Biochemical Pharmacology 82 (2011) 567–585

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Commentary

Asthma translational medicine: Report card

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ARTICLE INFO

Article history:
Received 10 June 2011
Accepted 24 June 2011
Available online 2 July 2011

Keywords:
Cytokines
Chemokines
T helper 2 cells
Asthma therapeutics
Eosinophils
Animal models

ABSTRACT

Over the last 30 years, scientific research into asthma has focused almost exclusively on one component of the disorder – airway inflammation – as being the key underlying feature. These studies have provided a remarkably detailed and comprehensive picture of the events following antigen challenge that lead to an influx of T cells and eosinophils in the airways. Indeed, in basic research, even the term "asthma" has become synonymous with a T helper 2 cell-mediated disorder. From this cascade of cellular activation processes and mediators that have been identified it has been possible to pinpoint critical junctures for therapeutic intervention, leading experimentalists to produce therapies that are very effective in decreasing airway inflammation in animal models. Many of these compounds have now completed early Phase 2 "proof-of-concept" clinical trials so the translational success of the basic research model can be evaluated. This commentary discusses clinical results from 39 compounds and biologics acting at 23 different targets, and while 6 of these drugs can be regarded as a qualified success, none benefit the bulk of asthma sufferers. Despite this disappointing rate of success, the same immune paradigm and basic research models, with a few embellishments to incorporate newly identified cells and mediators, continue to drive target identification and drug discovery efforts. It is time to re-evaluate the focus of these efforts.

1. Introduction

Asthma is a chronic respiratory disease characterized by recurring attacks of impaired breathing, of varying intensities. The definition of asthma has four cardinal components – variable airflow obstruction (bronchoconstriction), symptoms, airway inflammation, and airway hyper-responsiveness (AHR) [1]. Asthma affects over 8% of Americans and that number continues to rise. Despite its widespread prevalence, increasing severity and associated rising healthcare costs measured in billions of dollars, few new drugs representing novel modes of action have been introduced over the last 30 years [2]. Indeed the mainstays of treatment, in the form of inhaled corticosteroids, β2 adrenoceptor agonists and cholinergic antagonists, have a long history and were first used clinically more than 50 years ago [2]. None of these drugs prevent asthma, and, while most patients obtain some level of symptomatic relief, a significant proportion continues to suffer, and new therapies are required urgently. The goal of therapy is two-fold – to limit the current impairment or symptoms, and to reduce the risk for a severe attack (exacerbation) in the future [1]. Since even patients with mild asthma have evidence for inflammation of the large and small airways, and the severity of the inflammation often correlates with the severity of the disease, attention in the last 30 years has focused on the mechanisms of airway inflammation and its suppression as a means of identifying new therapeutics.

Despite the dearth of new medications, asthma research is alive and well. Type the search term "asthma" into PubMed and there are almost 100,000 articles since 1980 (99,817 at the time of writing). The pathway of events which follow antigen sensitization and challenge have been painstakingly mapped in exquisite detail, and critical sites of regulation of the process have been identified involving both the innate and adaptive immune systems. The cellular pathology, recognition receptors, co-stimulatory molecules, key transcription factors, cytokines, chemokines, adhesion

