CD4^+$ Th2 memory T-cells to support their maintenance and further polarization,[22] partially through upregulation of IL-17RB, conferring Th2-T-cell responsiveness to IL-25.[23]

It was established that in the steady-state TSLP is primarily expressed by epithelial cells in the skin, gut, and lungs; however, under inflammatory conditions, several addition cell types, including bronchial smooth-muscle cells and lung fibroblasts, express TSLP.[19,24] In addition, TSLP protein was found to be highly expressed in the lesional skin of patients with AD, but absent in normal skin or in skin samples from patients with Th1-type skin diseases, providing the first human disease association identified for TSLP.[19] TSLP expression in acute and chronic lesions of AD skin is also associated with DC activation and migration.[19] Together these seminal findings established TSLP as the first epithelium-derived cytokine with the capacity to skew the developing immune response toward a proallergic state through its direct actions on DCs.

TSLP EXPRESSION INITIATES THE DEVELOPMENT OF ALLERGIC INFLAMMATORY DISEASE *IN VIVO*

Human TSLP is found on chromosome 5q22.1, neighboring the atopy and asthma-associated cytokine cluster on 5q31,[25] which encodes IL-3, IL-4, IL-5, IL-9, IL-13, and the IL-4 receptor.[26] Like other Th2 cytokines its expression is associated with allergic inflammation in both mice and humans. The development of TSLP transgenic mice demonstrated that aberrant expression of this potent cytokine has dramatic local and systemic effects. Transgenic TSLP expression under the control of the *lck*-proximal promoter, which is preferentially active during early lymphocyte development,[27] results in systemic inflammatory disease involving the kidney, liver, spleen, lungs, and skin, and formation of cryoglobulins. Interestingly, these mice demonstrated progressive ulcerative lesions of the ears and a mixed perivascular leukocyte infiltrate in the lungs, ultimately leading to occlusion of the alveoli and death.[28] Mice expressing TSLP under the control of the ubiquitous β-actin promoter also developed lethal systemic inflammation involving the bone marrow, spleen, thymus, and lungs. These mice display elevated serum IL-5 levels and myeloid hyperplasia as evidenced by the presence of myeloperoxidase-positive granulocytes in the spleen. As observed in *lck*-TSLP transgenic mice, the principal cause of death in β-actin TSLP transgenic mice was attributed to severely compromised lung function.[29]

Keratin-specific overexpression of TSLP in the skin under control of inducible K5[30] or constitutive K14[31] promoters lead to an AD-like phenotype, with the development of skin lesions containing inflammatory cell infiltrate, increased Th2 cyokines and chemokines in affected skin, and systemic increases in IgE. The results from inducible K5-TSLP transgenic animals are notable in their demonstration that mice born with normal TSLP levels may develop severe allergic inflammatory disease upon induction of TSLP. Similarly, in non-transgenic models, aberrant TSLP expression can be induced by keratinocyte-specific ablation of retinoid X receptors (RXR) or topical application of vitamin D3 and its low-calcemic analogues. This expression leads to the development of an AD-like phenotype comparable to that seen in TSLP transgenic mice.[31,32] Interestingly, the phenotype seen in both TSLP transgenic and vitamin D3-driven models was reported to be independent of T and B-cells,[30,32] suggesting that the *in vivo* targets of TSLP at the sites of inflammation are likely myeloid-derived cells, which are then capable of initiating the disease process.[30]

As overexpression of TSLP in the skin leads to an AD-like phenotype, lung-specific overexpression of TSLP results in severe allergic airway inflammatory responses with asthma-like features. Mice overexpressing TSLP under the control of the lung-specific surfactant protein-C promoter (SPC-TSLP) develop Th2-biased $CD4^+$ T-cell airway infiltrates, eosinophilia, increased serum IgE, airway hyper-responsiveness, and remodeling.[33] A substantial reduction in airway inflammation and remodeling was observed in IL-4- or STAT6-deficient mice crossed with SPC-TSLP mice, or following blockade of IL-4 and IL-13 in SPC-TSLP mice with established airway disease. These results demonstrate that intact Th2 responses are necessary for the development of TSLP-induced airway inflammatory disease in this system.[34]

TSLP also plays a role in allergen-driven models of airway inflammation. In the ovalbumin-induced model of mouse asthma (OVA-asthma), TSLP expression is increased in response to antigen challenge and correlates with inflammatory cell infiltrates[33] and IL-5 levels in the broncho-alveolar lavage fluid.[35] In this model TSLPR-deficient mice display greatly reduced airway inflammatory responses[33,36] unless reconstituted with

wild-type $CD4^+$ T-cells.[36] As well, blockade of TSLP using a TSLPR-Fc fusion protein[36] or a TSLPR-blocking antibody[35] significantly alleviated the allergic airway inflammatory response. Together these results highlight the involvement of TSLP signaling in this common model of asthma.

The profound phenotypes observed in mice overexpressing TSLP demonstrate the consequences of aberrant TSLP expression *in vivo*. Interestingly, administration of recombinant TSLP protein to normal mice reveals differences in systemic inflammatory responses and required mediators when compared with endogenous TSLP-induced phenotypes. Repeated intradermal TSLP administration over a 2-week time frame resulted in the development of a systemic Th2 response and a significant, diffuse inflammatory cell infiltrate in the skin containing eosinophils and mast cells, with subcutaneous fibrosis.[37] In contrast to mice with dysregulated TSLP expression in the skin,[30–32] in this model no skin lesions developed even after 6 weeks of TSLP administration (unpublished observations). Interestingly, while T-cells were found to be unnecessary for the phenotypes observed in mice expressing TSLP in the skin,[30,32] they were a required component for the response induced following TSLP injection in the skin.[37] In an acute model of lung inflammation, intranasal TSLP administration over a 2-week period induced only a mild inflammatory response in the lung, unless co-administered with OVA as a model antigen[38] upon which mice develop a robust airway inflammatory response. As well, OVA administration to SPC-TSLP transgenic mice prior to onset of spontaneous disease resulted in accelerated disease development. Taken together these results demonstrate the capacity of TSLP to drive the development of allergic inflammatory responses upon exposure to normally innocuous antigens. As observed in experiments involving injection of recombinant TSLP in the skin,[37] T-cells were found to be required for the TSLP + OVA-induced responses and contribute significantly to the phenotype that develops in the SPC-TSLP transgenic mice, indicating the requirement of an adaptive immune response for the complete TSLP-driven inflammatory response to develop in this model.[38] Multiple factors could account for the differential responses seen in mice expressing native TSLP versus those receiving recombinant protein. Native mouse TSLP protein may have intrinsically different activities as compared with recombinant protein, as has been demonstrated with human TSLP.[39] As well, the recombinant TSLP used in both exogenous administration studies contains 10 histidine residues at the C-terminus of the protein, which may be seen as antigenic in mice repeatedly exposed to the protein. Alternatively, the constant systemic exposure to high levels of TSLP that occurs in transgenic and overexpression models may drive innate responses not seen with pulsatile delivery of recombinant protein. We have observed that intraperitoneally administered TSLP protein is no longer detectable in the circulation 2 h post injection (unpublished results). These caveats aside, both chronic and acute exposure to TSLP above homeostatic levels influences multiple aspects of the *in vivo* immune response, leading to the development of inappropriate allergic inflammatory responses.

Eczema in early life is often associated with the development of asthma and allergic rhinitis later in life, and this progression is referred to as the "atopic march".[40] As demonstrated in transgenic mice, dysregulated TSLP expression can lead to the development of widespread and multi-focal inflammation, which may initiate in one organ and ultimately lead to disease in another tissue. The first data implicating TSLP with human asthma pathogenesis was provided by *in situ* hybridization studies. Increased numbers of TSLP-expressing cells were found in the epithelium and sub-mucosa of bronchial biopsies of patients with asthma as compared with normal controls, and this expression correlated with disease severity.[41] An additional study of broncho-alveolar lavage fluid from moderate-to-severe asthmatics demonstrated elevated TSLP protein levels in asthma patients as compared with normal controls,[42] suggesting that TSLP mRNA expression may translate into the presence of protein in patients with asthma.

Recently, a large international study of four heterogeneous asthma populations identified a genetic variant in the promoter region of the TSLP gene that was associated with protection from asthma, atopic asthma, and airway hyper-responsiveness. Associations between TSLP and asthma-related phenotypes were the most statistically significant observation in the study, which included over 5,500 genotyped individuals.[43] An additional study identified a TSLP-gene variant associated with lower levels of allergen-specific IgE and total IgE in a gender-specific manner.[44]

Collectively, these studies provide compelling human disease association data and demonstrate that TSLP is necessary and sufficient for the initiation and development of allergic inflammation in rodents *in vivo*. The possibility that genetic variations affecting TSLP expression may influence asthmatic and allergic phenotypes across widespread populations, suggests an essential and central role for TSLP in the development of allergic inflammatory diseases.

REGULATION OF TSLP EXPRESSION IN THE PERIPHERY

Factors modulating TSLP expression encompass a wide variety of stimuli, which may be of cellular, microbial, or environmental origin, many of which are relevant to allergic inflammation. Constitutive TSLP expression is increased by the classic proinflammatory cytokines TNF-α and IL-1α (or IL-1β) in multiple cell types and tissues,[19,39,45–49] and Th2 cytokines have also been shown to influence TSLP expression. When used alone, IL-4 and IL-13 are minimally effective at inducing TSLP expression, but when added in combination with either TNF-α or IL-1 demonstrate considerable synergy in human skin, lung, and gut samples.[45,48,50] In contrast, IL-13 stimulation alone has recently been shown to induce robust TSLP production in mouse skin, lung, and nasal tissue cultures, suggesting that TSLP may be a downstream target of IL-13.[51] Interestingly, IL-13 does not induce significant TSLP production in cultures of mouse small and large intestine.[52] The promoters of both mouse and human TSLP contain nuclear factor-κB-binding sites that were found to be critical for TNF-α and IL-1β-induced TSLP transcription,[46] and mice with intestinal epithelial cell-specific deletion of IKK-β show reduced TSLP expression,[53] highlighting the importance of

the nuclear factor-κB pathway in the regulation of TSLP expression. Additionally, members of the nuclear receptor superfamily are involved in the regulation of TSLP expression. In the skin, keratinocytes-specific ablation of the retinoid X nuclear receptor α and β chains was shown to result in increased TSLP production in mice, leading to an AD-like phenotype, a response also seen in mice topically treated with RXR agonists, vitamin D3, or its low-calcemic analogues.[31,32]

Microbial infections, pollution, and allergens are all known to exacerbate allergic responses. Consistent with this, epithelial TSLP expression has been shown to be increased following incubation with bacteria,[24] infection of mice with the intestinal nematode *Trichuris*,[54] stimulation with ligands for toll-like receptors (TLR) TLR2, TLR3, TLR8, and TLR9,[39,45,46] rhinovirus infection,[45] diesel exhaust particles,[55] or cigarette smoke extract.[56] While IgE-activated mast cells induce TSLP production from airway smooth-muscle cells in a TNF-α-dependent manner, other products of mast cell activation, as well as common allergens and TLR ligands, fail to induce TSLP from these cells.[49] Mediators of allergic responses are also known to regulate TSLP expression. Mast cells express TSLP mRNA, which is upregulated upon cross-linking of the IgE receptor,[19] and pre-incubation with IL-4 results in significant upregulation of IgE-mediated TSLP protein and mRNA expression.[57] Recently, the contribution of proteases in regulating TSLP expression from both epithelial and hematopoetic cells has been reported. Proteases are a component of certain allergens and are also secreted by helminths.[58] Proteases promote the development of Th2 reactions,[59] and they are thought to provide a key link between Th2 immune responses in anti-helminth immunity and allergic responses.[60] A study using the model protease allergen papain demonstrated its capacity to activate mouse basophils and induce TSLP mRNA and protein expression from these cells.[61] Similarly papain and trypsin were shown to induce TSLP production from a human airway epithelial cell line. TSLP expression in this system was amplified in the presence of IL-4 and dependent upon the protease-activated receptor, PAR-2.[60] Airborne allergens such as house dust mite are also associated with protease activity and administration of house dust mite to the airways of mice results in significant accumulation of TSLP in the broncho-alveolar lavage fluid.[62]

These recent advances in the understanding of factors and pathways that regulate TSLP have provided significant insight into scenarios that may lead to dysregulated TSLP expression in allergic disease, and demonstrate that numerous microorganisms and their products, known to be exacerbating factors for allergic disease, may induce TSLP in sufficient amounts to activate innate immune responses.

TSLP AND EFFECTOR CELLS OF THE ALLERGIC RESPONSE

Initially the contributions of TSLP to the development of Th2-biased immune responses were focused on its DC-specific activities. It is now clear that several other cell types involved in the allergic response are capable of responding to TSLP under permissive conditions (**Figure 1**).

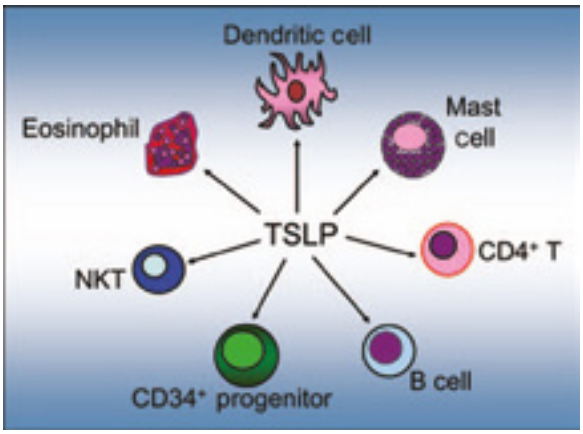

Figure 1 The constellation of TSLP-responsive cell types. TSLP derived from epithelial, stromal, and granulocytic cells acts on a variety of human and mouse cell populations, influencing multiple aspects of the allergic inflammatory response. TSLP, thymic stromal lymphopoietin.

TSLP AND Th2 T CELL RESPONSES

Allergic inflammatory responses involve Th2 cell-derived cytokines and a common hallmark of affected tissues is the infiltration of $CD4^+$ Th2 cells.[2] In addition to influencing Th2-T-cell differentiation indirectly through activation and programming of DCs, TSLP directly influences $CD4^+$ T-cell differentiation into Th2 cells. TSLP was initially shown to induce proliferation of $CD4^-CD8^-$ adult mouse thymocytes synergistically with IL-1β[10] and TSLPR has been cloned from a Th2-skewed mouse T-cell library.[15] Subsequently it was demonstrated that upon T-cell receptor (TCR) engagement mouse and human $CD4^+$ T-cells respond to TSLP stimulation,[63–65] a response likely mediated through TCR-mediated upregulation of TSLPR expression.[65] In experiments using cells sorted to greater than 99% purity, TSLP was shown to induce the differentiation of mouse splenic $CD62L^{high}$ $CD4^+$ naïve T-cells into Th2 cytokine-producing cells that rapidly induced STAT5 phosphorylation and IL-4 production in response to TSLP.[64] These data convincingly demonstrate that in the mouse, T-cells are direct targets of TSLP capable of responding in the absence of DCs. A recent study showed that TSLPR mRNA is not expressed in freshly isolated naïve, central memory, $CRTH2^+CD4^+$ memory Th2, or effector memory human T-cell populations.[66] In this study the authors did not determine whether TCR engagement conferred responsiveness to TSLP with these isolated T-cell populations as demonstrated in previous reports[63–65] Although the contribution of contaminating DCs in T-cell experiments has recently been called into question,[67,68] collectively these data suggest that T-cell activation is an additional mechanism by which TSLP is able to contribute to the developing immune response in the absence of DCs.

BEYOND DCs; TSLP IS A POTENT ACTIVATOR OF HUMAN MAST CELLS

Along with DCs, mast cells are located at sites exposed to the external environment such as the skin, airways, and gut, where they serve as crucial sentinel cells in host defense.[69] Originally thought of only in terms of their contributions to immediate

hypersensitivity reactions, the involvement of mast cells in innate and adaptive immune responses is now evident.[70–74] Mast cell numbers in normal tissues vary depending on their anatomic location, and the severity of allergic responses are influenced by their concentration in tissues.[3] Additionally, increased numbers of mast cells have been demonstrated in a variety of autoimmune and inflammatory conditions.[75–77] Among human hematopoietic cells examined to date, mast cells are one of the only identified non-epithelial sources of TSLP. In addition to providing a potential source of TSLP in response to IgE-receptor cross-linking,[19,57] mast cells express the functional TSLPR complex and respond to TSLP in the presence of IL-1 and TNF-α[39] or IL-33,[78] suggesting that a proinflammatory environment is necessary to confer mast cell responsiveness to TSLP. Interestingly, native TSLP protein derived from both primary lung epithelial cells and lesional skin samples from AD patients has been shown to potently activate mast cells without inducing degranulation. TSLP stimulation induced the release of several cytokines (IL-5, IL-6, IL-13, TNF-α, and granulocyte–macrophage colony-stimulating factor) and chemokines (CCL1 and CXCL8), but did not induce the release of pre-formed, granule-associated mediators such as β-hexosaminidase, histamine, leukotriene C_4, and prostaglandin-D_2.[39] These data implicate TSLP in IgE-independent forms of asthma and eczema, and suggest that in addition to DCs, mast cells at epithelial surfaces may be activated by TSLP, thereby contributing to both the initiation and perpetuation of innate immune responses.

Extending these findings, a potential role for TSLP as a mediator of cross-talk between bronchial smooth-muscle and mast cells was identified. In asthmatic patients, mast cells are the predominant inflammatory cells that accumulate within airway smooth-muscle-cell bundles.[79] Mast cells play a key role in the orchestration of airway inflammation through their release of mediators capable of inducing bronchoconstriction, smooth-muscle-cell proliferation, and recruitment and activation of inflammatory cells.[80,81] TSLP mRNA is expressed constitutively in cultured human bronchial airway smooth-muscle cells,[19] while protein expression is increased upon stimulation with the proinflammatory cytokines TNF-α and IL-1,[47] but not with Th2 cytokines IL-4 and IL-13.[49] Additionally, individually or in combination, TNF-α and IL-1 induce TSLP production from a variety of primary human cell types and tissues[19,39,47–49] Unlike other cellular sources of TNF-α in allergic inflammation, mast cells are known to contain abundant pre-formed TNF-α stores, which are available for immediate release upon appropriate stimulation,[6,82] suggesting that in IgE-dependent reactions mast cells may represent a critical initial source of this proinflammatory cytokine.[5] Supernatants from IgE-activated mast cells induce bronchial smooth-muscle cell production of TSLP in a TNF-α-dependent manner and in turn bronchial smooth-muscle cell -derived TSLP is sufficient to induce mast cell production of IL-5 and IL-13.[49] Consistent with these data, mast cells were shown to be necessary for TSLP expression in a model of allergic rhinitis using mast cell-deficient mice.[83] Collectively these data suggest a potential feedback loop where in an allergic state, IgE-activated mast cells may provide the proinflammatory environment necessary for TSLP production possibly through release of TNF-α. In this environment TSLP may activate bystander mast cells contributing to the exacerbation of allergic inflammatory responses (**Figure 2**).

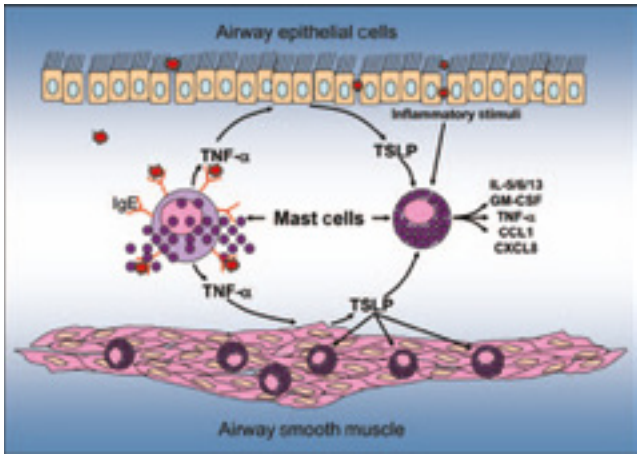

Figure 2 TSLP-mediated cross-talk between mast cells and airway epithelial and smooth-muscle cells in both IgE-dependent and independent inflammatory responses. Proinflammatory stimuli leading to epithelial cell production of TSLP directly activates mast cells, inducing release of multiple proinflammatory cytokines and chemokines independently of IgE, whereas IgE-mediated mast cell activation leads to release of TNF-α, which may drive TSLP production from smooth-muscle cells in the airway. TNF-α, tumor necrosis factor-α; TSLP, thymic stromal lymphopoietin.