Abbreviations: ACQ, asthma control questionnaire; AHR, airway hyper-responsiveness; AP-1, activator protein 1; APC, antigen-presenting cell; AQLQ, asthma quality-of-life questionnaire; BALF, bronchoalveolar lavage fluid; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; cysLT, cysteinyl leukotrienes; DC, dendritic cell; EAR, early airway response; FEV1, forced expiratory volume in 1 second; FLAP, 5-lipoxygenase activating protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule 1; ICOS, inducible costimulator; ICS, inhaled corticosteroid; IFN, interferon; IgE, immunoglobulin E; IL, interleukin; LAR, late asthmatic response; LPS, lipopolysaccharide; LT, leukotriene; mAb, monoclonal antibody; Maf-1, musculoaponeurotic fibrosarcoma 1; MHC, major histocompatibility complex; mRNA, messenger ribonucleic acid; NF-IL-6, nuclear factor interleukin-6; NKT, natural killer T cell; PD-1, programmed death 1; PEFR, peak expiratory flow rate; PG, prostaglandin; SCID, severe combined immunodeficiency; STAT, signal transducer and activator of transcription factor; TCR, T cell receptor; TGFβ, transforming growth factor β; Th, T helper cell; TLR, toll-like receptor; TNF, tumor necrosis factor; Treg, regulatory T cell; TSLP, thymic stromal lymphopoietin; VCAM-1, vascular cell adhesion protein 1; VLA-4, very late antigen 4.

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doi:10.1016/j.bcp.2011.06.038

molecules, and other mediators, have been investigated and incorporated into a comprehensive, detailed, unifying model of the events that translate into asthma. Such achievements seemed to hold great promise. As concluded in an article in *Nature Biotechnology* in 2000 [3] "Significant recent advances on all fronts suggest that significant new and better treatments are on their way." The start of the last decade seemed to hold much optimism and promise. What happened?

1.1. Purpose of this review

The goal of this article is to review the evidence for the research paradigm of asthma developed over the last 30 years as an immune response gone awry, and evaluate the clinical success of this model. Based on the mechanistic pathway that has emerged from research, a number of key targets have been identified and new therapeutics developed (Fig. 1). A significant number of these have now completed Phase 2 clinical trials in asthmatic subjects, such that the translational success of the mechanisms of airway inflammation can be evaluated. Not included in this commentary are studies with therapeutic agents that, while intended to target the inflammatory component of asthma, could also influence other features of the disorder thereby adding a degree of confusion to the interpretation of the results. These agents include iloprost, prostaglandin D2 antagonists, 5-lipoxygenase activating protein (FLAP) inhibitors, phosphodiesterase 4 inhibitors, and inhibitors of various kinases. This article focuses on agents that relate unambiguously to the immune paradigm defined by basic research and complemented by clinical observations in asthma patients.

As a number of clinical studies are ongoing, it is highly likely that new results will emerge in the period between writing this commentary and its publication, which could modify the conclusions reached. Indeed, let us hope that is the case since the clinical results obtained so far are disappointing.

2. Defining asthma pathophysiology: basic research studies

2.1. Introduction

Frequently, clinical asthma has been associated with atopy and elevated immunoglobulin E (IgE) levels, typifying an allergic component. However, murine "asthma" models do not show a distinct role for IgE in the pathogenesis of asthma. Bronchial inflammation and AHR occur to the same extent in $IgE^{-/-}$ and wild-type mice subjected to antigen challenge, while mast cell activation and mediator release in response to ovalbumin challenge are also observed in IgE deficient mice and those lacking the active IgE receptor, FcεRI (see [4]). While this might be due, in part, to a species-specific role for the IgG1 isotype, at a basic research level the term asthma has now become synonymous with a T helper 2 (Th2) cell-mediated disorder. According to this paradigm, allergic sensitization and subsequent challenge results