THE EFFECTS OF TSLP ON PROGENITOR CELLS IN THE PERIPHERY

The bone marrow is an active participant in systemic allergic inflammatory responses as several granulocytic effector cell types involved in allergic inflammation develop from $CD34^+$ bone marrow-derived progenitor cells.[84] While differentiation and maturation of eosinophils and basophils from these progenitors primarily occurs within the bone marrow, mast cell maturation typically occurs in peripheral tissues.[5] $CD34^+$ progenitor cells are normally present in circulation due to constant release from the bone marrow,[85] and in allergic individuals increased numbers of progenitor cells are present in both the bone marrow and peripheral blood. Upon allergen exposure these progenitors traffic into local tissues where they are capable of maturing into mast cells, eosinophils, and basophils depending on their local environments.[84,86,87] In mouse, TSLPR mRNA is expressed in hematopoietic progenitor cells and is upregulated in response to granulocytic differentiation signals.[88] In humans, $CD34^+$ progenitor cells express TSLPR mRNA[78] and protein.[89] As observed with mature mast cells,[39] these progenitors respond to TSLP in the presence of co-stimulation with TNF-α and IL-1 or IL-33,[78] and rapidly produce abundant amounts of cytokines (IL-5, IL-13, granulocyte–macrophage colony-stimulating factor, IL-6) and chemokines (CXCL8, CCL1, CCL17, CCL22). Supernatants from nasal explant cultures of chronic rhinosinusitis patients have also been shown to induce the production of IL-5 from $CD34^+$ progenitor cells in a TSLP-dependent manner.[89] $CD34^+$ progenitor cells residing in or recruited to tissues exposed to allergens may, therefore, contribute to proinflammatory

processes following exposure to locally expressed, epithelium-derived cytokines such as TSLP and IL-33.[89]

BASOPHILS AND EOSINOPHILS

Along with mast cells, basophils and eosinophils are the primary effector cells of immediate hypersensitivity reactions and allergic disease.[3] In addition, basophils and eosinophils are implicated in the pathogenesis of numerous inflammatory processes, including parasitic helminth infections. Present as mature cells found primarily in circulation, these cells can respond to a variety of activating stimuli and rapidly migrate to sites of inflammation. Both cell types are capable of a variety of immune functions and release an array of cytokines, chemokines, toxic granule proteins, and lipid mediators, thus participating as potent effector cells in the exacerbation of inflammatory responses.[90–92] That TSLP is capable of activating mast cells and the common progenitors of eosinophils and basophils in the presence of proinflammatory signals, suggests there may be similar cofactors or scenarios where eosinophil and basophil responses to TSLP might occur. Indeed two recent reports have emerged describing direct activities of TSLP on human eosinophils. Peripheral blood eosinophils were found to express TSLPR and IL-7Rα at the message and protein level, and respond to TSLP in a dose-dependent and specific manner.[93] As well, eosinophil responses to TSLP were synergistically enhanced in the presence of IL-3 and TNF-α.[94] Interestingly, although TSLP induced the release of inflammatory cytokines (TNF-α IL-6, IL-8) and chemokines (CCL2, CCL3, CCL4, CXCL1, CXCL8), it did not induce degranulation of eosinophils,[93,94] similar to what has been observed with mast cells.[39] Additionally, both local and systemic allergic inflammatory responses to exogenous TSLP administration in the mouse were shown to involve eosinophils. Repeated intradermal administration of TSLP protein induces a systemic inflammatory response that is largely Th2 in nature[37] and is characterized by systemic increases in circulating IgE, local inflammatory cell infiltrates, and increased Th2 cyokines and chemokines in the skin. Eosinophil-deficient dblGATA[95] mice failed to develop both local and systemic responses to TSLP[37] in this system. Collectively these recent data imply that in both humans and mice, eosinophils have the capacity to contribute to TSLP-driven allergic responses.

Although basophils constitute less than 1% of circulating leukocytes, these rare cells have potent effects on multiple aspects of the allergic inflammatory response. Recent findings have provided new insight into the role of basophils in allergic disease and immunity to helminths, suggesting that these cells may provide unique functions unmet by other hematopoietic cells.[92,96] Although no direct responses to TSLP have yet been described, basophils have been identified as a potentially important source of TSLP *in vivo.* Mice exposed to the model protease allergen papain developed Th2 inflammatory responses with increased systemic IgE levels and transient appearance of IL-4 and TSLP-producing basophils in lymph nodes. Depletion of basophils in this model demonstrated their necessity in the differentiation of naïve CD4 T cells to Th2 cells. Additionally, basophils stimulated *in vitro* with papain expressed TSLP mRNA, while neutralization of TSLP *in vivo* inhibited papain-induced Th2 responses.[61] As well delivery of recombinant TSLP protein leads to robust elicitation of basophils and their accumulation in the periphery unlike other epithelium-derived cytokines such as IL-25 and IL-33.[97] Taken together, these results suggest that in the context of protease allergens, basophil-derived TSLP is necessary for initiation of Th2 responses along with IL-4, and ascribe a potentially unique attribute to TSLP in comparison with other epithelium-derived cytokines that influence allergic inflammatory responses. Although no examination of basophils in mice overexpressing TSLP has been reported, these results suggest that they are likely a component of the lethal systemic phenotype observed in these animals. Thus, expansion of basophils under conditions that lead to dysregulated expression of TSLP may be a crucial early cellular response in the cascade of events leading to allergic inflammation.

NKT CELLS

A role for CD1d-restricted natural killer (NK)T cells has been proposed in asthma and although their contribution remains controversial, a significant body of supporting data provides a compelling case.[98] Mouse invariant NKT (iNKT) cells were found to express both chains of the TSLPR and in the presence of TCR stimulation, proliferate in response to TSLP.[99] While the proliferative response required TCR stimulation, similar to the TCR engagement required to confer TSLP responsiveness to $CD4^+$ T-cells,[63–65] TSLP alone was able to induce IL-13 production from iNKT cells.[99] While iNKT cells preferentially produced IL-13 upon TCR engagement, IL-4 and interferon-γ were also abundantly produced in the presence of TSLP. When SPC-TSLP transgenic mice were crossed with mice lacking iNKT cells, no difference in the spontaneous development of allergic inflammatory responses was observed, suggesting that iNKT cells are not required for the phenotype observed in the TSLP transgenic mice. Interestingly, in a standard OVA-asthma model iNKT-deficient SPC-TSLP mice demonstrated significantly reduced airway hyperresponsiveness and IL-13 production, but no difference in pulmonary eosinophilia or IgE when compared with controls.[99] It was therefore concluded that iNKT cells are required for SPC-TSLP mice to develop AHR in response to allergen challenge. Future studies will be required to determine whether human iNKT cells respond to TSLP in a similar manner.

A ROLE FOR TSLP IN GUT HOMEOSTASIS

Although allergic reactions to normally innocuous antigens can result in damaging inflammation, Th2 immune responses are thought to have developed partially in order to protect the host from parasitic helminths,[100] clearly a beneficial aspect of this arm of the immune response. TSLP is constitutively expressed by epithelial cells in the intestine, with the highest levels found in the proximal large intestine.[24,53,54,101] A critical role for TSLP in the development of protective immunity to *Trichuris muris* infection was identified in mice with reduced TSLP expression due to an intestinal epithelial cell-specific deletion of IKK-β.[53] Reduced epithelial TSLP expression in these mice was associated

with increases in pathogen-specific IL-12/23p40, IL-17, and interferon-γ, and increased worm burdens as compared with those in control mice. Similar Th1 responses were observed in TSLPR-deficient mice, which also failed to expel worms.[53] Extending these observations, it was reported that TSLP neutralization in normally genetically resistant mice also resulted in increased susceptibility to *Trichuris* infection and reduced Th2 cytokine production in the gut. Notably, blockade of interferon-γ in TSLPR-deficient mice restored Th2 responses and immunity to *Trichuris*, demonstrating that TSLP is dispensible for the generation of protective Th2 cytokine responses in the intestine.[101] Although the contribution of TSLP was less pronounced, similar results were obtained in two additional studies using helminth infection models.[102,103] Consistent with a role for TSLP at limiting inflammation in the intestine, TSLPR-deficient mice develop more severe inflammation, increased weight loss, and elevated Th1 cytokines in the dextran sodium sulfate model of colitis.[101] Interestingly, TSLPR-deficient mice also demonstrate reduced Th2 responses coupled with exaggerated IL-12 mRNA expression in the OVA-asthma model.[36] Collectively these results suggest a principal function of TSLP in the intestine is to limit the production of non-protective Th1 cytokines and inflammation.

SPECIES-SPECIFIC ACTIVITIES

The IL-7Rα chain is required for TSLP and IL-7 signaling as it is used by both cytokines in their receptor complexes. Mice deficient in the IL-7Rα chain demonstrate impaired T and B-cell development,[104] leading to deficiencies in both populations, and treatment of mice with neutralizing antibodies to IL-7 results in a similar phenotype.[105] In contrast, mice lacking the TSLPR exhibit normal T and B-cell development and cellularity.[63,106] IL-7 uses the common cytokine-receptor γ-chain (γ_c) as an additional component of its receptor complex.[107,108] Injection of TSLP into γ_c-deficient mice enhances the expansion of both T and B-cells, and interestingly, mice lacking both the TSLPR and γ_c display more severe lymphoid defects than γ_c-deficient mice.[63] As IL-7 and TSLP both use the IL-7Rα chain, these results suggest there may be a role for TSLP in mouse T and B-cell lymphopoiesis, although IL-7 likely plays the dominant role. Additionally, humans with severe combined immunodeficiency due to IL-7Rα mutations lack T-cells but have normal B-cell numbers,[109] a significant difference when compared with mice lacking the IL-7Rα chain. While most of the identified TSLP activities have been demonstrated in both mouse and human systems, these findings suggest that like IL-7, species-specific activities exist for TSLP.

At the amino-acid level there are additional differences in TSLP to note between species. While the position of the six cysteine residues involved in disulfide bond formation are conserved across species, mouse and human TSLP share only 43% amino-acid identity overall.[10,16] We have determined that this low level of conservation is also seen in additional rodent TSLP sequences, including rat (45%) and rabbit (58%) (**Figure 3**). Not surprisingly, non-human primate TSLP displays a much higher level of sequence conservation, with an amino-acid

Figure 3 Species differences in the amino-acid sequences of TSLP. Primate TSLP contains a putative furin cleavage site upstream of the final conserved cysteine residue that is not present in rodent sequences. TSLP, thymic stromal lymphopoietin.

identity of 93% for chimpanzee and 90% for the cynomolgus monkey. A comparison of TSLP amino-acid sequences from several species reveals an interesting conservation in the primate sequences of seven basic amino acids (KKRRKRK) upstream of the final cysteine residue near the C-terminal end of the protein[16] that is not present in any rodent sequence identified to date (**Figure 3**). This stretch of amino acids encodes a putative furin cleavage site.[110] Furin is a proprotein convertase enzyme that is typically involved in the post-translational processing of inactive precursor proteins into their biologically active forms, an ancient mechanism that enables cells to regulate the levels of bioactive proteins.[110] The presence of this conserved furin site in primate TSLP leads one to speculate on what purpose it may serve, if any, in the proteins biological activity. In an inflammatory state, furin cleavage may be a mechanism used by the primate immune system to limit the levels of bioactive TSLP protein in order to prevent inappropriate inflammatory responses. In order to determine whether this cleavage occurs and has any effect on TSLP activity, native TSLP expression would need to be examined both in the steady state and under inflammatory conditions where TSLP is thought to play a role.

CONCLUDING REMARKS

Much progress has been made in the understanding of the biological responses mediated by TSLP in the approximately 10 years since this cytokine was first cloned. Future studies of the interplay of TSLP with additional epithelium-derived cytokines such as IL-25 and IL-33 will likely reveal additional cellular targets and exciting novel findings. The regulation of TSLP by disease-relevant environmental factors, endogenous proinflammatory cytokines, and effector cells of the allergic response highlight the relevance of this epithelium-derived cytokine in innate and adaptive immunity (**Figure 4**) and make it an especially attractive target to consider for therapeutic intervention under both atopic and non-atopic conditions.

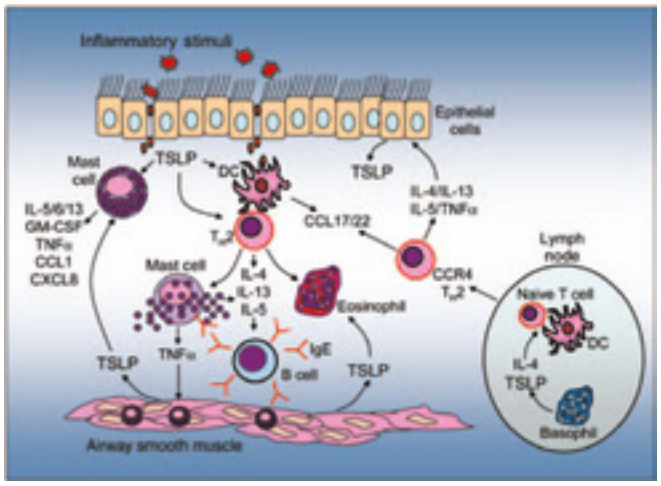

Figure 4 TSLP activation of key immune cells supports the development of allergic inflammatory responses. Multiple environmental and pathogenic factors are capable of inducing TSLP production from epithelial cells, which may act as both an upstream and downstream mediator of inflammatory responses through activation of inflammatory cells of the innate and adaptive immune response. Recently, activated basophils have been identified as an early source of IL-4 and TSLP, which may serve to influence the differentiation of Th2 cells from naïve T-cells. IL, interleukin; Th2, type-2 helper T-cells; TSLP, thymic stromal lymphopoietin.

ACKNOWLEDGMENTS

We thank Heidi K. Jessup for excellent scientific contributions and critical review of the manuscript.

DISCLOSURE

Michael R. Comeau is an employee and shareholder of Amgen Inc.

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torna all'abstract

EPIDEMIOLOGY

Bronchial hyperresponsiveness and the development of asthma and COPD in asymptomatic individuals: SAPALDIA Cohort Study

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Thorax 2006;**61**:671–677. doi: 10.1136/thx.2005.052241

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Received 4 October 2005
Accepted 18 April 2006
Published Online First 2 May 2006

Background: Bronchial hyperresponsiveness (BHR) is a common feature of asthma. However, BHR is also present in asymptomatic individuals and its clinical and prognostic significance is unclear. We hypothesised that BHR might play a role in the development of chronic obstructive pulmonary disease (COPD) as well as asthma.

Methods: In 1991 respiratory symptoms and BHR to methacholine were evaluated in 7126 of the 9651 participants in the SAPALDIA cohort study. Eleven years later 5825 of these participants were re-evaluated, of whom 4852 performed spirometric tests. COPD was defined as an FEV_1/FVC ratio of <0.70.

Results: In 1991 17% of participants had BHR, of whom 51% were asymptomatic. Eleven years later the prevalence of asthma, wheeze, and shortness of breath in formerly asymptomatic subjects with or without BHR was, respectively, 5.7% *v* 2.0%, 8.3% *v* 3.4%, and 19.1% *v* 11.9% (all $p<0.001$). Similar differences were observed for chronic cough (5.9% *v* 2.3%; $p=0.002$) and COPD (37.9% *v* 14.3%; $p<0.001$). BHR conferred an adjusted odds ratio (OR) of 2.9 (95% CI 1.8 to 4.5) for wheezing at follow up among asymptomatic participants. The adjusted OR for COPD was 4.5 (95% CI 3.3 to 6.0). Silent BHR was associated with a significantly accelerated decline in FEV_1 by 12 (5–18), 11 (5–16), and 4 (2–8) ml/year in current smokers, former smokers and never smokers, respectively, at SAPALDIA 2.

Conclusions: BHR is a risk factor for an accelerated decline in FEV_1 and the development of asthma and COPD, irrespective of atopic status. Current smokers with BHR have a particularly high loss of FEV_1.

Bronchial hyperresponsiveness (BHR) is a common finding in asthma[1] and has also been observed in patients with chronic obstructive airways disease (COPD).[2] Cross sectional studies have found significant associations between BHR and respiratory symptoms, including wheezing, cough and shortness of breath.[3–5] Population based studies, including the first cross sectional Swiss study on Air Pollution and Lung Diseases in Adults (SAPALDIA), suggest that 11–20% of individuals have BHR.[4 6] However, a significant proportion of individuals with BHR do not suffer from respiratory symptoms, asthma or other obstructive airways diseases. It is thought that the proportion of asymptomatic individuals with BHR ranges from 19% to 62% in the general population.[7] Thus, many subjects with BHR are asymptomatic or ''silent''. Although the presence of BHR has been positively associated with the development of respiratory symptoms and negatively associated with symptom remission in a longitudinal study of 2684 adults,[8 9] the relevance and long term impact of BHR in the absence of symptoms has not been fully elucidated.

Prevailing current opinion is that classical asthma is characterised by two main features that occur together: (allergic) inflammation with airway thickening and mucus formation,[10] and airway smooth muscle dysfunction with BHR.[11] BHR or airway inflammation alone are probably not sufficient to cause asthma, but might be independent risk factors for the development of symptomatic airway dysfunction.[12] Indeed, there is good evidence that allergic sensitisation is a risk factor for the development of asthma.[13 14] In a relatively small study of 194 adults, Segala *et al*[15] found that BHR to methacholine was a predictor of wheezing in a 5 year follow up study, independent of atopic status. In asthmatics, prolonged treatment with inhaled or systemic corticosteroids can reduce BHR but often does not abrogate it.[16] Thus, although often associated with airway inflammation,[17] it has not yet clear whether BHR is truly an independent risk factor for the development of asthma.

Even less is known about the association between BHR and COPD. The probability of a decline of 20% or more in FEV_1 in response to a provoking concentration of <3000 µmol inhaled methacholine is partially dependent on baseline lung function,[18] which must be addressed in studying the relation between BHR and COPD. However, apart from its role in the modern dual feature asthma hypothesis, BHR could also be a risk factor for the development and progression of COPD, particularly in situations where BHR occurs alongside a non-allergic, typically cigarette smoke induced airway inflammation. The potential interaction between smoking and BHR was documented in the Lung Health Study,[19] a 5 year randomised prospective clinical study of 4201 patients with mild COPD. The study showed that BHR improved after smoking cessation. Similarly, the European Community Respiratory Health Survey,[20] a large epidemiological study of random population samples of 22 European regions, found that smoking was a risk factor for an increase in BHR over time in 3993 participants. On the other hand, the Normative Aging Study,[21] which studied 435 men and excluded those

Abbreviations: BHR, bronchial hyperresponsiveness; COPD, chronic obstructive pulmonary disease; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity

with symptoms, found no relation between change in BHR over 3 years and smoking status. In a study of bronchial biopsy specimens, Willemse *et al*[22] found that bronchial inflammation was similar in current smokers with COPD and zasymptomatic smokers but lower in non-smoking patients with COPD. The authors concluded that the inflammatory effects of current smoking may mask the underlying ongoing inflammatory process pertinent to COPD. Thus, smoking could play the role of a non-specific ''amplifier''.

The current population based longitudinal survey investigates the relevance of BHR in asymptomatic adults with respect to a range of prospective clinical outcomes. We hypothesised that asymptomatic BHR in 1991 is a risk factor for the development of respiratory symptoms, and a risk factor for asthma and COPD 11 years later.