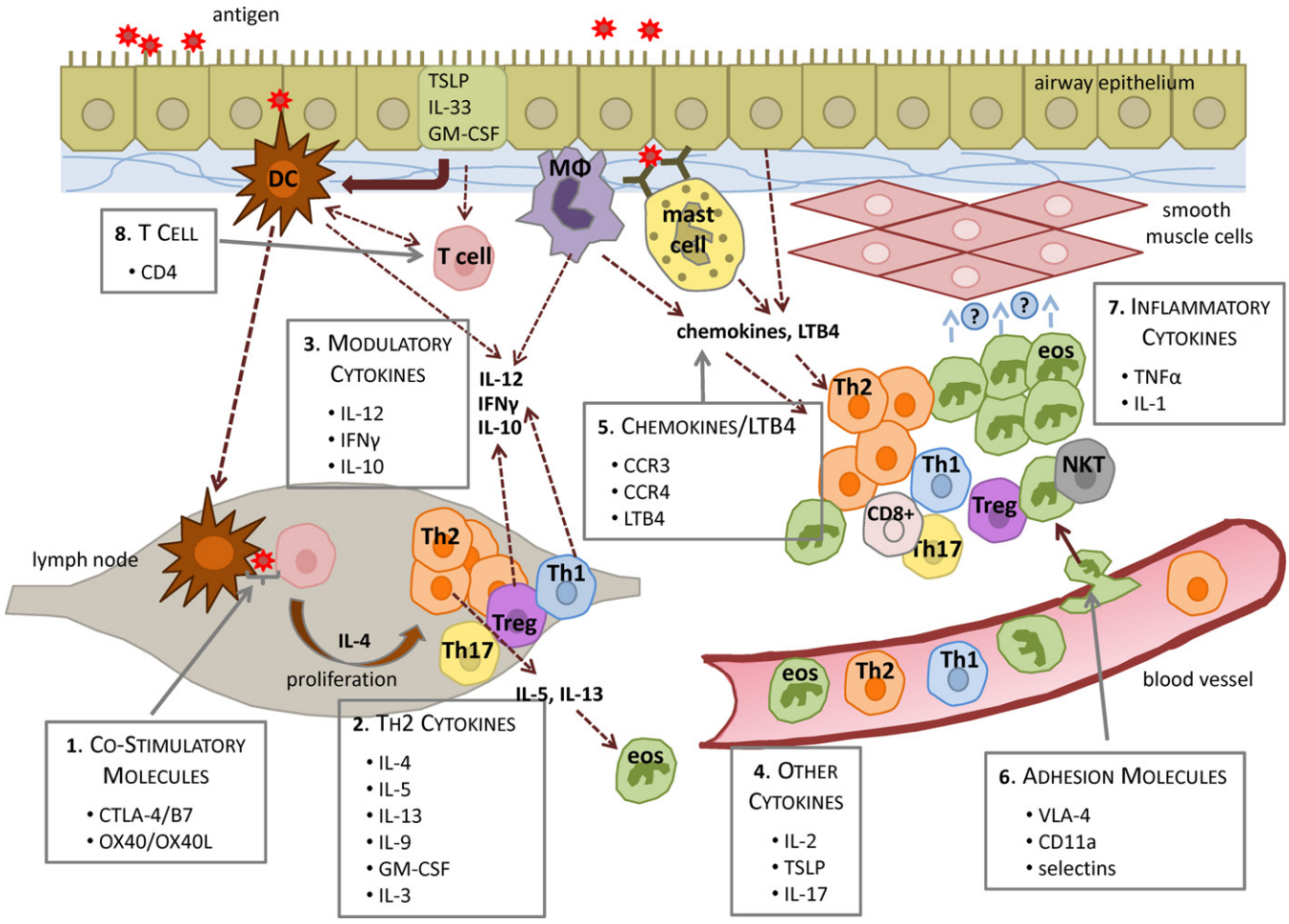

Fig. 1. Basic research has provided a detailed map of the events following antigen challenge that culminate in an asthma-like response in animal models. An abbreviated version is depicted, highlighting the components within this immune cascade that have targeted with novel therapeutics to treat the clinical condition (shown in the grey boxes). The numbers of each box relate to the sequence in which they are discussed in the text. DC, dendritic cell; Mϕ, macrophage; eos, eosinophil. Other abbreviations as defined in the text.

in T cell activation and transformation to a Th2 phenotype. The release of the Th2 pattern of cytokines, including interleukin (IL)-4, IL-13, IL-9, and, particularly, IL-5, promote airway inflammation rich in eosinophils, that are considered responsible for the asthmatic response. The sequence of events and the importance of dendritic cells, Th2 cells and eosinophils to the allergic response, have been mapped out primarily in the mouse model, utilizing techniques of selective cell depletion [5–7], passive or adoptive cell transfer [8–13], transgenic animals [14–16] targeted gene deletion [17], or pharmacological manipulation of their products (e.g. specific cytokines or chemokines) [4,18–20]. This has resulted in a comprehensive, detailed model of pathways leading to airway inflammation and AHR (Fig. 1).