METHODS

Study design and population

The methodology and selection of the participants of the SAPALDIA prospective cohort study have been described in detail elsewhere.[23 24] The population was a random sample (18–60 years) recruited from eight areas of Switzerland using population registries in 1990. Health examinations were conducted for SAPALDIA 1 in 1991. The second round of health examinations in 2002 (SAPALDIA 2) included identical protocols to those in the first survey. Of the 9651 participants in 1991, 7126 had a methacholine challenge. Subjects were included in the present analysis if spirometric and bronchial challenge data were available from SAPALDIA 1 and questionnaire data were available from both surveys (n = 5825). Of these, 4852 performed spirometric tests at both surveys.

Ethical approval for the study was given by the central ethics committee of the Swiss Academy of Medical Sciences and the Cantonal ethics committees for each of the eight examination centres.

Respiratory symptoms, phenotype definitions, and smoking habits

Information about respiratory symptoms, smoking habits, and other risk factors was gathered through an interview administered questionnaire based on the European Community Respiratory Health Survey (ECRHS) questionnaire.[25] Symptoms examined were ''wheeze without cold in the last 12 months'', ''shortness of breath when hurrying on level ground or walking up a slight hill'', ''chronic cough—cough during the day or night on most days for as much as 3 months each year for more than 2 years'', and ''chronic phlegm—phlegm during the day or night on most days for as much as 3 months each year for more than 2 years''. Asymptomatics were defined as participants without wheeze, shortness of breath, chronic cough, chronic phlegm, or physician-diagnosed asthma at SAPALDIA 1. Participants with a forced expiratory volume in 1 second/forced vital capacity (FEV_1/FVC) ratio of <0.70 without a physician's diagnosis of asthma were classified as having evidence of COPD.[26] Asthma was defined as physician-diagnosed asthma. Smokers were participants who had smoked $\geqslant$20 packs of cigarettes or $\geqslant$360 g of tobacco in their lifetime. Former smokers were smokers who had quit smoking at least 1 month before examination in 2002 and current smokers were participants who reported active smoking at the interview in 2002. The cigarette exposure of participants was assessed by pack-years. Participants were asked not to smoke in the hour before the examination and recent exposure to smoking was validated by the measurement of carbon monoxide (CO) concentration in exhaled air using an EC50 Micro-Smokerlizer.

Assessment of pulmonary function, bronchial responsiveness, and atopy

The same spirometers (SensorMedics 2200 SP Yorba Linda, USA) were used in 1991 and in 2002.[24 27] The protocol for the measurement of lung volumes and flows was identical to that in the ECRHS and complied with American Thoracic Society recommendations.[5] Participants were requested not to use short acting inhalers 4 hours and long acting inhalers 8 hours before the examination appointment.

Participants able to produce technically satisfactory spirometric values and who satisfied health inclusion criteria were invited to undergo a bronchial challenge. Non-specific bronchial responsiveness was assessed by bronchial challenge with methacholine chloride administered by MEFAR aerosol dosimeters.[23] The challenge schedule started with an inhalation of saline followed by increasing concentrations of methacholine up to a cumulative dose of 8.37 μmol. The test was stopped when either the maximum cumulative dose had been reached or FEV_1 had fallen by 20% or more.[6] BHR to methacholine was defined as a fall of 20% or more in FEV_1 compared with the highest FEV_1 value measured during the test in response to inhalation of methacholine to the maximum dose, and the degree of bronchial reactivity was measured by calculating a dose-response slope.[28] The slope was defined as the ratio between the percentage decline in FEV_1 and the total cumulative dose administered. Since the distribution of the slopes was skewed, data were transformed using natural logarithms before analysis. A small constant (0.01) was added before transformation in order not to lose observations with zero slope.

Skin prick tests were conducted in 1991 in accordance with the ECRHS allergy testing protocol and included testing sensitisation to house dust mite (*Dermatophagoides pteronyssinus*), cat, dog, fungi (*Cladosporium* and *Alternaria* spp), timothy grass, birch and parietaria pollen.[23 25] Participants were classified as atopic if they developed a skin wheal to one or more of the allergens with a mean diameter exceeding the negative control wheal by at least 3 mm.

Statistical analysis

Univariate and bivariate analyses were conducted initially to provide descriptive statistics. Logistic regression was used to model relations between the presence and absence of BHR in asymptomatic subjects in 1991 and new symptoms at follow up in 2002 while adjusting for potential confounders. Factors tested as potential confounders included age, sex, atopy, smoking status, pack years, FVC at baseline, height, body mass index at baseline, change in weight, level of education, exposure to environmental tobacco smoke (ETS), exposure to dust and fumes at work and study area. FVC was included as a proxy for lung size and airway calibre.[6 29] In addition, effect modification of the relation between BHR and new symptoms by sex, atopy, and smoking status (current, former or never smoker at SAPALDIA 2) were investigated. The relation between responsiveness to methacholine and symptoms and COPD 11 years later was also examined with responsiveness measured by the continuous variable ''slope''. Potential non-linearities in the associations between methacholine slope at SAPALDIA 1 and outcomes at SAPALDIA 2 were tested by adding the square and the cube of slope as covariates. Percentage risks of new symptoms or COPD at follow up associated with the presence or absence of BHR were estimated from the logistic regression models upon adjusting covariates to their population means.

The effect of BHR at baseline on change in FEV_1 was modelled by linear regression adjusting for the relevant confounders listed above and for baseline FEV_1. Analyses were conducted using Stata Special Edition release 8.2 (Stata Corporation, Texas, USA). p values of <0.05 and <0.1 were

Table 1 Characteristics of subjects with bronchial challenge at baseline according to whether they participated in both surveys or only in the first one

	SAPALDIA 1 & 2 (n = 5825)	SAPALDIA 1 only (n = 1301)	p value
Women (%)	50.3	45.5	0.002
Mean (SD) height (cm)	169 (9)	169 (9)	0.14
Mean (SD) weight (kg)	69 (13)	69 (14)	0.12
Mean (SD) FEV_1 (l/s)	3.6 (0.8)	3.6 (0.8)	0.378
Mean (SD) FVC (l)	4.6 (1.0)	4.5 (1.0)	0.284
Atopic (%)	23.5	23.2	0.794
$FEV_1/FVC \geqslant 0.70$ (%)	92.4	90.8	0.066
Bronchial hyperresponsiveness (%)	16.7	16.8	0.928
Geometric mean methacholine dose-response slope*	1.1	1.1	0.860
Severe respiratory infection as an infant (%)	7.5	6.1	0.081
No professional education (%)	13.3	22.0	<0.001
Exposed to dust and fumes at work (%)	31.1	36.2	<0.001
Mother smoked (%)	12.5	15.1	0.012
Father smoked (%)	53.9	56.5	0.085
Current smokers (%)	31.8	40.6	<0.001
Geometric mean pack-years in current smokers	11.2	12.8	0.071
Never smokers (%)	45.5	36.3	<0.001
Physician-diagnosed asthma (%)	5.5	6.9	0.050
Wheeze in last 12 months without cold (%)	6.1	8.8	0.001
Shortness of breath while walking (%)	21.8	27.7	<0.001
Chronic cough (%)	4.1	6.0	0.002
Chronic phlegm (%)	5.6	8.6	<0.001

*Percentage decrease in FEV_1 from its maximum level per μmol methacholine.

interpreted as statistically significant for main and interaction effects, respectively.

RESULTS

Population characteristics

Of the initial 9651 participants in SAPALDIA 1, 7126 underwent a methacholine challenge. Reasons for lack of a challenge included technically poor baseline spirometry, refusal, exclusions on the basis of health criteria including heart disease, epilepsy, pregnancy, lactation, and use of β blockers,[23] and 135 (1.4%) participants were excluded because of a baseline FEV_1 <70% predicted or <1.5 l.[30] Of the 7126 participants with a valid bronchial challenge test in 1991, 5825 were re-evaluated in 2002 and are included in the current analyses. Of these, 4852 performed spirometric tests and 3931 were asymptomatic at SAPALDIA 1. A total of 222 participants were excluded from the multivariate analyses because of inconsistent information about smoking habits between surveys or exhaled carbon monoxide concentrations of more than 10 ppb, despite claiming to be a never or former smoker.

Non-participants in the second evaluation in 2002 (n = 1301) were compared with the participants in both evaluations (table 1). Slightly more men, smokers, persons with low educational background, with professional exposure to fumes and dust, and more individuals with respiratory symptoms were non-participants in the follow up evaluation.

Demographic characteristics, respiratory symptoms, and lung function measured in 1991 in participants without BHR compared with hyperreactive symptomatic or asymptomatic persons are shown in table 2. In 1991, 970/5825 (17%) subjects had BHR, of which 492/970 (51%) were

Table 2 Symptoms and lung function at baseline by presence or absence of bronchial hyperresponsiveness (BHR)

		BHR		
Baseline measures	No BHR (n = 4855)	Silent (n = 492)	Symptomatic (n = 478)	p value*
Proportion of total population (%)	83	9	9	–
Sex (% female)	47	67	64	<0.001
Mean (SD) age (years)	40 (11)	40 (11)	41 (12)	0.41
Current smokers (%)	31	29	40	<0.002
Never smokers (%)	46	51	38	0.0001
Atopic	20.8	32.3	42.3	<0.001
Physician-diagnosed asthma (%)	3.2	–	34.3	–
Wheeze in last 12 months without cold (%)	4.7	–	26.8	–
Shortness of breath while walking (%)	20.3	–	60.0	–
Chronic cough (%)	3.5	–	14.0	–
Chronic phlegm (%)	5.3	–	15.1	–
COPD† (%)	5.8	13.4	12.8	<0.001
Mean (SD) FEV_1 (% pred)[31]	103 (11)	96 (11)	93 (12)	<0.001
Mean (SD) FVC (% pred)	102 (12)	100 (12)	97 (13)	<0.001
Mean (SD) FEV_1/FVC	80 (6)	78 (7)	77 (8)	<0.001
FEV_1/FVC <0.7 (%)	6	13	20	<0.001
Geometric mean methacholine dose-response slope‡	0.7	6.5	10.0	<0.001 <0.001

*Significance test between individuals without BHR and individuals with asymptomatic BHR.
†COPD was defined as FEV_1/FVC <0.70 and no physician's diagnosis of asthma at either survey.
‡Percentage decline in FEV_1 per μmol methacholine relative to maximum FEV_1.

Table 3 New reports of respiratory symptoms and prevalence of COPD at SAPALDIA 2 in formerly asymptomatic participants with and without BHR at SAPALDIA 1

	Asymptomatic at baseline		
Symptoms developed between surveys	No BHR (n = 3439)	BHR (n = 492)	p value
Physician-diagnosed asthma (%)	2.0	5.7	<0.001
Wheeze in last 12 months without cold (%)	3.4	8.3	<0.001
Shortness of breath while walking (%)	11.9	19.1	<0.001
Chronic cough (%)	2.3	5.9	0.002
Chronic phlegm (%)	4.8	4.9	0.964
COPD* (%)	14.3	37.9	<0.001

*COPD was defined as $FEV_1/FVC < 0.70$ and no physician's diagnosis of asthma.

asymptomatic. The proportion of women was almost 20% higher than men in both BHR groups, and symptomatic individuals with BHR were more likely to be current smokers. The prevalence of atopy was higher in subjects with BHR, especially when symptomatic. In individuals with BHR, particularly in those with respiratory symptoms, the proportion with abnormal lung function and the degree of functional impairment was slightly higher. As expected, FEV_1, FEV_1/FVC, and methacholine dose-response slope showed a trend across categories with the poorest results in individuals with symptomatic BHR.

Longitudinal results and multivariate analyses

Longitudinal results are given in table 3. Reported new symptoms at SAPALDIA 2 in previously asymptomatic participants with and without BHR are compared. Participants with BHR were at greater risk of developing respiratory symptoms and asthma, as well as COPD.

The results of multivariate logistic regression analysis are presented in table 4 and fig 1. Participants diagnosed with asthma between 1991 and 2002 were excluded from analyses for chronic cough, phlegm, and COPD, allowing us to focus on the role of BHR in the onset of those conditions. Silent BHR conferred an increased risk of newly diagnosed asthma, new symptoms of wheeze, chronic cough, and COPD 11 years later. Excluding subjects with COPD in 1991 reduced the association between the presence of BHR and COPD in 2002 slightly, but a significant association remained (adjusted OR 4.0, 95% CI 2.9 to 5.6, p<0.001). There was no relation with new reports of chronic phlegm. Despite the differences observed in the prevalence of BHR between sexes at SAPALDIA 1, there was no evidence of interaction by sex in the effect of BHR on symptoms. However, there is some evidence for sex differences in the association between the presence of BHR and the risk of COPD (p = 0.087), with slightly lower risks in women (OR 4.1 (95% CI 2.8 to 6.1) than in men (OR 5.4 (95% CI 3.4 to 8.5).

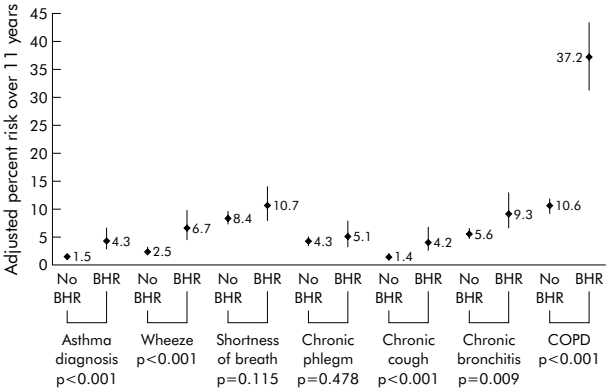

Figure 1 Adjusted risk for subsequent respiratory symptoms, asthma and COPD among subjects who were symptom-free at baseline according to the presence or absence of BHR. Estimates are derived from a logistic regression model upon adjusting covariates listed in the footnote to table 4 to their mean values. Participants diagnosed with asthma between 1991 and 2002 were excluded from the analysis of chronic cough, chronic phlegm, chronic bronchitis, and COPD.

The associations between responsiveness and the clinical phenotypes persisted when responsiveness was quantified by slope. There were highly significant non-linear relations between methacholine slope and new wheeze, physician-diagnosed asthma, and COPD (all p<0.01), whereas new symptoms of cough, phlegm, or shortness of breath were not associated with the degree of bronchial responsiveness. There was a non-linear relation between methacholine slope at SAPALDIA 1 and change in FEV_1/FVC over the follow up period in men and a linear relation in women (p<0.001 for differences between men and women).

Silent BHR and decline in FEV_1

In asymptomatic individuals, BHR was associated with an accelerated decline in FEV_1. Our linear regression analyses adjusting for potential confounders (from baseline assessment: FEV_1, age, age squared, height; change between surveys: weight; from the follow up assessment: exposure to environmental tobacco smoke and exposure to dust and fumes at work) showed that this effect was significantly modified by smoking status (p = 0.02), but not by sex (p = 0.17). Silent BHR was associated with an additional decline in FEV_1 by 12 (5–18) ml/year (p = 0.038), 11 (5–16) ml/year (p<0.001), and 4 (2–8) ml/year (p<0.001) in current smokers, former smokers, and never smokers, respectively, at SAPALDIA 2 compared with asymptomatic participants without BHR. Figure 2 shows the relationship of the observed annual loss of FEV_1 as a function of the methacholine response slope. Although the variability in the observed change in FEV_1 over time is significant, the modelled adjusted mean follows the same lines—current and former smokers with BHR have a greater loss in FEV_1 than lifelong non-smokers.

DISCUSSION

This prospective population based study confirms that BHR is associated with the development of respiratory symptoms, asthma, and COPD. Active smoking in individuals with BHR conferred a synergistic detrimental effect on the loss of lung function. The effects were observed in a population defined as asymptomatic at baseline.

Interpretation of our results warrants careful consideration. Some selection bias cannot be excluded since participation in the bronchial challenge required fulfilment of health criteria as well as satisfactory spirometry. Non-participants in the follow up survey who had a challenge at baseline were significantly more likely to be smokers, symptomatic, and of

Table 4 Risk for the development of respiratory symptoms and for the presence of COPD at SAPALDIA 2 related to bronchial hyperresponsiveness (BHR) in asymptomatic individuals

	Unadjusted OR (95% CI)	Adjusted OR (95% CI)*	p value†
Asthma phenotypes			
Physician-diagnosed asthma	3.0 (1.9 to 4.7)	3.0 (1.8 to 5.0)	<0.001
Wheeze in last 12 months without cold	2.7 (1.8 to 3.9)	2.9 (1.8 to 4.5)	<0.001
Shortness of breath while walking	1.8 (1.4 to 2.4)	1.3 (0.9 to 1.8)	0.115
COPD phenotypes‡			
All subjects			
Chronic phlegm	1.0 (0.7 to 1.6)	1.2 (0.7 to 2.0)	0.478
Chronic cough	2.7 (1.7 to 4.3)	3.0 (1.7 to 5.2)	<0.001
Chronic bronchitis¶	3.0 (1.5 to 6.3)	2.6 (1.1 to 6.0)	0.023
COPD§	3.7 (2.9 to 4.7)	4.5 (3.3 to 6.0)	<0.001

OR, odds ratio; CI, confidence interval.
*From logistic regression with adjustments for sex, age, FVC in 1991, BMI in 1991, change in weight, exposure to environmental tobacco smoke reported in 2002, smoking status in 2002, pack years in 2002, atopy at baseline, exposure to dust and fumes at work in 2002, level of education at baseline, and study area.
†For effect estimates in adjusted analyses.
‡Participants diagnosed with asthma between 1991 and 2002 were excluded from analyses for chronic cough, phlegm, chronic bronchitis, and COPD.
§COPD was defined as $FEV_1/FVC < 0.70$ and no physician's diagnosis of asthma.
¶Chronic bronchitis defined as the presence of chronic cough or phlegm.

lower educational background, although they were similar to participants in terms of lung function characteristics.

The definitions of asthma and COPD are controversial, especially in the context of epidemiological studies.[26 32] In order to address the problems of misclassification in reports of new symptoms, we used a rather sensitive definition for asymptomatic (absence of all symptoms) at baseline to exclude as many participants as possible with undetected but existing respiratory symptoms. On the other hand, we used more specific definitions of symptoms at follow up which should have minimised false positive reports of new symptoms.

In addition, we found significant dose-response relations between new symptoms and BHR as a continuous variable. The coherence of our findings strongly supports the importance of BHR as a risk factor for the development of asthma as well as COPD, and argues against misclassification explaining the results. An additional potential limitation to the study is the fact that we did not measure post-bronchodilator lung function. As a consequence, our definition of COPD may be somewhat imprecise because we may have underestimated lung volumes in participants with reversible airway obstruction. However, we have tried to address this possible bias by excluding all individuals with physician-diagnosed asthma.[33]

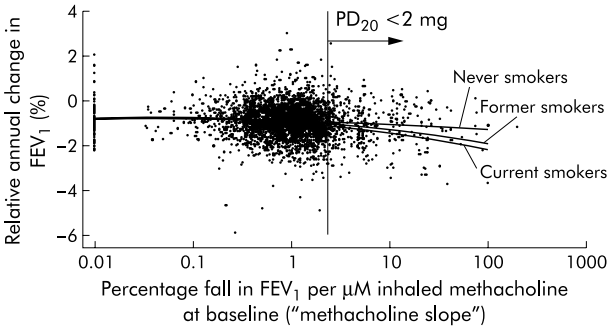

Figure 2 Relative annual change in FEV_1 (expressed as percentage of baseline) and responsiveness to methacholine in 1991. The scatter plot shows the unadjusted relative annual change in FEV_1 in participants who underwent a bronchial challenge at baseline and spirometric tests at both surveys. The line plot shows the mean annual change in FEV_1 by methacholine slope adjusted for FEV_1 at baseline, sex, height, pack years, exposure to environmental tobacco smoke, area, weight change, and occupation exposure to dust and fumes by smoking status at SAPALDIA 2. The vertical line indicates the PD_{20} at 2 mg methacholine and thus separates participants with and without bronchial hyperresponsiveness to methacholine.

BHR at baseline was associated with an increased risk of COPD (defined as $FEV_1/FVC < 0.70$) at follow up. This observation confirms the finding of a longitudinal Dutch study[8 9] which found a positive association between BHR and the development of respiratory symptoms, and a negative association with the resolution of such symptoms. However, in their study subjects with asthma were not systematically excluded from the primary analyses. In order to reduce the risk of contamination between asthmatic and COPD phenotypes, we excluded subjects with physician-diagnosed asthma when assessing BHR as a predictor for COPD. In the multivariate analyses we also adjusted for FVC, since responsiveness is affected by lung size and airway calibre.[6 29]

Since individuals with BHR at baseline had an increased risk for COPD at follow up, BHR may precede the development of COPD and not be just a consequence of it. The highest annual losses of FEV_1 were observed in current smokers with BHR, which suggests that BHR is not only an independent risk factor for the development of COPD but also increases the detrimental effect of cigarette smoking.

Interestingly, the same holds true for asthma: BHR is an independent risk factor for the development of asthma. There is good evidence of an interaction between BHR and airway inflammation derived from cross sectional and longitudinal studies as well as from pharmacological intervention trials.[34–36] The prevalence of atopy was higher in subjects with BHR at baseline, especially when symptomatic. However, in our adult study population aged 30–72 years, there was no evidence of a modification of the effect of BHR by atopy. It could be that atopy plays a more important role in the development of respiratory disease in younger adults and children than later in life.[37]

The common mechanism triggering the interaction between BHR and either the (atopic) inflammation leading to asthma or the smoke induced inflammation leading to COPD remains speculative. The abnormal airflow resulting from BHR might alter the deposition profile of both allergen and cigarette smoke derived particles in the central and peripheral airways. Indeed, Kohlhäufl *et al*[38] found that

women with BHR have increased deposition of fine particles compared with women without BHR, independent of their smoking habits. As a result of an increased exposure to allergen derived particles, sensitisation to airborne allergens would be more likely. Different studies have shown a dose-response relation between allergen exposure and sensitisation rates.[39 40] In addition, once sensitised, the ongoing increased exposure fuels atopic airway inflammation.[36] Similarly, increased airway deposition of cigarette smoke derived particles could increase local toxicity and gradually worsen airway inflammation and dysfunction. There is evidence from studies in bronchial biopsies[41] and sputum markers[42] that, even in individuals with asymptomatic BHR, there are signs of active inflammation. The dose-response relation between the quantity of cigarettes smoked and BHR,[43] airway inflammation,[44] and the risk for COPD[45] are well known and overtly visible in daily clinical practice. In fact, an altered deposition profile would render subjects with BHR more vulnerable to any sort of particulate inhalation irritants. Further investigations are needed to analyse whether subjects with BHR are more vulnerable to air pollutants in general.

Women had a 20% higher prevalence of BHR at baseline, which is in line with the findings of the ECRHS study.[20] There was no evidence in this population sample of a sex difference in the effect of BHR on the development of symptoms 11 years later. However, there were some differences in the effect of BHR on decline in FEV_1/FVC between men and women, with slightly stronger effects observed in men.

In conclusion, BHR is a risk factor for the development of respiratory symptoms, asthma and COPD, and is associated with an increased annual loss of FEV_1. Particularly at risk for COPD are active smokers with BHR. The combination of BHR and smoking confers a detrimental synergistic effect on the decline in FEV_1. Further studies are needed to elucidate the exact pathogenesis underlying this phenomenon.

ACKNOWLEDGEMENTS

The study could not have been conducted without the help of the study participants, technical and administrative support and the medical teams and field workers at the local centres. We particularly thank the SAPALDIA participants and their continued participation. *Local fieldworkers:* Aarau: M Broglie, M Bünter, G Drita; Basle: R Armbruster, T Damm, M Gut, L Maier, A Vögelin, L Walter; Davos: D Jud; Geneva: M Ares, M Bennour, B Galobardes, E Namer; Lugano: B Baumberger, S Boccia Soldati, E Gehrig-Van Essen, S Ronchetto; Montana: C Bonvin; Payerne: S Blanc, A V Ebinger, M L Fragnière, J Jordan; Wald: N Kourkoulos, U Schafroth. *Software technicians:* S Baur, P Frankenbach, D Burkhard. *Administrative assistants:* D Baehler, N Bauer, R Nilly.

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The SAPALDIA study was supported by the National Science Foundation of Switzerland (grant no 32-65896.01, NF 32-59302.99, 3247B0-102081), the Federal Office for Forest, Environment and Landscape, the Federal Office of Public Health, the Federal Office of Roads and Transport, the Cantons Basel-Stadt, Basel-Land, Geneva, Zurich, Ticino, Aargau, Luzern, the Swiss Lung League and the Lung League of Ticino, Zurich and Basel Stadt/Basel Landschaft. SAPALDIA Basel is part of the European Respiratory Health Survey.

Competing interests: none declared.

The following are members of the SAPALDIA Team. The letters in parentheses indicate their specialties, defined below.

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LUNG ALERT

When should we use inhaled steroids in 'asthmatic' infants?

▲ Bisgaard H, Hermansen MN, Loland L, *et al*. Intermittent inhaled corticosteroids in infants with episodic wheezing. *N Engl J Med* 2006;**354**:1998–2005

It is unclear when to treat wheezy infants with inhaled corticosteroids. In this single centre, prospective, double blind, randomised, placebo controlled trial, the authors hypothesised that the development of asthma in infants is predated by episodes of wheezing during early life. Furthermore, treatment with inhaled corticosteroids early in life would prevent the establishment of asthma as defined by persistent wheezing.

One month old infants born to mothers with a history of asthma were recruited. If the infants developed an episode of wheezing lasting more than 3 days they were randomised to receive either inhaled budesonide (400 μg/day) or placebo for 2 weeks. The primary end point was the number of symptom-free days, with a secondary end point being the development of persistent wheezing. Follow up was for 3 years.

Of 411 infants enrolled, 294 were randomised (149 to budesonide and 145 to placebo). There was no difference in the number of symptom-free days between the groups (83% budesonide *v* 82% placebo, absolute difference 1%, 95% CI −4.8 to 6.9), and similar numbers of infants had to discontinue the study because of persistent wheezing (24% budesonide *v* 21% placebo, hazard ratio 1.22, 95% CI 0.71 to 2.13). The mean duration of a wheezing episode was identical in both groups (10 days) and symptoms were likewise similar.

The authors conclude that the early use of intermittent inhaled corticosteroids does not reduce progression to persistent wheezing (or asthma) and, moreover, does not lead to any short term improvement in episodes of wheezing. Therefore, with current evidence, the use of this treatment strategy cannot be recommended. However, an accompanying editorial emphasises that it is not possible to exclude a beneficial effect in a smaller subgroup, and the lack of effect on symptom duration may reflect the introduction of steroids after 3 days of illness.

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torna all'abstract

The role of the mast cell in asthma: Induction of airway hyperresponsiveness by interaction with smooth muscle?

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In a recent study, the difference between asthma and eosinophilic bronchitis (a condition characterized by cough but not airway hyperresponsiveness or airflow obstruction) was infiltration of airway smooth muscle (ASM) by mast cells. Mast cells produce a variety of lipid mediators, chemokines, cytokines, and enzymes that may interact with ASM cells to cause hyperreactivity to constrictive stimuli and proliferation, and activated ASM can produce stem cell factor and other chemokines, cytokines, and growth factors that may act in recruitment, differentiation, and retention of mast cells. Mast cell infiltration of the airways in asthma is T-cell–dependent, and T_H2 cytokines from T cells and other sources act in mast cell expansion from circulating and tissue precursors. The recent data on interactions of mast cells and ASM suggest that this could be an important contributor to airway hyperresponsiveness in asthma. Why this occurs in asthma and how it is sustained remain to be established. (J Allergy Clin Immunol 2004;114:58-65.)

Key words: *Mast cells, asthma, chemokines, airway smooth muscle*

Abbreviations used
AHR: Airway hyperresponsiveness
ASM: Airway smooth muscle
EB: Eosinophilic bronchitis
HASM: Human airway smooth muscle
MC_T: Tryptase-only mast cell
MC_{TC}: Tryptase-chymase mast cell (human)
PAR-2: Protease-activated receptor
SCF: Stem cell factor
TLR: Toll-like receptor
VCAM-1: Vascular cell adhesion molecule 1

Although the mast cell has long been associated with asthma, recent studies have added to our knowledge of the biology of this intriguing cell type and raised questions about how mast cells contribute to asthma pathophysiology. In particular, Brightling et al[1] conducted a study comparing bronchial biopsy specimens from asthmatic volunteers with biopsy specimens obtained from patients with eosinophilic bronchitis (EB). EB is characterized by chronic cough and sputum eosinophilia in the absence of the airway hyperresponsiveness (AHR) and reversible airflow obstruction that characterizes asthma.[2] Interestingly, both groups of patients shared many of the immunopathologic features considered important in asthma, namely mucosal infiltration with eosinophils, T_H2 cytokine expression, and subepithelial collagen deposition. The only difference found between the 2 conditions was increased mast cell filtration of the airway smooth muscle (ASM) layer in asthma, which was not seen in EB.[1]

MAST CELLS AND IgE IN ALLERGIC ASTHMA

Mast cells were first described as tissue resident cells with prominent granules that were characteristic of allergic diseases. The description of IgE and its association with mast cell histamine release in the Gel and Coombs type I hypersensitivity reaction formed the basis for the initial understanding of the role of mast cells in asthma and acute allergic reactions. Indeed, in the 1970s, the cross-linking of IgE on high-affinity receptors on mast cells was regarded as the mechanism for the variable airflow obstruction that occurs in asthma and was the major target for therapeutic development of drugs such as cromoglycate.[3] However, asthma treatment with antihistamines and cromones was disappointing, particularly compared with responses to inhaled corticosteroids. The emergence of the eosinophil and later T_H2 hypotheses together with results from animal models then eclipsed the mast cell's role in asthma, which was considered relevant to acute symptoms and the early asthmatic response to allergen challenge, but less involved in chronic asthma.[4] Recent data must call for a re-evaluation of such a view. Effector functions of mast cell mediators have been extensively reviewed.

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Supported by a Wellcome Trust United Kingdom Research Leave Grant for Clinical Academics.
Received for publication March 1, 2004; revised March 1, 2004; accepted for publication March 8, 2004.
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0091-6749/$30.00

doi:10.1016/j.jaci.2004.03.034

This article focuses on mast cell function in the context of interaction with ASM.

MAST CELL DEVELOPMENT AND DIVERSITY

In common with other leukocytes, mast cells develop from $CD34^+$ bone marrow progenitors.[5-7] However, unlike other leukocytes, these cells mature in the tissue and circulate as committed immature precursor forms. An obligate factor in development of the mast cell lineage is stem cell factor (SCF) acting on its receptor, Kit.[6-11] This is confirmed by mast cell deficiency in mouse strains deficient in Kit (W/Wv) or the membrane-bound form of SCF: the Sl/SLd strain.[8,9] Mouse committed mast cell precursors were isolated as Kit^{hi}, Thy-1^{lo} from fetal blood.[12] These cells lacked the high-affinity IgE receptor (FcεR1) but expressed mRNA for mast cell proteases. They could reconstitute mast cells in W/Wv mice and gave rise to immature mast cells when cultured in the presence of SCF. Human mast cell precursors circulate at the $CD34^+Kit^+Fc\varepsilon R1^-$ promastocyte stage and express CD13 but not CD14, which distinguishes them from monocytes.[13,14]

It is suggested that there is a constitutive population of mast cells found in connective tissues, which are controlled by SCF.[5] This constitutive mast cell population appears to have an important role in innate immunity, and can be activated via Toll-like receptor (TLR) 2 and TLR4 (mouse mast cells) or TLR1, TLR2, and TLR6 (human cells) and CD48.[7,15,16] Although IFN-γ reduces mast cell growth, it increases expression of the high-affinity IgG receptor FcγR1, which, together with TLR signals, acts to promote TNF and β-tryptase production, both of which play roles in antibacterial defense.[7] This is most graphically demonstrated by the rapid demise of W/Wv mice after cecal puncture.[17,18]

The increased numbers of mast cells seen during inflammation at mucosal sites (such as the respiratory mucosa in asthma and allergic diseases) are expanded in response to SCF together with T-cell–dependent cytokines including IL-3, IL-5, IL-6, IL-9, IL-10, IL-13, and, in some situations, IL-4.[5-7]

There is now evidence of considerable diversity of mast cells in terms of protease and surface receptor expression, and this is likely controlled by the local tissue milieu including cytokines, growth factors, chemokines, adhesion molecules, and extracellular matrix proteins.

Human mast cells have been subdivided on the basis of their content of either tryptase and chymase (MC_{TC}) or tryptase only (MC_T).[19] The predominant mast cell type in the lung is MC_T, although MC_{TC} is also present, and the mast cells localized to the smooth muscle layer in asthma were of the MC_{TC} phenotype.[1] Although this division is a useful concept and correlates with other functional differences, it is likely an oversimplification, because mouse mast cells show great diversity of protease expression depending on *in vitro* culture conditions or *in vivo* location, and multiple human mast cell tryptases are described.[20,21] The crystal structure of human β-tryptase reveals that it is a tetramer[22] that depends on heparin for stabilization and activity.[23] The tetrameric structure explains relative resistance to serine protease inhibitors but also limited substrate specificity. Tryptases activate protease-activated receptors (PAR-2),[24] which may be important for activity on smooth muscle. In addition, they have differential activity on neuropeptides, which may favor bronchoconstriction in asthma.[25] Mast cell phenotype in terms of tryptase expression appears plastic, varying with the tissue localization, as most elegantly shown in studies of mast cells in the villi of the mouse intestine.[26] TGF-β regulates expression of the mouse tryptase mouse mast cell protease 1,[27] but how relative expression of tryptases and chymase in human mast cells is regulated is unknown.

Much has been learned from *in vitro* studies of human mast cell development from $CD34^+$ precursor cells by using SCF with IL-6 and IL-10, which results in approximately 60% mature mast cells by 10 weeks of culture.[5] Similar culture systems have been used to study mouse mast cells. However, there is still much to learn about the control of migration and phenotypic differentiation of mast cells in different tissue environments.

REGULATION OF MAST CELL PHENOTYPE AND TISSUE SURVIVAL IN ASTHMA

Both mouse and human studies implicate T_H2 cytokines in control of the mucosal and epithelial mast cell infiltration that characterizes asthma.[5] Interestingly, coculture of human cord blood–derived mast cells with SCF and IL-4 increased FcεR1 expression, IgE-mediated histamine release, and cytokine mRNA transcripts for TNF, IL-5, IL-13, CCL3 (MIP-1α), and GM-SCF (compared with culture in SCF alone), whereas IL-5 did not alter FcεRI or IgE-mediated histamine release and modestly increased transcripts for TNF, IL-5, CCL3, and GM-SCF but not IL-13.[28] This work has recently been extended by gene array analysis to describe the patterns (signatures) of gene activation in response to IgE cross-linking after culture in SCF alone or with IL-4, IL-5, or IL-9.[29] These cultured human mast cells, like dispersed gut mast cells, did not express transcripts for IL-4. This result contrasts with the mast cells described by Brightling et al[1] infiltrating ASM in asthma: these MC_{TC} mast cells expressed IL-4.

What, then, are the specific factors regulating the recruitment of precursors from the bone marrow, expansion and survival in the tissues, and specific tissue-dependent mast cell phenotype in asthma? Chemokines may play an important role in each of these steps. Cultured human mast cell precursors expressed functional CXCR2, CXCR4, CCR3, and CCR5 at 4 weeks of culture, but only CCR3 at the mature 9-week stage.[30] Thus, ligands for these receptors may act in recruitment of mast cell progenitors to specific tissue sites: whether receptors are

selectively expressed by differentiating subtypes of mast cells that are thus selectively recruited or differentiation occurs from a common precursor in the tissues remains uncertain, though the latter is perhaps more likely. CCR3 expression by both cultured human mast cells and nasal polyp mast cells was further upregulated from intracellular stores by FcεRI cross-linking.[31]

Data from the CCR3 knockout mouse are complex: when animals were sensitized by intraperitoneal ovalbumin with alum adjuvant followed by inhaled challenge, there was an increase in intraepithelial mast cell numbers in the trachea of the $CCR3^{-/-}$ mice compared with the wild-type litter mates, and AHR was increased.[32] However, when the animals were sensitized by epicutaneous exposure to ovalbumin, inhaled challenge did not result in mast cell recruitment to the airway epithelium in knockout or wild-type mice, and the AHR seen in the wild-type mice did not occur in the $CCR3^{-/-}$ mice.[33] These findings likely result from differential activation of T-cell cytokines acting on mast cell precursors, but the reasons for the observed difference require further elucidation.

Coculture of human mast cell precursors with fibroblasts results in mature mast cells of MC_{TC} phenotype, and because fibroblasts are a rich source of the CCR3 ligand CCL11 (eotaxin), it is possible that CCR3 plays a role in differentiation of the MC_{TC} phenotype.[34] ASM can also produce chemokines, including CCL11.[35] Whether this is involved in mast cell infiltration of smooth muscle in asthma and why it should differ from EB remains to be determined.

Adhesion molecules will clearly play an important role in recruitment of mast cell precursors to the airway, and possibly in selective localization and retention of mast cells with the tissues. Cultured human mast cells expressed α4β1, β7, α1β2, and αMβ2 integrins and P-selectin ligand, and bound to E-selectin, P-selectin, and vascular cell adhesion molecule 1 (VCAM-1).[35] Interestingly, binding to IL-4–activated human umbilical vein endothelial cells was completely blocked by antibodies to α4β1, although binding to TNF-activated human umbilical vein endothelial cells was not.[35] Thus, α4β1 integrin may play an important role in mast cell progenitor recruitment to the allergic asthmatic airway, where IL-4 (and IL-13) upregulate VCAM-1 expression.

Mast cell survival is dependent on SCF; however, additional factors may be involved, including binding of IgE itself to the high-affinity IgE receptor. Recent data suggest that monomeric IgE/FcεRI interactions are important for several mast cell functions, including inhibition of BCL_{xl} expression and, hence, survival.[36] In addition, T_H2 cytokines act in mast cell survival,[5-7] which may thus be promoted by T_H2 cells, but also by other cells producing T_H2 cytokines in the asthmatic airway, including basophils, eosinophils, epithelial cells, fibroblasts, and smooth muscle and mast cells.

One important question for understanding regulation of tissue mast cell numbers is whether the increase seen in the airway mucosa in asthma or rhinitis results from increased differentiation and recruitment from bone marrow, increased local development and expansion of tissue precursors, or increased survival or proliferation of an existing mast cell pool. These may not be separate processes, but, for example, factors acting in traffic of precursors from bone marrow to tissues may differ from those driving expansion and survival in the mucosa. There is remarkably little information on this subject, but 1 article does suggest an increase in circulating SCF-responsive $CD34^+$ cells in peripheral blood from asthmatic children compared with normal donors.[37] It is of note that mast cells cultured *in vitro* from cord blood precursors are IL-5–responsive and express IL-5Rα.[7] It has been assumed in many studies (including our own) that $CD34^+$IL-$5R\alpha^+$ cells in bone marrow, blood, or tissue represent potential eosinophil precursors,[38,39] but it is also possible that these could be mast cell precursors, and further characterization of such cells in terms of receptor expression and plasticity of differentiation potential is required.

MAST CELL EFFECTOR FUNCTIONS IN ASTHMA

Mast cells release preformed, stored mediators and synthesize other factors de novo on activation. The classical type 1 hypersensitivity reaction in acute asthma or the early response to allergen challenge results from IgE cross-linking by allergen and, thus, FcεRI signaling. It is now clear that this results in release of histamine, tryptases and chymase, and heparin, and synthesis of lipid mediators including leukotriene C4 and prostaglandin D2, which have roles in bronchoconstriction, edema, and recruitment of inflammatory cells.[6] Studies have defined additional roles for histamine in activation of inflammatory cells, dendritic cells, and T cells.[40] Further, leukotriene and prostaglandin receptors are also described: CysLT1 and CysLT2 receptors for cysteinyl leukotrienes, BLT for leukotriene B4 (LTB4), and CrT_H2 for PGD2.[41-43] Both CysLT1 and CysLT2 are expressed by mast cells, and the balance between these is differentially regulated by IL-4 and IL-5.[44] Recently, BLT receptors (for LTB4) have been implicated in mast cell–mediated $CD8^+$ T-cell recruitment,[45] and the PGD2 receptor CrT_H2 is selectively expressed by T_H2-type T cells[43]: mast cells may thus interact with T cells in asthma through histamine, leukotrienes, and PGD2. In addition, mast cell activation by IgE cross-linking results in release of preformed cytokines, chemokines, and growth factors and de novo synthesis of others.