Since most animals do not develop asthma per se, models of pulmonary allergic reactions are used, particularly in the mouse. However, even antigen challenged mice do not develop bronchoconstriction or other clinical signs of asthma apart from AHR. Consequently, the main endpoints used in the majority of research studies are airway inflammation (measured as leukocyte numbers in bronchoalveolar lavage fluid (BALF)) and AHR. Whether the antigen-induced AHR is the same phenomenon as that described in asthmatic subjects has not been adequately evaluated (see Section 3.3), while its association with airway inflammation has resulted in it being considered by some as merely a surrogate marker of the inflammation. Thus, pulmonary inflammation has become the critical endpoint in most research studies, and all potential therapeutic agents are evaluated and interpreted in terms of their effects on this response.

There are a number of important qualifications to establish before discussing the role of the various cells and cytokines in the process leading to an asthma-like syndrome produced in animals, and one note of caution. Although regarded as critical components of airway inflammation, Th2 cells are not the predominant cells in BALF of asthma patients. Intracellular cytokine staining indicates that while the number of IL-4 positive cells increases after allergen challenge, the percent of these cells is unchanged from baseline, indicating that other cells are also recruited to the airways [24]. Similarly in asthma basic research it has become increasingly recognized that it is not only a Th2 phenomenon, but other T cell repertoires, including Th1, Th17, regulatory T (Treg) cells, cytotoxic CD8+ T cells, natural killer T cells (NKT) and γδ T cells are also incriminated and have been incorporated into a holistic view of asthma. While many of these cells represent alternative sources of the cytokines previously featured in the response, they also offer some new cell-specific mediators (e.g. IL-33, IL-25), such that some 30+ cytokines have now been implicated. At one level, these observations represent embellishments and extensions to the original Th2 archetype, while, significantly for this commentary, therapeutics targeting these newly implicated cell-specific mediators have yet to enter clinical evaluation, so they will not be considered in detail here. For additional information in these areas, the reader is directed to several excellent reviews [18,21–23].

Cytokines implicated in the response to antigen challenge can be produced by a variety of cells, and their presence or resultant activity does not infer a role for any particular cell-type. These cytokines are listed in this commentary under a particular heading for convenience, and to avoid repetition. For example, the "Th2 cytokines," (IL-3, IL-4, IL-5, IL-9, IL-13 and granulocyte macrophage colony-stimulating factor (GM-CSF)), are historically associated with, and therefore discussed under Th2 cells, but some of which can also be produced by the airway epithelium, eosinophils, mast cells, and other T cell populations (including CD8+, NKT, and γδ T cells).

Another significant development is that for a long time it was believed that once a proliferating CD4+ T cell became committed to a distinct lineage – Th1 or Th2 for example – it was an irreversible process, while more recently it has become recognized that the cells retain a level of plasticity, and have the capacity to be re-differentiated depending on mediators present in the local microenvironment [21]. Many of the molecular details have been defined in *in vitro* systems, which might not readily translate into complex *in vivo* systems, so the significance of any such interconversion has yet to be established.

In order to adequately address how effectively research in the field of asthma has translated to the clinical condition, it is necessary to acknowledge the inconsistencies and contradictions that remain outstanding in basic research. This is exemplified by studies with IL-18, where IL-18-deficient mice show enhanced [25], diminished [26], or no difference [27] in airway inflammation. Unfortunately this field is rife with such anomalies, and while attempts to rationalize such inconsistencies have been made based on differences in strains of mice, sensitization agents and protocols, timing of treatment, other mediators present in the microenvironment, etc. [19,21], they all beg the question of which research setting or finding, if any, are relevant to the clinical condition. Probably this commentary should come with a public service announcement that "viewer discretion is advised."

2.2. T Cell activation: sequence of events

T lymphocytes are a key component of the adaptive immune response geared towards defense of the host against infection. Within the airways is a large network of antigen-presenting cells (APCs), which process the antigen and present it to T cells in the adjacent lymphoid tissue located in the lung parenchyma and mediastinum. Although the lung contains T cells (predominantly memory cells, including those produced in response to the first introduction of an allergen), spread over various locations including the bronchial wall, the airway lumen and the alveoli, the dramatic increase in the number of T cells in the airways after antigen challenge is not due to local proliferation, but recruitment into the lung from regional lymph nodes (see [24]). This offers multiple sites where T cell activation, proliferation, and migration can be targeted pharmacologically (Fig. 1). The aberrant accumulation of large numbers of activated T cells in and around the airways of asthmatics is thought to orchestrate the inflammatory response, where the ultimate effector cell is considered to be the eosinophil.

2.3. Airway epithelial cells: the gatekeepers

Aeroallergens first come into contact with airway epithelial cells as the initial barrier against pathogens invading the lung. Interest in immune defense mechanisms localized in the airway epithelium stems from the recognition that these cells could provide an important link between environmental stimuli and the determination of the ensuing response in the host. When activated the epithelial cells provide immune defense by producing an array of cytokines that include IL-1, IL-6, IL-8, IL-25, IL-33, GM-CSF, IFNα and β, TNFα and thymic stromal lymphopoietin (TSLP). TSLP and GM-CSF have been implicated in the activation of dendritic cells that lie just beneath the epithelium, and help define whether the reaction to the allergen is the development of either a Th2 response or tolerance [24]. Moreover, IL-33 and TSLP can induce many of the features of an antigen response, including eosinophilia, production of IgE antibodies, and formation of IL-4, IL-5 and IL-13.

2.3.1. Thymic stromal lymphopoietin (TSLP)

Particular interest in TSLP lies in its proposed role as a "master switch" for promoting Th2 cell activation. While TSLP is upregulated in lungs of ovalbumin-challenged mice [28], inducing the lung-specific expression of a TSLP transgene leads, of itself, to a

mild, spontaneous asthma-like condition, and markedly enhances both airway inflammation and AHR when combined with antigen challenge [29]. This augmented reaction requires CD4+ T cells, but not B cells. TSLP receptor-deficient mice do not develop airway inflammation in response to ovalbumin but mount a Th1 response with high levels of IFNγ and IL-12 [28,30], reinforcing the concept that TSLP can direct the response to antigen. Finally, bronchial biopsies from patients with asthma also show an increased number of TSLP mRNA+ epithelial and submucosal cells compared to controls, which correlate inversely with lung function [31].

2.4. Dendritic cells: surveillance and initiation

The development of an allergic response is divided into two phases – sensitization to a particular allergen, and subsequent challenge where the same antigen is re-introduced. Airway epithelial cells and dendritic cells (DCs) carry surface pattern-recognition receptors for pathogens, including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors and retinoic inducible gene (RIG) receptors, capable of recognizing surface characteristics of pathogens and initiating the response of the innate immune system. DCs are among the most powerful APCs, and located just below the epithelial layer of the airways (see [24]) where they extend processes through the epithelial cell layer into the airway lumen for immune surveillance of inhaled pathogens.