It is now clear that mast cell effector function can also be stimulated by stimuli that do not cross-link IgE, including IgE binding to FcεR1, IgG receptors (FCγR1 and III activate, whereas FcγRII is inhibitory), complement receptors, histamine-releasing factors, and TLRs.[7] Because different mast cells express different patterns of cytokines and other mediators, it will be important to clarify whether different signals result in differential

effector functions, and which signaling cascades are involved.

MAST CELL–ASM INTERACTIONS

If localization of mast cells to the ASM is important for the asthma phenotype, how might these 2 cell types interact, and how does this contribute to the features of asthma?

First, how might ASM cells recruit, retain, and activate mast cells in asthma? It is now clear the smooth muscle cells have the capacity to produce a wide variety of proinflammatory cytokines and chemokines. For example, IL-1β or TNF-activated human ASM (HASM) produces CCL11, CCL5 (RANTES), and CCL2 (monocyte chemoattractant protein 1) as well as GM-CSF, IL-6, CXCL8 (IL-8), and IL-11.[46-49] IL-4 and IL-13 can activate HASM CCL11 production.[50] Thus, in the inflammatory setting of asthma, HASM chemokine release may act in recruitment and retention of mast cells. This may then be amplified by mast cell products (such as IL-4 or TNF). In addition, activated HASM produces both soluble and membrane-bound SCF, which may thus play an important role in recruitment, survival, and differentiation of mast cell precursors and mature mast cells: to some extent, this is constitutive and may contribute to localization of mast cells in smooth muscle reported in normal airways.[51,52] Recent data suggest that HASM may attract mast cells though release of TGF-β in addition to SCF.[53] In a culture system, the HMC-1 cell line showed chemotaxis to HASM culture supernatants after these had been activated with tryptase.[53] Both factors have been localized to smooth muscle in asthmatic airways.

Human ASM expresses a variety of adhesion molecules that could act in recruitment and retention of mast cells and their precursors, including VCAM-1 and intercellular adhesion molecule 1.[48] More recently, a novel mast cell adhesion molecule, spermatogenic immunoglobulin superfamily (SgIGSF), was described that acts in adhesion of mouse mast cells to fibroblast cell lines and was controlled by the transcription factor MITF. Whether this or other specific mast cell adhesion pathways act during mast cell/HASM interaction will be of interest.[54]

Clearly, mast cell infiltration of the ASM in asthma could lead to direct activation of airway narrowing via release of histamine, leukotrienes, or other mediators. In addition, leukotrienes may play a role in HASM proliferation and other changes in remodeling.[55] Mast cells also interact with HASM through the effects of chemokines, cytokines, proteases, and growth factors. Tryptase and TNF can both increase HASM responsiveness to histamine and other constrictors; tryptases may do this partly through activation of PAR-2 receptors.[56,57] Transmembrane tryptase but not β-tryptase could induce AHR when administered by aerosol to mice.[58] This effect was dependent on signal transduction and activator of transcription 6 activation by IL-4 and/or IL-13. Whether this was via an effect on smooth muscle or other cell types remains to be determined. Mast cells may also play a role in HASM proliferation through tryptases[59] and growth factors such as platelet-derived growth factor.[60] In contrast, other mast cell–derived factors such as chymase,[61] heparin,[62] and IL-4 inhibit HASM proliferation,[63] whereas TGF-β inhibits proliferation to some growth factors, such as EGF, but enhances responses to leukotriene D4.[64,65] TGF-β induces HASM production of extracellular matrix proteins.[64,66] Activin A is also released from mast cells and was recently shown to induce smooth muscle proliferation *in vitro*.[67]

The interaction of mast cells and HASM may also influence a wide variety of other inflammatory and structural cells in the asthmatic airway. For example, IL-6 induced from HASM will potentiate IgE synthesis and T_H2 cell development, whereas GM-SCF and CCL11 will amplify eosinophilic inflammation. Putative interactions between mast cells and HASM are summarized in Fig 1.

MAST CELL INVOLVEMENT IN ASTHMA

Numerous human studies have demonstrated increased mast cell numbers in the airway in asthma and have detected histamine, PGD2, and tryptase in bronchoalveolar lavage fluid both in symptomatic asthma and after allergen inhalation challenge, suggesting mast cell degranulation.[68-70] Mast cell numbers in bronchoalveolar lavage and airway biopsies could be related to AHR in some studies.[68] However, it is of note that other studies did not find increased mast cell numbers in either the airway epithelium or submucosa in asthma.[71] Mast cell infiltration of ASM was reported in postmortem studies of both fatal and nonfatal asthma and in isolated tracheal rings from asthmatics,[52,72] and was the major difference between asthma and EB in the study by Brightling et al.[1]

Results from a mouse model of asthma showed that AHR and tissue eosinophils could still be evoked if the mast cell-deficient W/Wv mice underwent sensitization with intraperitoneal ovalbumin with aluminium hydroxide adjuvant (which favors a T_H2 response), but not if sensitization was without adjuvant.[73] Thus, mast cells can cause AHR, but it can also be elicited without these cells.

Mast cell infiltration of the airway mucosa is also a feature of nonatopic asthma.[74] How mast cells might be activated without a role for allergens is uncertain, but 1 possibility is that IgE is in fact involved, because local airway IgE synthesis was detected in nonatopic as well as atopic asthma,[75] and it has been suggested that an autoallergen termed Hom s 1 might activate IgE in atopic dermatitis.[76] Alternatively, mast cells might be activated by mechanisms that do not involve IgE cross-linking. It is of note that serum total IgE concentration in nonatopic asthma is often higher than in nonasthmatic, nonatopic subjects. This raises a potential role for monomeric IgE in sensitizing mast cells for activation in nonatopic asthma.

Mast cells are well established as profibrotic cells and release a variety of growth factors.[77] These cells are thus

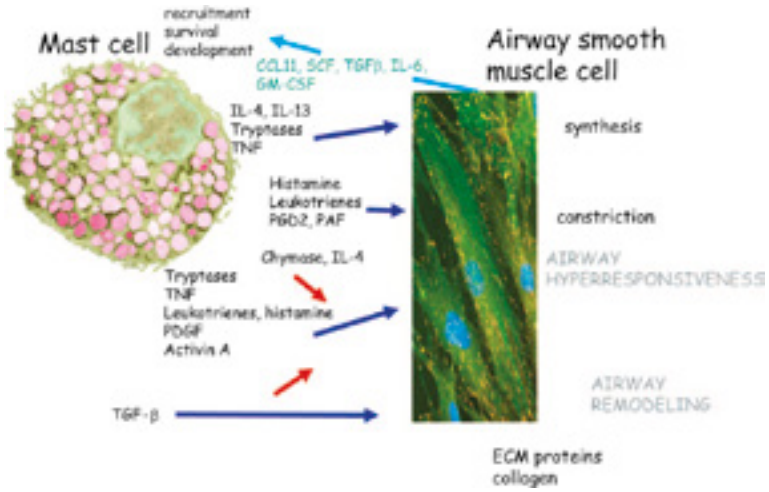

FIG 1. Mast cell/ASM cell interactions in asthma. ASM cells can produce SCF, cytokines, and chemokines active in recruitment and survival of mast cells, whereas mast cells produce mediators that directly constrict ASM, potentiate their constrictor response, cause ASM proliferation, and alter ASM to a synthetic phenotype that may contribute extracellular matrix proteins and collagens in airway remodeling. *PDGF,* Platelet-derived growth factor. Tania Vitalis and Linda Sharp/Wellcome Photo Library.

implicated in airway remodeling in asthma. The effect of airway remodeling on airway function and hyperresponsiveness remains uncertain. Subepithelial basement membrane deposition of extracellular matrix proteins occurs in both asthma and EB, and recent evidence suggests that this is at least partly dependent on IL-5 and eosinophils via TGF-β production.[78] However, patients with EB do not have AHR. Whether these patients have the smooth muscle accumulation or epithelial changes seen in asthma will be an important future question.

Clearly, other cell types play important roles in asthma pathogenesis, and much debate has occurred about the relative importance of mast cells and others cell types such as eosinophils and T cells. This partly resulted from work on the model of inhaled allergen challenge and dissection of the early and late asthmatic response. Although there is wide agreement that the early asthmatic reaction (EAR) is IgE-dependent and mast cells play a pivotal role,[6] the late asthmatic reaction (LAR) can be IgE-dependent or IgE-independent, as seen by isolated LAR induced by intradermal injection or inhalation of allergen-derived peptide fragments that activate T cells but not IgE; there was no evidence of mast cell activation in such reactions.[79-81]

EFFECTS OF ASTHMA THERAPY ON MAST CELLS

Steroid treatment of asthma reduces airway mast cells numbers, presumably in part through reduction in T_H2 cytokine expression. Other treatments have specific effects on mast cell function, including β_2 agonists, leukotriene antagonists, antihistamines, and cromones.[82] More specifically, anti-IgE treatment is shown to inhibit both the early- and late-phase response to allergen challenge and reduce symptoms and steroid requirement in moderate to severe asthma.[83,84] These effects may be through reduction in mast cell activation (and presumably mast cell numbers in the airway, although this has yet to be confirmed), although other cells are activated through IgE. The effects of asthma treatment on mast cell infiltration of ASM will be of great interest.

REMAINING QUESTIONS

The study by Brightling et al[1] refocuses on the mast cell as an important determinant of the asthma phenotype. It is now clear that these cells are pleiotropic, multipotential, and complex. Further understanding of airway mast cell biology should open novel avenues for therapy. However, specific and pressing research questions remain:

The findings of Brightling et al[1] need to be confirmed and extended, preferably with more reliable ways of sampling ASM.

What is the situation in nonatopic asthma?

It remains to be shown why mast cells behave differently in these 2 related conditions: is this an effect of differences in mast cell or smooth muscle biology? Further analysis of airway samples is required.

Are there genetic determinants of airway mast cell-ASM interactions in asthma as opposed to other conditions?

What is the role of mast cell infiltration of HASM in airway remodeling, and what are its consequences?

What signals in and from ASM activate mast cells?

How do these changes respond to treatment?

CONCLUSION

The differences between EB and asthma in terms of HASM infiltration by mast cells, together with many mechanisms by which mast cells can stimulate and potentiate smooth muscle contraction and proliferation,

suggest that this interaction is an important determinant of AHR in asthma. It is of note that cough and other shared features of asthma and EB may be more associated with eosinophil activation. These different cell types may contribute to different aspects of airway remodeling in asthma, with HASM accumulation more dependent on mast cells, whereas extracellular matrix protein deposition may result more from eosinophil and myofibroblast activation. Future development of specific therapy to target these cell types will be of interest.

I thank Prof Tim Williams and Drs Adele Hartnell, Charlotte Weller, and Stuart Hirst for reading the manuscript, and Stuart Hirst for the picture of HASM in Fig 1.

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torna all'abstract

ORIGINAL ARTICLE

A Longitudinal, Population-Based, Cohort Study of Childhood Asthma Followed to Adulthood

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ABSTRACT

BACKGROUND

The outcome of childhood asthma in adults has been described in high-risk cohorts, but few population-based studies have reported the risk factors for persistence and relapse.

METHODS

We assessed children born from April 1972 through March 1973 in Dunedin, New Zealand, repeatedly from 9 to 26 years of age with questionnaires, pulmonary-function tests, bronchial-challenge testing, and allergy testing.

RESULTS

By the age of 26 years, 51.4 percent of 613 study members with complete respiratory data had reported wheezing at more than one assessment. Eighty-nine study members (14.5 percent) had wheezing that persisted from childhood to 26 years of age, whereas 168 (27.4 percent) had remission, but 76 (12.4 percent) subsequently relapsed by the age of 26. Sensitization to house dust mites predicted the persistence of wheezing (odds ratio, 2.41; P=0.001) and relapse (odds ratio, 2.18; P=0.01), as did airway hyperresponsiveness (odds ratio for persistence, 3.00; P<0.001; odds ratio for relapse, 3.03; P<0.001). Female sex predicted the persistence of wheezing (odds ratio, 1.71; P=0.03), as did smoking at the age of 21 years (odds ratio, 1.84; P=0.01). The earlier the age at onset, the greater the risk of relapse (odds ratio, 0.89 per year of increase in the age at onset; P<0.001). Pulmonary function was consistently lower in those with persistent wheezing than in those without persistent wheezing.

CONCLUSIONS

In an unselected birth cohort, more than one in four children had wheezing that persisted from childhood to adulthood or that relapsed after remission. The factors predicting persistence or relapse were sensitization to house dust mites, airway hyperresponsiveness, female sex, smoking, and early age at onset. These findings, together with persistently low lung function, suggest that outcomes in adult asthma may be determined primarily in early childhood.

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N Engl J Med 2003;349:1414-22.

THE INCREASE IN THE PREVALENCE OF wheezing disorders, whether or not they are labeled as asthma, could be related to an increased incidence or an increased persistence of asthma.[1,2] Studies of the natural history of asthma have often focused on selected populations. However, the outcomes in children referred to university clinics[3,4] or selected in high-risk cohorts[5] may not reflect the outcomes in the general population, since the initial selection criteria may predetermine the risk factors for persistence or relapse.[6]

Most children attending asthma specialty clinics who have been followed have had atopy, with frequent symptoms and airway hyperresponsiveness, indicating severe disease that is likely to persist. The effects of sex, age at onset, and smoking on the outcome have been uncertain in such children[7,8] and in high-risk cohorts selected according to the presence of parental atopy.[5,9] The few population-based epidemiologic studies of outcomes of childhood asthma differ in the frequency of assessment, cohort-retention rates, and the use of quantitative measurements.[10-13] In particular, repeated lung-function measurements have been reported infrequently.[14-16] We report outcomes in an unselected, population-based birth cohort of over 1000 New Zealand children followed to adulthood.

METHODS

STUDY MEMBERS

The Dunedin Multidisciplinary Health and Development Study is a longitudinal investigation of health and behavior in a complete birth cohort.[17-21] The study members were born in Dunedin, New Zealand, between April 1972 and March 1973. Of 1139 children born during that period and residing in the province of Otago, New Zealand, at the age of three years, 1037 (91 percent, 52 percent of whom were boys) participated in the first follow-up assessment at three years of age. The cohort families represented the full range of socioeconomic status in New Zealand's South Island and were primarily of European extraction.

The children were seen every 2 years between 3 and 15 years of age and then were seen at 18, 21, and 26 years. Respiratory questionnaires were completed and lung-function measurements were performed at the ages of 9, 11, 13, 15, 18, 21, and 26 years; airway hyperresponsiveness to methacholine was determined at the ages of 9, 11, 13, 15, and 21 years; atopy was determined at 11 years (IgE levels only), 13 years (skin tests only), and 21 years (IgE levels and skin tests); and responsiveness to a bronchodilator was determined at 18 and 26 years.

The research ethics committee of the Otago Hospital Board approved each assessment. The participants gave written informed consent from the age of 18 years; before that age, a parent or guardian gave written informed consent, and the child gave oral assent.

FOLLOW-UP

For accurate classification of remission, relapse, or persistence of asthma, study members who completely missed any assessment were excluded from the analysis. For an assessment to be included, at least a respiratory questionnaire had to be completed. The characteristics of the study members who were included were compared with the characteristics of those not included. The outcome characteristics of those included and all study members participating at the age of 26 years were also compared.

QUESTIONNAIRES

When the child was nine years old, the accompanying adult (usually the mother) answered detailed questions about the child's symptoms and illnesses, providing a retrospective history of respiratory events between birth and the age of nine, including frequency, severity, trigger factors, and treatment (specifically, the use of bronchodilator and corticosteroid medications).[19] Subsequently, similar questions were answered by the study member. From the age of 18 years, the study member completed the self-administered questionnaire used in the European Community Respiratory Health Study[22] and questions from the American Thoracic Society questionnaire[23] before answering the interviewer-administered questionnaire.

SKIN TESTS

Skin-prick testing was performed at the ages of 13 years (714 participants) and 21 years (885 participants) to determine sensitivity to house dust mites (*Dermatophagoides pteronyssinus*) (Bencard), grass, cat dander, dog dander, horse dander, kapok, wool, *Aspergillus fumigatus*, alternaria, penicillium, and cladosporium (Hollister–Stier).[20] The positive and negative controls were 0.1 percent histamine and diluent, respectively. The response was measured as the mean diameter of the resulting wheal.

LUNG-FUNCTION TESTS

Three measurements of forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1), obtained in the absence of recent use (within six hours) of a bronchodilator, were recorded on a Godart water-sealed spirometer at the ages of 9, 11, 13, 15 and 21 years (when methacholine challenges were also performed), a Morgan rolling-seal spirometer at the age of 18 years, and a SensorMedics body plethysmograph at the age of 26 years. The instruments were calibrated daily with a 3-liter syringe. The measurements were made between 1 p.m. and 4 p.m. The predicted values at 26 years of age were based on a study of New Zealand adults.[24]

AIRWAY RESPONSIVENESS

Methacholine challenge was performed in all consenting study members at the ages of 9, 11, 13, 15, and 21 years with the use of an abbreviated validated protocol[21] modified from that of Chai et al.[25] Challenge was not performed in those with airflow obstruction (FEV_1 of less than 75 percent of the FVC at 9 and 11 years of age and of less than 70 percent of the FVC at older ages), but spirometry was repeated 10 minutes after they had inhaled nebulized albuterol (5 mg per milliliter) for 2 minutes. At 18 and 26 years of age, bronchodilator responsiveness was determined in all consenting study members.

DEFINITIONS

Figure 1 illustrates the definitions of different patterns of wheezing. All wheezing, irrespective of causal factors, was included except for wheezing occurring only once or twice annually and lasting less than one hour. Wheezing reported at every assessment after its first mention was classified as persistent wheezing. Remission was defined as the absence of wheezing after wheezing had been reported at two or more successive prior assessments. Relapse was recorded if wheezing was reported at two or more successive assessments, then was absent at one or more successive assessments, and then was reported at all subsequent assessments. Intermittent wheezing was characterized by the presence of symptoms at some assessments but not others and not at two consecutive assessments and not fitting the patterns described above. Wheezing reported at one assessment only was classified as transient wheezing.

Airway hyperresponsiveness was defined by a value for methacholine PC_{20} (the concentration of methacholine causing a 20 percent decrease in the FEV_1) of 8 mg per milliliter or less or an increase in the FEV_1 of at least 10 percent from base line in response to a bronchodilator. Atopy was defined by a wheal diameter at least 2 mm greater than that of the wheal produced by the diluent control.

STATISTICAL ANALYSIS

The data were analyzed with SAS software. The characteristics of the study members and the prevalences of persistence, remission, and relapse were described with summary statistics. Logistic regression was used to estimate unadjusted and adjusted odds ratios, significance levels, and confidence intervals for factors associated with persistence or relapse. Trends and differences between outcome groups in mean measures of pulmonary function were assessed with generalized estimating equations incorporating the repeated nature of these data.

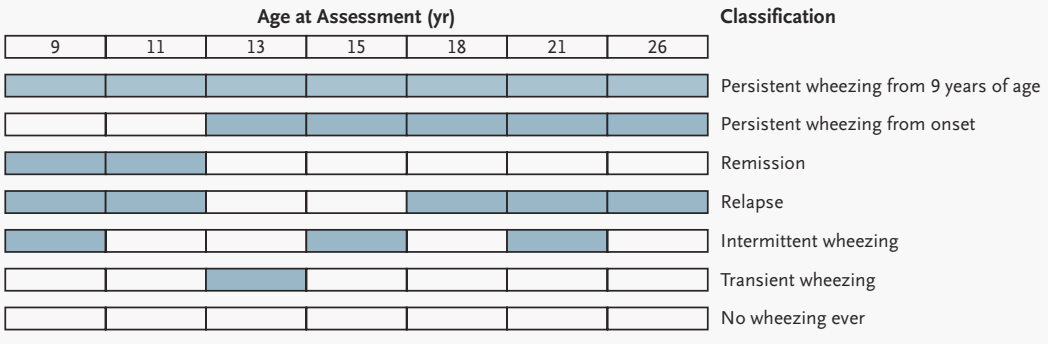

Figure 1. Patterns of Wheezing (Shaded Bars) in Childhood Reported by Study Members or Their Parents, Illustrating Definitions of Persistent Wheezing, Remission, Relapse, Intermittent Wheezing, Transient Wheezing, and No Wheezing Ever.

RESULTS

STUDY SAMPLE

At the age of 9 years, 815 study members (78.6 percent of the cohort of 1037) completed respiratory questionnaires, as did 802 (77.3 percent) at 11 years, 735 (70.9 percent) at 13 years, 972 (93.7 percent) at 15 years, 868 (83.7 percent) at 18 years, 957 (92.3 percent) at 21 years, and 954 (92.0 percent) at 26 years. Because of the reduced numbers at 11 and 13 years, only 613 study members (59.1 percent of the total, of whom 317 were male) provided respiratory data at every assessment. These 613 make up the sample for the analysis of persistence, remission, and relapse of wheezing, since those with missing data cannot be accurately classified.

REPRESENTATIVENESS OF THE SAMPLE

There were no significant differences in sex ratio, family history of asthma and hay fever, symptoms, proportion with atopy, lung-function measurements, or prevalence of airway hyperresponsiveness between the 613 study members with complete respiratory data and the original cohort of 1037, or between the 613 study members and all study members undergoing these investigations at particular ages. As compared with those not attending every assessment, the 613 were more likely to report current asthma or wheezing at 9 years and were more likely to be sensitive to house dust mites or to any allergen at 13 and 21 years. However, they were not more likely to have airway hyperresponsiveness at 9 years and were less likely to have airway hyperresponsiveness at 21 years (Table 1). At 26 years, there were no significant differences in the prevalences of asthma, wheezing, asthma treatment, or smoking or in lung-function measurements between those seen at every assessment and those not seen at every assessment (Table 1). Hence, the 613 with complete outcome data are generally representative of the base cohort.

PERSISTENCE OF WHEEZING

Of the study members, 72.6 percent had reported wheezing during at least one assessment by the age of 26 years, and 51.4 percent had reported such wheezing at more than one assessment. At this age, 26.9 percent of the study members were currently wheezing. In 14.5 percent, wheezing had persisted from onset, whereas 12.4 percent had had a remission followed by a relapse by the age of 26 years (Table 2). Another 15.0 percent remained in remission, 9.5 percent had intermittent symptoms, and 21.2 percent had reported symptoms at only one assessment.

TRANSIENT WHEEZING

Wheezing at only one assessment (transient wheezing) was reported by 130 of the 613 study members (21.2 percent) (Table 2), including 28 (4.6 percent) who reported wheezing only at the age of 26 years. As compared with the group of study members who never reported wheezing, the group with transient wheezing had a significantly higher prevalence of atopy for house dust mites at the age of 13 years (23.3 percent vs. 12.7 percent, $P=0.02$), and nonsignificant trends toward an increased prevalence of atopy at 21 years of age and toward smoking.

RELATION OF PERSISTENCE AND REMISSION TO RISK FACTORS

Table 3 shows the relations between outcomes at the age of 26 years and atopy, airway hyperresponsiveness, parental and personal smoking, birth order, and whether the study member had been breastfed. At the age of 26 years, study members with persistent or relapsing wheezing had higher prevalences of sensitivity to house dust mites ($P<0.001$) and cat allergen ($P<0.001$) and of airway hyperresponsiveness ($P<0.001$) and lower lung-function measurements ($P<0.001$) than those whose wheezing did not persist or relapse.

Table 4 shows the odds ratios for persistence and relapse of wheezing according to univariate and multivariate models. The highest odds ratios associated with either persistence or relapse were for airway hyperresponsiveness (determined as either a value for methacholine PC_{20} that was less than 8 mg per milliliter or an increase in the value for FEV_1 of more than 10 percent from base line in response to a bronchodilator) between the ages of 9 and 21 years and for a positive skin test for house dust mites at the age of 13 years. Female sex and smoking also predicted persistence, whereas an early age at onset predicted relapse. Other factors that were significant in the univariate analysis were not independently significant in multivariate analyses.

Throughout childhood and into adulthood, study members with persistent wheezing had consistently lower lung-function measurements, expressed as the ratio of FEV_1 to FVC, than study members who never reported wheezing (Fig. 2). The slopes of change in the FEV_1:FVC ratio over time from the ages of 9 to 26 years in any outcome category for ei-

Table 1. Characteristics of the 613 Study Members Who Provided Respiratory Data at All Assessments from 9 to 26 Years of Age, as Compared with the Characteristics of Those Not Providing Data at All Assessments.*

Characteristic†	Prevalence in 613 Study Members Seen at All Assessments	Prevalence in Study Members Not Seen at All Assessments	Study Members Included in Comparison
	% (*no. of study members with data*)		
Male sex	51.7 (613)	51.4 (424)	Full cohort
Maternal asthma	9.0 (589)	5.9 (271)	Seen at 7 yr
Paternal asthma	9.1 (583)	9.0 (266)	Seen at 7 yr
Maternal hay fever	21.8 (588)	16.9 (272)	Seen at 7 yr
Paternal hay fever	12.6 (581)	11.7 (265)	Seen at 7 yr
Asthma at 9 yr	9.0 (613)	3.5 (202)‡	Seen at 9 yr
Wheezing at 9 yr	21.7 (613)	14.4 (202)‡	Seen at 9 yr
Atopy at 13 yr (any skin-test wheal ≥2 mm)	46.9 (597)	33.3 (117)§	Skin-tested at 13 yr
Positive for house-dust-mite allergen at 13 yr	31.3 (597)	22.2 (117)‡	Skin-tested at 13 yr
Positive for house-dust-mite allergen at 21 yr	58.6 (577)	49.7 (308)‡	Skin-tested at 21 yr
PC_{20} ≤8 mg/ml at 9 yr	15.6 (578)	11.0 (191)	Methacholine-challenged at 9 yr
PC_{20} ≤8 mg/ml at 21 yr	6.5 (543)	10.3 (301)‡	Methacholine-challenged at 21 yr
Asthma at 26 yr	20.7 (613)	16.4 (367)	Seen at 26 yr
Wheezing at 26 yr	36.1 (613)	37.6 (359)	Seen at 26 yr
Asthma treatment at 26 yr	17.8 (612)	13.2 (348)	Seen at 26 yr
Smoking at 26 yr	33.8 (612)	40.5 (348)	Seen at 26 yr
FEV_1 at 26 yr (% of predicted)	101.5 (597)	101.1 (335)	Spirometry at 26 yr
FEV_1:FVC at 26 yr (%)	82.0 (597)	82.7 (335)	Spirometry at 26 yr

* Study members are described as "seen" if they attended the assessment and provided respiratory data.
† PC_{20} denotes the concentration of methacholine causing a 20 percent decrease in forced expiratory volume in one second (FEV_1), and FVC denotes forced vital capacity.
‡ P<0.05.
§ P<0.01.

Table 2. Outcomes at Age 26 Years among 613 Study Members Who Provided Respiratory Data at Every Assessment, According to Sex.

Outcome	Male Study Members (N=317)	Female Study Members (N=296)	Total (N=613)
		% (*no. of study members*)	
Persistent wheezing (from onset to 26 yr)	12.6 (40)	16.6 (49)	14.5 (89)
Relapse (wheezing stopped then recurred)	12.9 (41)	11.8 (35)	12.4 (76)
In remission (free of wheezing at 26 yr)	15.5 (49)	14.5 (43)	15.0 (92)
Intermittent wheezing	9.5 (30)	9.5 (28)	9.5 (58)
Transient wheezing (reported at only one assessment)	19.9 (63)	22.6 (67)	21.2 (130)
Wheezing never reported	29.7 (94)	25.0 (74)	27.4 (168)

Table 3. Characteristics of Study Members with Different Patterns of Wheezing.*

Characteristic	Wheezing Pattern						P for Trend†
	Persistent from Onset	Relapse	Remission	Intermittent	Transient	Never Wheezed	
	percent (number of study members with data)						
Male sex	44.9 (89)	53.9 (76)	53.3 (92)	51.7 (58)	48.5 (130)	56.0 (168)	—
Smoking at 18 yr	40.5 (89)	35.5 (76)	31.5 (92)	37.9 (58)	30.8 (130)	14.9 (168)	—
Smoking at 26 yr	46.1 (89)	43.4 (76)	35.9 (92)	43.1 (58)	32.3 (130)	19.6 (168)	—
Father smoked when study member was a child	39.8 (88)	56.6 (76)	54.4 (92)	62.1 (58)	50.0 (130)	44.1 (168)	—
Mother smoked when study member was a child	37.1 (89)	40.8 (76)	46.7 (92)	50.0 (58)	36.9 (130)	38.7 (168)	—
Positive skin test for house-dust-mite allergen at 13 yr	55.7 (88)	54.9 (71)	35.6 (87)	30.4 (56)	23.3 (129)	12.7 (166)	<0.001
Positive skin test for cat allergen at 13 yr	28.4 (88)	26.8 (71)	21.8 (87)	14.3 (56)	7.8 (129)	4.2 (166)	<0.001
Positive skin test for house-dust-mite allergen at 21 yr	77.5 (80)	73.9 (69)	64.8 (88)	55.6 (54)	54.8 (124)	43.2 (162)	<0.001
Positive skin test for cat allergen at 21 yr	53.8 (80)	47.8 (69)	35.2 (88)	24.1 (54)	18.6 (124)	11.7 (162)	<0.001
PC_{20} ≤8 mg/ml or BDR ≥10% at 9 yr	42.5 (87)	43.1 (72)	23.9 (92)	15.5 (58)	5.6 (126)	3.6 (165)	<0.001
PC_{20} ≤8 mg/ml or BDR ≥10% at any assessment from 9–21 yr	52.8 (89)	56.6 (76)	31.5 (92)	27.6 (58)	8.6 (128)	7.2 (167)	<0.001
FEV_1 at 26 yr (% of predicted)	96.6 (85)	95.7 (76)	100.6 (89)	103.7 (58)	102.5 (126)	105.6 (161)	<0.001
FEV_1:FVC at 26 yr (%)‡	78.0 (86)	79.1 (76)	83.1 (89)	82.2 (58)	83.4 (126)	83.7 (162)	<0.001
Firstborn	41.6 (89)	32.9 (76)	32.6 (92)	34.5 (58)	36.2 (130)	43.5 (168)	—
Breast-fed ≥4 wk	57.3 (89)	52.6 (76)	59.8 (92)	37.9 (58)	49.2 (130)	51.2 (168)	—

* PC_{20} denotes the concentration of methacholine causing a 20 percent decrease in the forced expiratory volume in one second (FEV_1), BDR the response of FEV_1 to a bronchodilator (increase from base line), and FVC the forced vital capacity.
† The trend is across categories of frequency from persistent from onset to never wheezed.
‡ FEV_1 (% of predicted) was based on a prediction formula from a New Zealand population[24] and was measured without the use of a bronchodilator.

ther sex were not significantly different from those for study members who never reported wheezing. When generalized estimating equations incorporating the repeated nature of the data in the analysis were used, the mean FEV_1:FVC ratio for male study members with persistent wheezing was 6.8 percent less than the mean for male study members who never reported wheezing (P<0.001); for male study members who had a relapse, the difference was –6.5 percent (P<0.001). The differences in the mean FEV_1:FVC ratio between those with remission, intermittent wheezing, or transient wheezing and those who never reported wheezing were nonsignificant (–0.8 percent, –1.9 percent, and –1.1 percent, respectively). Among female study members, the differences in the FEV_1:FVC ratio, as compared with those who never reported wheezing, were –4.7 percent for persistent wheezing (P<0.001), –2.7 percent for relapse (P=0.003), –2.3 percent for remission (P=0.022), –1.8 percent for intermittent wheezing, and –0.1 percent for transient wheezing.

The lung-function measurements in study members with persistent wheezing who reported having used inhaled corticosteroids at any time were substantially lower at all ages and for both sexes than they were in those with persistent wheezing who had never used inhaled corticosteroids; the mean difference in the FEV_1:FVC ratio was –7.2 percent in male study members and –8.2 percent in female study members. Similarly, in the group with persistent wheezing, lung-function measurements were lower in study members who had airway hyperresponsiveness on three or more occasions than in those who had hyperresponsiveness less often (mean difference, –7.4 percent for male and female study members combined).

DISCUSSION

Our study of an unselected, population-based birth cohort with the use of seven respiratory assessments from childhood into adulthood provides insights into the risk factors for the persistence and relapse of childhood asthma and for pulmonary-function

Table 4. Odds Ratios for Factors Predicting Persistence of Wheezing from Onset to the Age of 26 Years or Relapse, by the Age of 26 Years.*

Model	Persistence		Relapse	
	OR (95% CI)	P Value	OR (95% CI)	P Value
Univariate				
PC_{20} or BDR at 9 yr	4.32 (2.64–7.06)	<0.001	6.82 (3.89–11.95)	<0.001
PC_{20} ≤8 mg/ml at any assessment from 9–15 yr	4.24 (2.64–6.79)	<0.001	6.93 (4.07–11.77)	<0.001
PC_{20} ≤8 mg/ml or BDR at any assessment to 21 yr	4.13 (2.59–6.59)	<0.001	7.22 (4.29–12.17)	<0.001
Positive skin test for house-dust-mite allergen at 13 yr	3.38 (2.12–5.37)	<0.001	4.17 (2.49–7.01)	<0.001
Positive skin test for cat allergen at 13 yr	2.81 (1.65–4.79)	<0.001	3.27 (1.78–6.03)	<0.001
Smoking at 21 yr	2.05 (1.30–3.24)	0.002	1.84 (1.11–3.04)	0.02
Father smoked when study member was a child	0.63 (0.40–1.00)	0.05	1.29 (0.79–2.11)	0.31
Mother smoked when study member was a child	0.84 (0.53–1.37)	0.46	0.98 (0.60–1.61)	0.93
Family history of wheezing	1.44 (0.92–2.27)	0.11	1.59 (0.98–2.60)	0.06
Age at onset of wheezing†	0.97 (0.94–1.01)	0.11	0.87 (0.83–0.91)	<0.001
Female sex	1.37 (0.87–2.16)	0.17	0.95 (0.58–1.55)	0.84
Multivariate (significant factors only)				
PC_{20} ≤8 mg/ml or BDR >10% at any assessment from 9 –21 yr	3.00 (1.71–5.26)	<0.001	3.03 (1.65–5.55)	<0.001
Positive skin test for house-dust-mite allergen at 13 yr	2.41 (1.42–4.09)	0.001	2.18 (1.18–4.00)	0.01
Female sex	1.71 (1.04–2.82)	0.03	—	—
Smoking at 21 yr	1.84 (1.13–3.00)	0.01	—	—
Age at onset of wheezing†	—	—	0.89 (0.85–0.94)	<0.001

* The odds ratio (OR) for persistence of wheezing is for the comparison with all other study members except those who never reported wheezing. The OR for relapse is for the comparison with all other study members except those with persistent wheezing and those who never reported wheezing. CI denotes confidence interval, PC_{20} the concentration of methacholine causing a 20 percent decrease in the forced expiratory volume in one second (FEV_1), and BDR the response of the FEV_1 to a bronchodilator (increase from base line).

† The OR was calculated for persistence or relapse per year of increase in the age at onset (i.e., a later age at onset was protective).

outcomes. There were significant differences in the prevalences of childhood asthma, wheezing, and atopy between the 613 study members for whom respiratory data were available at all assessments (59.1 percent) and those for whom respiratory data were not available at all assessments, but there were no differences in outcome characteristics at the final assessment, at the age of 26 years. Because our multidisciplinary study evaluated many aspects of health and development other than asthma and allergy, this approach reduced the likelihood that the decision to return for each assessment was biased by the presence of these conditions, thus increasing generalizability.

Wheezing was common in this cohort, reported at some time by 72.6 percent of the 613 study members. This high cumulative prevalence, which may be slightly biased upward for reasons noted above, includes the 21.2 percent of study members who reported wheezing at only one assessment. The latter group differed from those who never reported wheezing in having almost double the prevalence of atopy to house dust mites, and therefore this group could not be ignored. We have previously compared the responses of 946 study members assessed at the age of 21 years with those of 991 subjects 20 to 22 years old who were enrolled in the cross-sectional European Community Respiratory Health Survey, performed elsewhere in New Zealand. This comparison showed no significant or systematic differences in the prevalence of reported wheezing in the previous 12 months, waking with chest symptoms, attacks of asthma, and use of asthma medication,[26] thus providing evidence that our high prevalence rates are not biased by the longitudinal design. In a birth cohort from the United Kingdom, 43 percent of the cohort members reported wheezing by the age of 33 years,[12] a result suggesting, as does our study, that wheezing is very common but is often mild and transient.

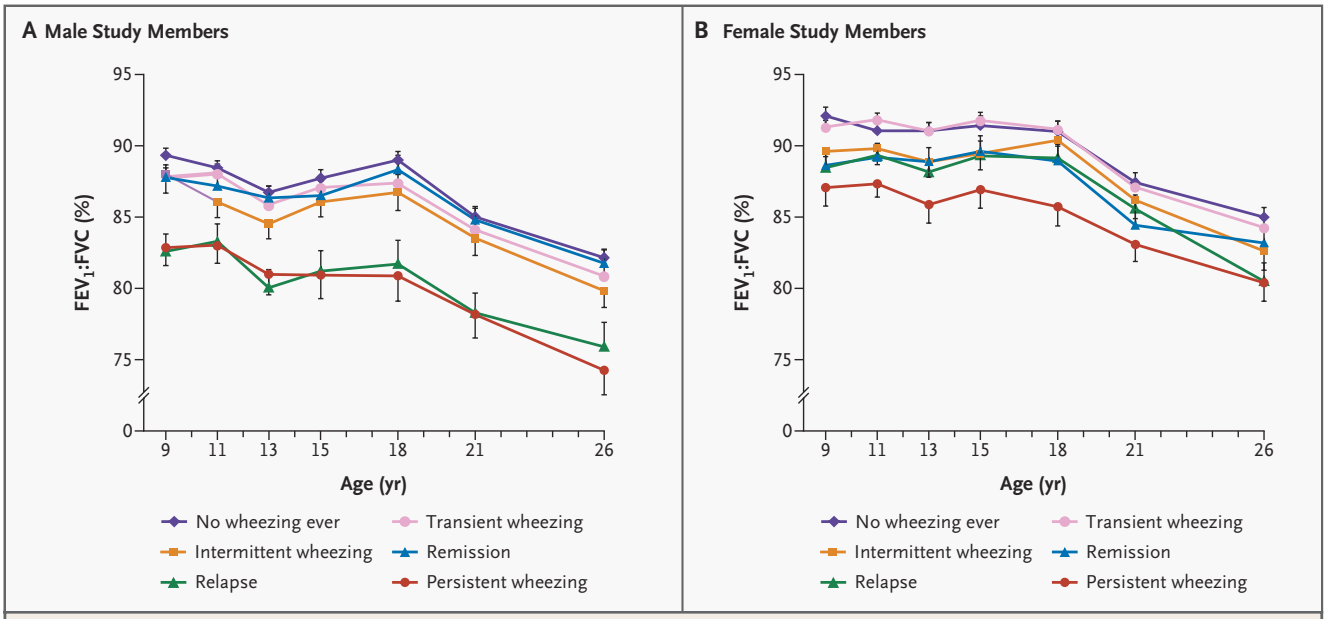

Figure 2. Mean (±SE) FEV_1:FVC Ratios Measured at 9, 11, 13, 15, 18, 21, and 26 Years in Male (Panel A) and Female (Panel B) Study Members, According to the Pattern of Wheezing.