Endocytotic uptake of the antigen by DCs is followed by migration of the cells to the mediastinal nodes where the antigen is processed into peptides for presentation on major histocompatibility complex (MHC) class II molecules. In subjects previously sensitized to a particular allergen, expression of the high affinity receptor for IgE (FcεR1) on DCs facilitates processing of the specific allergen captured by the bound IgE. Naive uncommitted CD4+ T cells, expressing the T cell receptor (TCR)/CD3 complex, bind to the class II MHC proteins, and along with engagement of the co-stimulatory molecules CD28 on the CD4+ T cell and CD80 (B7-1) or CD86 (B7-2) on the APC, undergo proliferation and develop a Th1, Th2, Th9, Th17 or Treg cell inducing phenotype, depending on the cytokine signals.

2.5. Co-stimulatory molecules

T cells require at least two signals to become activated. The first is antigen-specific and involves major histocompatibility class II-peptide complexes on the surface of APCs; while the second is a non-specific co-stimulatory signal. This second signal, involving molecules expressed on the surfaces of the two cell types, can either enhance or inhibit the immune cell response (see [32]). There are two families of co-stimulatory molecules – the B7 family, comprised of CTLA-4, CD80, CD86, ICOS (inducible costimulator), and PD-1 (programmed death-1); and the TNF receptor family made up of OX40, CD30, CD40, CD27, Fas and 4-1BB (CD137). This discussion will be limited to CTLA-4 from the B7 family and OX40 from the TNFR family, since these are the targets currently being explored in Phase 2 clinical studies.

APCs express CD80 (B7-1) and CD86 (B7-2) that bind the T cell counter-receptors designated CD28 or cytotoxic T lymphocyte-associated antigen (CTLA)-4. SCD28 promotes T cell activation and survival, while binding to CTLA-4 has the opposite effect to inhibit T cell responses and regulate peripheral T cell tolerance [32]. Although CD80 and CD86 have little sequence homology, the receptor binding domains are generally conserved and mutations of this region have similar effects on CTLA-4 and CD28 binding [33]. T regulatory cells also mediate the suppressive effects of CTLA-4, in part, since blocking CTLA-4 activity ablates the suppressive effects of CD4+CD25+ Treg cells [32]. CTLA4-Ig is a fusion protein comprised of the external domain of the human CTLA4 protein coupled to the heavy chain constant region of human IgG1, and is called abatacept [34]. Abatacept binds to both CD80 and CD86 on APCs to prevent their engaging the counter-receptors on the T cells, and thus the second signal required for full activation. Abatacept administered to ovalbumin-sensitized mice either before sensitization or before challenge decreases the ensuing airway eosinophilia, Th2 cytokine production and AHR [35]. More recent studies suggest that CTLA-4 might be more important in the early sensitization, rather than the established, phase [36].

Activated CD4+T cells express OX40, which interacts with its ligand, OX40L, expressed by APCs. This interaction results in increased IL-4 production by naïve T cells, and their development into committed Th2 effector cells producing high levels of IL-4, IL5 and IL-13 [37]. Blocking the OX40/OX40L interaction inhibits the production of these Th2 cytokines while increasing levels of the inhibitory cytokine, IL-10. Mice deficient in OX40 or OX40L show a reduced airway inflammation and Th2 responses after sensitization and challenge with ovalbumin [38]. OX40 can also inhibit the development of adaptive Foxp3+Treg cells [32], which normally act to put a brake on the Th2 response, so that this control mechanism is removed.

2.6. Th2 phenotype

For transformation to the Th2 phenotype, stimulation of the TCR of naïve CD4+ T cells induces the transcription factor NFAT1, which in the presence of IL-4 stimulates expression of the nuclear transcription factor, GATA-3, a critical regulator of Th2 differentiation [39]. GATA-3 is phosphorylated and activated by p38 MAPK, resulting in translocation of GATA3 from the cytoplasm to the nucleus to initiate the coordinated activation of genes encoding IL-3, IL-4, IL-5, IL-9, IL-13 and GM-CSF, which are clustered on chromosome 5q31–33 [40]. GATA-3 inhibits STAT-4, and consequently Tbet (the transcription factor regulating the TH1 phenotype) thereby maintaining the Th2 polarization [41], while, in turn, Tbet can inhibit GATA-3. Other transcription factors have also been implicated in the differentiation of Th2 cells, including NF-IL-6, AP-1 and c-Maf [42].