As young adults, 26.9 percent of our cohort had continuing symptoms of asthma; 14.5 percent had persistent wheezing from onset with no remission, and 12.4 percent had relapsed after remission. These study members represent over one third of the 72.6 percent who reported ever wheezing, a result consistent with Australian studies in Tasmania[10] and Melbourne,[13] in which two thirds of subjects with asthma "outgrew" their disease.[6]

An early age at onset was a risk factor for relapse. The odds ratio of 0.89 indicates the protective effect per year of increase in the age at onset. According to this model, the risk for children with a 10-year-later age at onset was 0.89^{10}, or 0.31 — that is, a 10-year-later age at onset reduced the risk of relapse by 69 percent.

Assessing outcomes relatively early in adult life may overestimate the number of remissions and underestimate the number of relapses, because symptoms may recur later. In a study in Tucson, Arizona, remission was most likely in adolescence and was uncommon in adulthood.[27] Another limitation of our study is that histories of wheezing in early childhood were obtained when the children were already nine years old. Our study therefore indicates the likelihood of persistence or relapse among children whose mothers recalled that they wheezed in early childhood or in whom wheezing developed subsequently. However, early-childhood wheezing not recalled by the mother had probably been mild and had remitted, since otherwise one would expect these symptoms to be recalled.

Lung function was persistently impaired throughout childhood in study members with persistent asthma in adulthood, a phenomenon known as tracking. However, the slopes of change in FEV_1:FVC were similar in each group, indicating that impairment of lung function occurred in early childhood, before our first measurements at the age of nine years. Children with atopy may have impaired lung function as early as three years of age.[28] Lung function in male study members with a relapse tracked closely with that in male study members with persistent asthma, whereas female study members with a relapse had lower lung function only as adults. This difference may be due to the greater severity of disease among young boys with asthma.

The impairment in lung function in the group with persistent asthma was greater in those with persistent airway hyperresponsiveness and in those treated with inhaled corticosteroids. This finding illustrates confounding by severity, since the use of inhaled corticosteroids is an indicator of more severe disease, not the cause of impaired lung function. In the Melbourne longitudinal study, adult lung function was impaired in subjects with current severe asthma, but not in those with milder asthma who were not using inhaled corticosteroids, whereas those who were asymptomatic for three years had normal lung function, even though they had had

asthma throughout childhood.[13,16] Our study extends the findings of the birth-cohort study from the United Kingdom[15] by showing that reduced lung function in adulthood among those with persistent asthma is evident early in childhood and has consistently been at this lower level throughout childhood and adolescence into adulthood.

In the United Kingdom birth cohort, smoking was a risk factor for the development of asthma between 17 and 33 years of age and was a strong predictor of relapse of earlier asthma by the age of 33 years.[12] In our study, smoking at 21 years of age was predictive of persistent wheezing in both univariate and multivariate analyses and of relapse of wheezing in univariate but not in multivariate analysis. In studies based on asthma-clinic populations, the effect of smoking on the persistence of asthma has been inconsistent, perhaps reflecting self-selection (the "healthy smoker" effect).[7]

In summary, in a population-based birth cohort of New Zealand children who were not selected because they had asthma or were at high risk, the independent risk factors for persistent asthma in adulthood included allergy to house dust mites, smoking, airway hyperresponsiveness, and female sex. The independent risk factors for relapse after remission included allergy to house dust mites, airway hyperresponsiveness, and early age at onset. Those with persistent or relapsing asthma had substantially impaired lung function at each assessment during childhood, adolescence, and adulthood, a result suggesting that these outcomes are determined early in childhood. The challenge is to develop identification and treatment strategies applicable to early childhood that will reduce these adverse outcomes.

Supported by funds from the Health Research Council of New Zealand, the Otago Medical Research Foundation, the New Zealand Lottery Grants Board, and the Asthma Foundation of New Zealand.

We are indebted to Air New Zealand; to the study members and their families for their ongoing commitment and support; to Dr. Wendy Lou for statistical assistance; and to Mrs. Pearl Davis and Mrs. Jan Kettink for secretarial and administrative assistance.

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Predictive Markers of Asthma Exacerbation during Stepwise Dose Reduction of Inhaled Corticosteroids

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To determine predictors for failed reduction of inhaled corticosteroids (ICS), in 50 subjects with well-controlled asthma (age 43.7 [18–69]; 22 males) taking a median dose of 1,000 μg ICS/d (100–3,600 μg/d), ICS were halved every 8 wk. Airway hyperresponsiveness (AHR) to a bronchial provocation test (BPT) with histamine was measured at baseline. AHR to BPT with mannitol, spirometry, exhaled nitric oxide (eNO), and, in 31 subjects, sputum inflammatory cells were measured at baseline and at monthly intervals. Thirty-nine subjects suffered an asthma exacerbation. Seven subjects were successfully weaned off ICS. Using a Kaplan–Meier survival analysis, the significant predictors of a failure of ICS reduction were being hyperresponsive to both histamine and mannitol at baseline ($p = 0.039$), and being hyperresponsive to mannitol during the dose-reduction phase of the study ($p = 0.02$). Subjects older than 40 yr of age tended to be at greater risk of ICS reduction failure ($p = 0.059$). Response to mannitol and percentage sputum eosinophils were significantly greater before a failed ICS reduction than before the last successful ICS reduction, whereas there were no significant differences in symptoms, spirometry, or eNO. These findings suggest that documentation of patient's AHR or sputum eosinophils may be useful in guiding the reduction of ICS doses.

Asthma is a prevalent disease in developed countries (1) and causes an economic burden to patients, their families, and society (2). In Australia the direct cost of asthma associated with medical care has been estimated at $A250 million (3). First line treatment for asthma is usually with inhaled corticosteroids (ICS) (4). There is clear evidence for the positive clinical effects of ICS in symptomatic patients taking bronchodilator therapy (5, 6). As ICS are expensive and can have systemic effects, it is important to keep the dose at the lowest level possible that maintains good asthma control and minimizes side effects. However, reduction in ICS dose may lead to unstable asthma, emergency visits and hospital admissions. Hospitalization accounts for 20–25% of the direct costs of asthma (6, 7). Because most hospitalizations for asthma are emergency admissions, inadequate disease control can be assumed to be present (6). Therefore to avoid this situation, it would be very helpful to have predictors for failure or success of a planned ICS reduction.

According to current asthma management guidelines (8), the level and adjustment of anti-inflammatory asthma treatment are guided by symptoms and lung function. However, there are patients in whom airway hyperresponsiveness (AHR) and airway inflammation persist, although they are apparently clinically well controlled (9). Airway hyperresponsiveness is a characteristic feature of asthma, is considered to be related to airway inflammation (10, 11), and its severity changes with ICS treatment (12). Airway hyperresponsiveness may be measured using challenge tests with direct agonists such as histamine (13) or indirect agonists such as mannitol (14). The severity of airway inflammation can be measured directly by counting the numbers of inflammatory cells in sputum (15). Sputum eosinophils are increased in patients with asthma exacerbations (16), and they are reduced following ICS treatment (17). Airway inflammation may also be reflected by the levels of exhaled nitric oxide (eNO). Exhaled NO is increased during exacerbations of asthma (18), and reduced in subjects taking inhaled corticosteroids (18, 19).

To identify predictors for failure of ICS dose reduction with loss of control, we measured AHR to histamine and mannitol and noninvasive markers of airway inflammation as measured by sputum eosinophils and eNO in patients with stable asthma.

METHODS

Subjects

The patients were recruited from the Asthma Clinic of the Royal Prince Alfred Hospital, Australia. Fifty subjects with asthma using ICS to control their asthma who had a past history of wheezing and chest tightness and who had asthma previously diagnosed by a physician were studied. The subjects' characteristics are summarized in Table 1. Asthma severity was graded on the basis of lung function (8). Information on atopic status was available for 44 subjects, 41 of whom (93%) were atopic. Eight subjects were using long-acting β-agonists (LABA) and all used short acting β-agonists when needed. All subjects were clinically stable. In the 4 wk before the study, subjects had asthma symptoms no more than twice a week, did not wake at night because of asthma, and had no respiratory tract infection. They had no changes in their dose of ICS in the last 4 wk and no major changes in dose (> 1,000 μg daily) in the last 3 mo. Exclusion criteria were current smoking and the use of oral steroids within the previous 6 mo.

The study was approved by the Central Sydney Area Health Service Ethics Committee (protocol no. X97-0230). The trial was carried out under the Clinical Trial Notification Scheme of the Therapeutics Goods Administration of Australia (CTN No. 1997/373). All subjects signed a consent form prior to commencement of the study.

Study Design

This was a prospective study including two study periods: A 4-wk run-in phase (baseline) and a dose-reduction phase, in which the subjects' current ICS dose was halved every 8 wk. Before the start of the study, subjects were screened for eligibility on the basis of the inclusion and exclusion criteria. On the screening day, the clinical diagnosis of asthma was confirmed by a staff physician by examination and history and informed consent was obtained. During the run-in period, subjects attended for two study visits within 2 wk at the end of the run-in period

(*Received in original form December 28, 1999 and in revised form July 15, 2000*)

Supported by the National Health and Medical Research Council, the Australian ARDS Association, and a grant-in aid from Rhone-Poulenc Rorer, Australia. Jörg Leuppi was funded by Swiss National Science Foundation, Novartis-Foundation, Switzerland, "Freiwillige Akademische Gesellschaft" Basel, Switzerland, and Swiss Respiratory Society.

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Am J Respir Crit Care Med Vol 163. pp 406–412, 2001
Internet address: www.atsjournals.org

and monthly during the dose-reduction phase. They were also asked to refrain from taking short acting β-agonists for 6 h, LABA for 24 h, and antihistamines for 3 d prior to each study day. No ICS were taken on the day of the study. An "indirect" challenge with mannitol powder was performed, at the first visit of the run-in period and a "direct" challenge with histamine was performed at the second visit. Exhaled NO and spirometry were measured before the challenge tests on both days and sputum was collected during or after the mannitol challenge.

After the run-in period of 4 wk, in which disease stability was monitored by peak flow and symptom diary cards, the current ICS dose was halved every eight weeks. The ICS treatment was stopped after a dose of 200 μg of budesonide or beclomethasone, or 125 μg of fluticasone was achieved after successive reductions in steroid dose. The subjects visited the laboratory at monthly intervals, and a BPT with mannitol was performed, eNO and spirometry were measured, and sputum was collected.

Throughout the study subjects were asked to record their asthma symptoms, β-agonist use, and peak expiratory flow (PEF), twice daily in a diary card before inhaling their asthma medication.

The clinician responsible for the steroid reduction and for identifying the asthma exacerbation was "blinded" to the results of the mannitol challenge test and sputum results.

Study Endpoints

The dose of ICS was halved every 8 wk, until the patient suffered an asthma exacerbation or was successfully weaned off ICS for 8 wk. An exacerbation was defined by at least one of the following criteria:

1. Reduction of PEF by more than 3 standard deviations from mean PEF value obtained during the run-in period (20), and
2. A sudden rapid decline in peak flow or deterioration in symptoms, suggestive in the physicians' opinion of the development of a severe exacerbation (21).

Lung Function Measurements

Spirometry was performed using a MicroLoop II Spirometer (Micro Medical Ltd, Kent, UK). Forced expiratory volume in 1 s (FEV_1) was used as an index of change in airway caliber. Forced expiratory maneuvres were repeated until two readings of FEV_1 within 100 ml were obtained, the largest of which was used in analyses. Values for FEV_1 and FVC were recorded as a percentage of the predicted values of Knudson and coworkers (22).

Bronchial Responsiveness

Histamine challenge. A bronchial challenge test with histamine was administered using the rapid method (23). Histamine diphosphate (ICN Pharmaceuticals Inc., Costa Mesa, CA) was administered using DeVilbiss No. 45 handheld nebulizers (DeVilbiss Health Care Inc., Somerset, PA), in doubling doses from 0.03 to 3.9 μmol. The test was stopped if the FEV_1 fell by 20% or more. Salbutamol aerosol was administered to aid recovery when necessary. The dose of histamine that provoked a 20% fall in FEV_1 ($PD_{20}FEV_1$) was estimated by interpolation. Dose–response ratio (DRR) was calculated for all subjects as the percentage fall in FEV_1 at the last dose, divided by the total dose administered, with a constant of 3 added to allow logarithmic conversion (24, 25). Airway hyperresponsiveness (AHR) was defined as $PD_{20}FEV_1 < 3.9$ μmol histamine, or a DRR of $> 8.1\%$.

Mannitol capsule challenge. A bronchial challenge test with a dry powder of mannitol was administered to all subjects using the protocol previously described by Anderson and coworkers (26). In brief, a noseclip was applied and subjects then performed the challenge with doses consisting of 0 (empty capsule acting as a placebo), 5, 10, 20, 40, 80, 160, 160, and 160 mg of mannitol via a Halermatic (Rhône-Poulenc Rorer, Collegeville, PA). The 80 mg and 160 mg were given in multiple doses of 40-mg capsules. At least two FEV_1 maneuvres were performed 60 s after each dose and the highest FEV_1 was used in the calculation. The FEV_1 value measured after the 0 mg capsule was taken as the prechallenge FEV_1 and used to calculate the percentage decrease in FEV_1 in response to the mannitol challenge. If the subject had a greater than 10% fall in FEV_1 in response to a single dose, the same dose was repeated for reasons of safety. The challenge ceased when a 15% fall in FEV_1 was documented or a cumulative dose of 635 mg had been administered. AHR was defined as a PD_{15} of 635 mg or less. Salbutamol aerosol was administered to aid recovery when necessary. DRR was calculated for all subjects as the percentage fall in FEV_1 at the last dose, divided by the total dose administered (24, 25). The provoking dose of mannitol to cause a 15% (PD_{15}) fall in FEV_1 was calculated by linear interpolation of the relationship between the precentage fall in FEV_1 and the cumulative dose of mannitol required to provoke this. A PD_{15} mannitol was equivalent to a DRR of 0.023% fall FEV_1/mg.

Nitric Oxide Measurement

Mixed expired nitric oxide was measured using a modification of the method of Massaro and coworkers (18). The measurement was performed with the subject standing, without wearing a noseclip. The patient took a deep breath and exhaled over 5–15 s to residual volume into an NO impermeable polyethylene bag (Scholle Industries Pty Ltd, Elizabeth West, Australia). The exhaled flow, measured by a rotameter (Dwyer Flowmeter Model VFASS-25; AMBIT Instruments Pty Ltd, Parramatta, Australia), was 10 L/min at a mouth pressure > 20 cm H_2O. The exhaled gas from a single breath was analyzed within an hour using a chemiluminescence analyzer (Thermo Environmental Instruments Model 42C in the Institute of Respiratory Medicine), which has a lower limit of detection of 1 ppb. Ambient NO in the laboratory was measured at the time of testing.

Sputum Collection

Sputum collection was carried out in conjunction with the mannitol challenge. If subjects had to cough during the mannitol challenge, we asked them to spit whatever they produced into a sterile container. At the end of the mannitol challenge, subjects were asked to cough and spit and we collected whatever was produced. All subjects rinsed their mouths with water at each collection point to remove any particles and reduce salivary contamination. All specimens were retained for later examination under the microscope, even if there were no obvious sputum plugs.

Sputum Preparation and Differential Cell Count

Sputum was processed as described by Pin and coworkers (15). Briefly, sputum plugs were picked up and four-times the volume of diluted

TABLE 1

CHARACTERISTICS OF THE 50 SUBJECTS

Age	43.7 yr (range: 18–69)
Sex	26 women–24 men
Ex-smokers	14 subjects
Mean asthma duration	24.2 yr (range: 2.5–60)
Asthma severity	Severe (FEV_1 or PEF $\leq$ 60% predicted): 4 subjects
	Moderate (FEV_1 or PEF $\geq$ 60%–$<$ 80% predicted): 11 subjects
	Mild (FEV_1 or PEF $\geq$ 80% predicted): 35 subjects
Mean duration of ICS use	6.2 yr (range: 1–18)
Mean ICS dose	1,344 μg beclomethasone equivalent dose (95% CI: 1,142–1546)
Median ICS dose	1,000 μg beclomethasone equivalent dose (range: 100–3,000)
ICS drug	fluticasone (n = 15), budesonide (n = 25), beclomethasone (n = 9)

Definition of abbreviations: ICS = inhaled corticosteroids; PEF = peak expiratory flow.

Sputolysin (0.1%) (Sputolysin Reagent; Calbiochem, San Diego, CA) was added. The samples were placed in a shaking water bath (37° C) for 30 min and then filtered through 50-μm nylon gauze. A total cell count was performed and cytocentrifuge slides were prepared (Shandon Cytospin II, Sewickery, PA). The inflammatory cells were expressed as a percentage of the total inflammatory cell count (400 cells) on slides fixed with methanol and stained with May–Grunwald–Giemsa.

Peak Flow Home Monitoring

Subjects were asked to perform peak expiratory flow (PEF) measurements twice a day before inhaling their medication for the whole study period. The subjects blew three times into the PEF meter (Mini-Wright; Clement Clarke International Ltd., Essex, UK) while standing and the best of three values was recorded. The lowest reading for each week was calculated as a percentage of the best peak flow value achieved during the 4 wk of the run-in period (27, 28).

Statistical Analysis

Data were analysed using the statistical package SAS (SAS Institute Inc., Cary, NC) and STATA (Stata Statistical Software, Release 6.0, 1997; Stata Corporation, College Station, TX). Analyses of PD_{15} mannitol, PD_{20} histamine, DRR values of both challenge tests, and eNO were carried out on log-transformed data. Summary values for both DRR values and eNO are geometric means, with their 95% confidence intervals (CI). Summary values for the other measurements are arithmetic means and 95% confidence intervals. Paired *t* test was used to compare the outcome measurements (DRR mannitol, eNO, FEV_1 percentage predicted, FVC percentage predicted, PEF percentage predicted, PEF lowest percentage best, sputum inflammatory cells) between run-in period and exacerbation period, and the outcome measurements between the visit before the last successful ICS reduction and visits before the failed ICS reduction. Unpaired *t* test was used for comparison between groups. Possible predictors for failure of ICS reduction were determined using log-rank test and Cox regression. Kaplan–Meier survival curves were used to demonstrate the probability of failure of ICS reduction between AHR positive and negative groups, which were defined at baseline or at the visit prior to exacerbation. Logistic parameters were estimated using maximum likelihood estimation and evaluated using the likelihood ratio test. Odds ratios (OR) with a confidence interval greater than 1 indicate a significantly increased risk for failure of ICS reduction. Significance was accepted at the 5% level. To examine the ability of the DRR mannitol and percentage eosinophils to predict failure of ICS reduction, receiver operator characteristic (ROC) curves were generated by plotting sensitivity against 1-specificity over a range of cut-points for both of these measures.

RESULTS

Subject characteristics at run-in period are summarized in Table 2. Apart from a small but significant difference in PEF values, there were no significant differences between the two study days in the run-in period. Of these 50 patients with clinically well-controlled asthma taking ICS, 15 had AHR to histamine, 24 to mannitol, and 13 to both histamine and mannitol in the run-in period. Sputum was successfully collected from 31 subjects (62%) during or at the end of the mannitol challenge at baseline. Subjects older than 40 yr had significantly lower FEV_1 percentage predicted than younger subjects (76% [67.7–84.3] and 98.9% [92.5–105.4]; p = 0.0001) and longer duration of asthma (29.1 yr [22.5–36.7] and 18.9 yr [14.4–23.4]; p = 0.017).

Study Endpoints: Exacerbations and Successfully Weaned off ICS

Thirty-nine subjects suffered an exacerbation of their asthma—13 subjects after the first ICS dose reduction, 19 after the second ICS reduction, 5 after the third reduction, and 2 after the fourth reduction. Seven subjects reduced their ICS dose to zero and remained well at the 2 mo follow-up appointment. Inhaled steroids were ceased after the first ICS reduction in one subject, after the second ICS reduction in two subjects, after the third reduction in one subject, and after the fourth reduction in three subjects. Four subjects dropped out of the study during the dose-reduction phase—two after the first ICS reduction (both noncompliant) and two after the second reduction (pregnancy; noncompliant). When comparing the changes between run-in visits and visits during an exacerbation there were significant differences in ICS dose, lung function (FEV_1 percentage predicted, PEF percentage predicted, and PEF lowest percentage best), and response to mannitol (Table 3). Sputum was able to be collected in 21 out of 39 subjects at the exacerbation visit and showed a significant increase in sputum eosinophils (Table 3).

Predictors for Failure of ICS Reduction

Kaplan-Meier survival analysis using baseline data as predictors. Figure 1 shows the survival curve using AHR measurements at baseline. Being hyperresponsive to both direct (hista-

TABLE 2

BASELINE VALUES FOR 50 SUBJECTS, SHOWING SPIROMETRY BEFORE THE MANNITOL CHALLENGE (VISIT 1) AND THE HISTAMINE CHALLENGE (VISIT 2), AND THE SPUTUM INFLAMMATORY CELLS AT VISIT 1*

	Mannitol Challenge	Histamine Challenge	p Value
Lung function (n = 50)			
DRR	0.012 (0.008–0.017)	8.5 (2.4–3.3)	
PD_{20} histamine, n = 15		1.4 μmol (1.06–1.68)	
PD_{15} mannitol, n = 24	117 mg (75.9–180.3)		
Exhaled NO, ppb	18.5 (16–21.4)	19 (16.4–21.9)	0.38
FEV_1 predicted, %	86.8 (81.7–92)	88.7 (83.4–94)	0.31
FVC predicted, %	92.3 (82.2–102.4)	90.1 (85.7–94.5)	0.651
PEF predicted, %	83.8 (79–88.5)	86.2 (81.2–91.3)	0.03
PEF, lowest % best		84.5 (82.2–86.8)	
Sputum (n = 31)			
Neutrophils, %	23.1 (15.2–31.1)		
Eosinophils, %	6.6 (2.8–10.3)		
Macrophages, %	63.6 (54.6–72.6)		
Lymphocytes, %	1.9 (0.6–3.2)		
Mast cells, %	0.1 (−0.003–0.2)		

Definition of abbreviations: DRR = % fall FEV_1 at the last dose/cumulative dose (for histamine +3 is added); PD_{15}/PD_{20} = provocation dose causing a 15% (mannitol) or a 20% (histamine) fall in FEV_1; PEF = peak expiratory flow.

* Mean values (95% confidence interval).

TABLE 3

MEASUREMENTS OF THE RUN-IN VISIT AND THE VISIT AT EXACERBATION IN 39 SUBJECTS*

	Run-in Period	Exacerbation	p Value
Lung function (n = 39)			
DRR mannitol	0.04 (0.02–0.07)	0.096 (0.05–0.17)	0.0009
ICS dose, μg	1,330 (1,131–1,529)	323 (256–390)	< 0.0001
Exhaled NO, ppb	17.6 (14.8–21)	20.6 (16–26.4)	0.16
FEV_1 predicted, %	88.1 (80.9–95.3)	84.2 (76.8–91.7)	0.02
FVC predicted, %	89.6 (83.9–95.3)	83.2 (77.1–89.3)	< 0.001
PEF predicted, %	84.6 (78.5–90.6)	79.8 (78.2–90.9)	0.02
PEF, lowest % best	85.1 (82.7–87.6)	75.2 (71.6–78.8)	< 0.0001
Sputum (n = 21)			
Neutrophils, %	25 (14.7–35.3)	15.6 (8.1–23.1)	0.55
Eosinophils, %	8 (2.9–13.1)	33.8 (21.8–45.9)	0.002
Macrophages, %	58.7 (47.4–70.0)	48.8 (38.4–59.3)	0.16
Lymphocytes, %	2.1 (0.3–4)	0.8 (0.02–1.2)	0.93
Mast cells, %	0.1 (−0.003–0.2)	0 (0–0)	0.17

Definition of abbreviations: DRR = maximal % fall FEV_1/cumulative dose mg; ICS = inhaled corticosteroids; PEF = peak expiratory flow.

* Mean values (95% confidence interval): paired data.

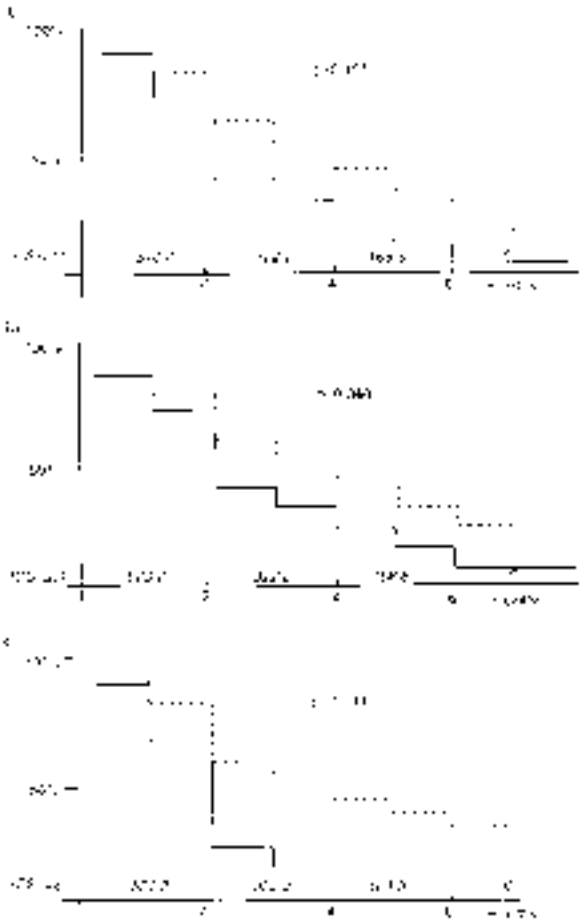

Figure 1. Kaplan-Meier survival curve based on AHR to histamine (*A*), mannitol (*B*), or both histamine and mannitol (*C*) at baseline. (*A*, *B*) The *dashed line* represents the AHR negative subjects and the *continuous line* represents the AHR positive subjects. (*C*) The *dashed line* represents the AHR negative and the AHR positive (to one test only) subjects to either of the challenge test and the *continuous line* represents the AHR positive subjects to both challenge tests.

mine) and indirect (mannitol) challenge test at baseline was a significant predictor for failure of ICS reduction, whereas being hyperresponsive to only a direct or indirect challenge test at baseline was not a predictor for failure of ICS reduction. The majority of exacerbations occurred following the first or second ICS reduction. The odds ratio for failure at or before the second ICS reduction was 2.38 (95% CI: 0.67–8.4; $p > 0.05$) for AHR to histamine, 2.27 (0.73–7.07; $p > 0.05$) for AHR to mannitol, and 4.38 (1.03–18.56; $p < 0.05$) for AHR to both a direct and indirect challenge test at baseline. Being older than 40 was a borderline significant predictor for failure of ICS reduction ($p = 0.059$). Nonpredictors for failure of ICS reduction at baseline were eNO (cut-off point 20 ppb; $p = 0.61$), ICS dose (cut-off point 800 μg; $p = 0.621$), sputum eosinophils (cut-off point 2.5%, $p = 0.445$, and PEF lowest percentage best (cut-off point 90%; $p = 0.162$; 85%; $p = 0.674$; and 80%; $p = 0.754$).

Survival analysis using the visits prior to each ICS reduction. Figure 2 shows the survival curve using AHR to mannitol measured at the visit prior to an ICS reduction. Being hyperresponsive to mannitol (DRR mannitol > 0.023 percentage fall FEV_1/mg) was a significant predictor for failure of ICS reduction. Failure of ICS reduction was not predicted by FEV_1 percentage predicted ($p = 0.46$), PEF percentage predicted ($p = 0.8$), or eNO ($p = 0.98$).

Comparison of the visit before the last successful ICS reduction with the visit before the failed ICS reduction. Twenty-six subjects had both one successful ICS dose reduction and one failed ICS reduction. There were significantly higher levels of airway responsiveness to mannitol, as described by the DRR, and sputum eosinophils before the last successful reduction compared with levels before the failed reduction. There were no significant differences in spirometric function and eNO (Table 4). Figure 3 shows the ROC curves that describe the performance of DRR mannitol and percentage eosinophils for prediction of failure of ICS reduction. DRR mannitol had 90% sensitivity at a cutpoint of 0.023% fall FEV_1/mg (equivalent to a PD_{15} value of 635 mg) and 90% specificity at a cutpoint of 0.27% fall FEV_1/mg (equivalent to a PD_{15} value of 53.6 mg). Sputum eosinophils had 90% sensitivity at a cutpoint of 6.3%, and 90% specificity at a cut-point of 13.3%.

DISCUSSION

The results of the present study suggest that measurements of AHR and sputum eosinophils can be used to predict the success or failure of reduction in ICS dose during backtitration. First, being hyperresponsive to both "direct" (histamine) and "indirect" (mannitol) challenges at baseline and hyperresponsive to mannitol during dose-reduction phase of the study were clear predictors for failure of ICS reduction. Second, an increase in sputum eosinophilia during backtitration, but not

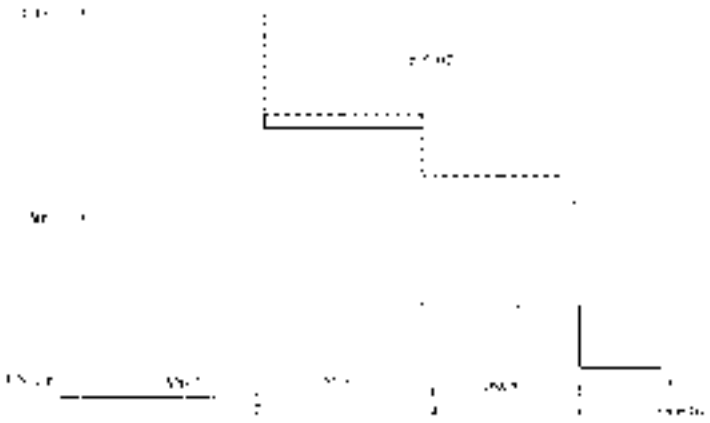

Figure 2. Survival curve using AHR to mannitol measured at the visit prior to an ICS reduction. The *dashed line* represents the norm-responsive subjects and the *continuous line* represents the hyperresponsive subjects.

TABLE 4

COMPARISON VISITS BEFORE THE LAST SUCCESSFUL ICS REDUCTION AND VISITS BEFORE THE FAILED ICS REDUCTION IN 26 SUBJECTS*

	Successful Reduction	Failed Reduction	p Value
DRR mannitol	0.05 (0.03–0.08)	0.09 (0.06–0.15)	0.003
Exhaled NO, ppb	18.4 (14.9–22.8)	18.5 (12.9–26.4)	0.95
FEV_1 predicted, %	87.4 (81.1–93.8)	86.9 (79.7–92.5)	0.84
FVC predicted, %	88.7 (82.7–92.7)	87.5 (81.2–94.2)	0.73
PEF predicted, %	84.3 (80.5–92.1)	82.6 (79.4–83.7)	0.65
PEF, lowest % best	85.5 (81.9–87.8)	82.9 (78.3–86.5)	0.57
Sputum neutrophils, %	26 (13.5–38.5)	18.6 (8.5–28.7)	0.23
Sputum eosinophils, %	7.9 (3.9–11.8)	22.5 (11.4–33.6)	0.03
Sputum macrophages, %	56.8 (45.5–68)	58.6 (47.5–69.7)	0.62
Sputum lymphocytes, %	3.2 (0.4–6.1)	0.53 (0.1–0.9)	0.13
Sputum mast cells, %	0.09 (−0.03–0.2)	0.4 (−0.4–1.2)	0.42

Definition of abbreviations: DRR = % fall FEV_1 at the last dose/cumulative dose mg; ICS = inhaled corticosteroids; PEF = peak expiratory flow.

* Mean values (95% confidence interval).

high levels at baseline, was a significant predictor for failure of ICS reduction. Third, subjects aged 40 and older seem to be at greater risk of exacerbation following ICS reduction. Finally, lung function and exhaled NO did not have predictive value for exacerbation following ICS reduction at any time points.

At baseline, having AHR to both histamine and mannitol was a clear predictor for failure of ICS reduction, whereas having AHR to either histamine or mannitol alone was not a significant predictor. Interestingly the combination of the tests was most strongly predictive at or before the second ICS reduction. Although the odds ratio for failure at or before the second ICS reduction was > 1, irrespective of which challenge test was used, only AHR to both challenges was a statistically significant risk factor for failure. Our findings are supported by Sont and coworkers (29), who demonstrated better asthma control if a decrease of AHR to mechacholine was used in addition to existing guidelines (optimizing symptoms and lung function). AHR probably reflects several different acute as well as chronic aspects of airway inflammation (30). There is also some evidence that direct and indirect challenge tests provide different, and probably complementary, information (12, 21, 24, 31), which can also be seen in our present study. The presence of AHR to both challenge tests in our study was a stronger predictor for failure of ICS reduction than either test alone.

We were able to collect sputum in more than 60% of the subjects, which is similar to other reported data (32). We may have been more successful had we used a wet aerosol challenge. With respect to assessment of AHR, mannitol acts as a hyperosmolar stimulus in the same way as hypertonic saline (26). There was no formal comparison made in the same subject using mannitol and hypertonic saline for inducing sputum. However, the mannitol is unlikely to have changed cell number as there is no difference in cell number reported when hypertonic and normal saline are used for sputum induction (33).

Levels of responsiveness to mannitol and of sputum eosinophilia are both predictors of the likelihood of success or failure of ICS dose reduction at any given time. Our subjects were clinically well controlled and symptom free before the failed ICS reduction, suggesting that mannitol responsiveness and sputum eosinophils provide information additional to that provided by symptoms. Our findings are supported by the study by in't Veen and coworkers (21), in which the baseline severity of AHR to hypertonic saline in clinically well controlled subjects was increased in the group of subjects with frequent exacerbations compared to those without exacerbations. Further support comes from the studies of Jatakanon and coworkers (34, 35). In one study (34), they reported dose-dependent changes in sputum eosinophils and PC_{20} methacholine during treatment with budesonide, with the maximum reduction at their highest dose of 1,600 μg budesonide. In the other study, Jatakanon and coworkers (35) induced an asthma exacerbation by reducing the dose of ICS to 200 μg budesonide and found that the change in sputum eosinophils could be a useful predictor of loss of asthma control. The predictive value for failure of ICS reduction by DRR mannitol and percentage eosinophils in sputum, however, is moderate in our study. A DRR mannitol of 0.035 percentage fall FEV_1/mg had a sensi-

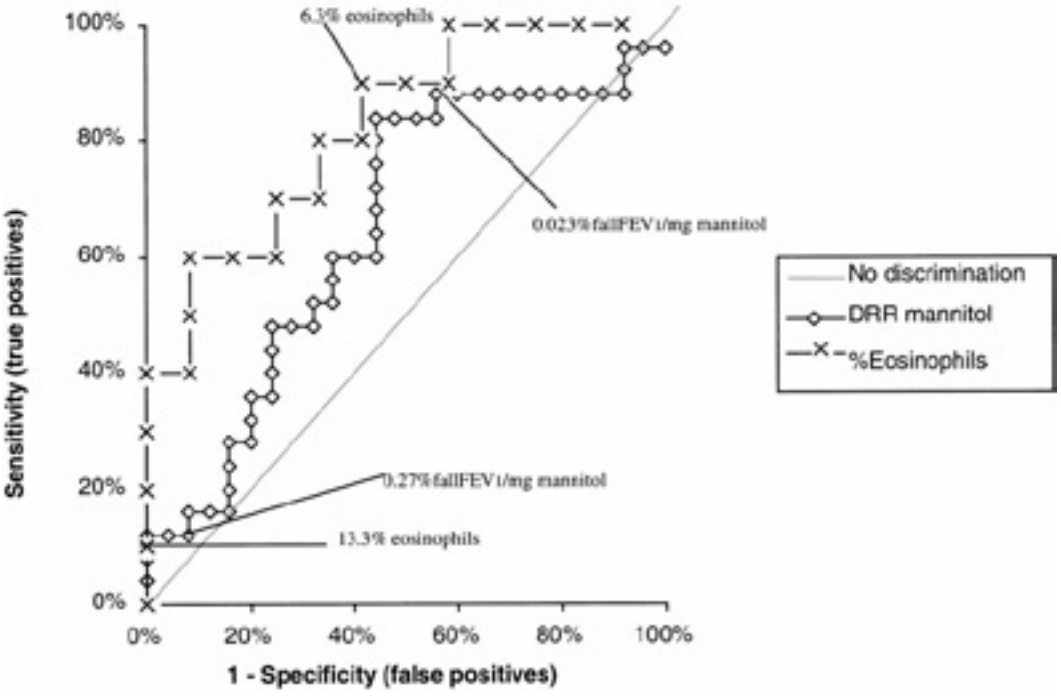

Figure 3. Receiver operator characteristic (ROC) curves showing the sensitivity and 1-specificity over a range of cut-points for DRR mannitol (*open triangles*) and % sputum eosinophils (*crosses*) for predicting failure of ICS reduction in 26 subjects The *solid line* indicates no discrimination.

tivity of 72% and a specificity of 56%, and sputum eosinophils of 9.3% had a sensitivity of 80% and a specificity of 66.7%. Unfortunately, we cannot calculate these figures for histamine, because we did not perform a histamine challenge during the ICS dose-reduction phase. A single challenge test and sputum eosinophils do not seem to have the same predictive power as the combination of a direct and indirect challenge test.

Age older than 40 yr was a predictor of borderline significance for failure of ICS reduction. In our study population, the subjects older than 40 yr of age had a significantly lower FEV_1 percentage predicted and longer duration of asthma than those younger than 40 yr. There is some evidence that longer duration of an inflammatory state of the airways may result in anatomical and functional changes (36), especially if we take into account that the duration of the ICS use was not different between the two age groups. These findings are supported by Quardelli and coworkers (37), who found that elderly patients with asthma (> 65 yr) had significantly fewer symptom-free periods and required more frequent systemic corticosteroids; their FEV_1 percentage predicted was significantly lower than those younger than 40 yr of age. In that study, increased asthma severity in the elderly group correlated with the duration of asthma.

Values for spirometric function and exhaled NO did not have any predictive value at any time points. Treatment with ICS causes an improvement of lung function and discontinuation of treatment is often accompanied by exacerbation of the disease and decline in lung function (38). FEV_1 and PEF in our study population were also significantly lower during exacerbation than at baseline. However, spirometric function was not a predictor of failure of ICS reduction. The study of Jatakanon and coworkers (34) found dose-dependent changes in eNO levels, with the maximum reduction evident at moderate doses of budesonide (400 μg). These data suggest that eNO may be more useful when the doses of ICS used are lower. Their subjects' eNO levels were decreased to the same level as the baseline eNO value in our clinically well-controlled study population. Although, eNO might be expected to be a good predictor of response to steroid reduction, it did not change significantly during the dose-reduction phase and it did not increase significantly during exacerbation. It could be hypothesized that long-term ICS treatment leads to sustained inhibition of inducible NO synthase (19), so that eNO does not necessarily increase during exacerbations if ICS have been taken recently.

We conclude that AHR to both a direct and indirect challenge test is a good predictor for failure of ICS reduction. Age older than 40 yr, positive response to an indirect challenge test, and sputum eosinophil numbers also have some predictive value for failure of ICS reduction. However, spirometric function and exhaled NO provide no information that predict failure of ICS reduction. Therefore, for an ICS reduction of more than one halving step in patients with asthma, we would recommend having information on AHR using both a direct and an indirect challenge test and sputum eosinophils.

Acknowledgment: The use applications for mannitol described in this study are covered in the United States by Patent No. 5,817,028 and internationally by PCT/AU 95/00086. The patent is owned by the Central Sydney Area Health Service.

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