A second route of Th2 transformation has also been proposed that is IL-4 independent. This pathway involves activation of Notch receptors on naïve T cells, with the release and nuclear translocation of the notch intracellular domain to induce the expression of GATA-3 [43].

2.7. Th2 cytokines

Following sensitization subsequent exposure to the same allergen epitopes provokes the release of a group of cytokines considered characteristic of Th2 cells and so referred to as the Th2 cytokines, comprised of IL-3, IL-4, IL-5, IL-9, IL-13 and GM-CSF. Although not the exclusive provenance of Th2 cells, these cytokines serve to promote the activation and proliferation of cells of the immune system, their recruitment into the airways, and are implicated in the accompanying pathophysiogical responses typical of asthma [4,19].

Patients with asthma show increased Th2 cytokine production in the airways, indicative of the significant role they play in the disorder [18,19]. Bronchial biopsies from patients with atopic asthma show increased expression of IL4, IL-4Rα, IL-5, IL5Rα, IL-9, and IL-9R mRNA together with increased IL-3, IL-4, IL-5, IL-13 and GM-CSF protein levels. Induced sputum or BALF from asthmatics also shows elevated levels of IL-4, IL-5, IL-13 and GM-CSF protein and/or mRNA. The increased expression in bronchial mucosa of IL-5 mRNA, or that encoding the alpha-chain of the IL-5R, correlates

with clinical indices of asthma severity, and the increased expression of IL-9 and IL-9R mRNA also correlates with declines in FEV1 and AHR. Asthma patients inhaling IL-5 exhibit increased sputum eosinophils, and enhanced AHR [18,19].

It is important to recognize that not only the Th2 cytokines are increased in asthma, but also a host of others, including IL-1β, IL-2, IL-6, TNFα, IL-17(A), IL-17F, IL-25, IL-33, TSLP, as well as the "inhibitory" cytokines IFNγ, and IL-16. However, the Th2 cytokines were among the first to be identified, demonstrate activities consistent with promoting recognized features of the cellular pathology of asthma, and manipulation of which ameliorates aspects of the disorder. Accordingly they became the center of attention.

2.7.1. IL-4

The importance of IL-4, both in regulating IgE production, and in directing Th2 cell development and recruitment, made it an attractive target. The over-expression of IL-4 in lungs produces an airway inflammation comprised predominantly of lymphocytes and eosinophils without AHR [44]. Mice deficient in IL-4, or administered an IL-4 neutralizing antibody, show diminished antigen-induced airway inflammation [4,45]. An alternative approach, using soluble IL-4 receptor α-chain to bind IL-4 and prevent it from exerting its biological effects, also blocks airway eosinophilia after antigen challenge, and reduces VCAM-1 expression and mucus hypersecretion [46]. Neither the neutralizing antibody nor the soluble receptor improve AHR elicited by the antigen, indicating AHR is independent of IL-4 and airway eosinophilia [4,46].

2.7.2. IL-4 receptor (IL-4R)

In contrast to the methods used to prevent the effects of IL-4, blocking IL-4Rα does inhibit AHR [47]. Since the IL-13R shares a common α-chain with the IL-4R, to activate a STAT6-dependent signal transduction pathway, there is some overlap in the activities of these two cytokines [48,49]. IL-4Rα$^{-/-}$, STAT6$^{-/-}$ or IL-13$^{-/-}$ mice, or those with combined IL-4/IL13 deletions show a reduced AHR after antigen challenge [4,48,49]. Consequently, interfering with the common pathway mediated by IL-4Rα and STAT6 is considered a superior target than IL-4 alone. Mutant IL-4 proteins have been developed that bind IL-4Rα without inducing signal transduction. Of particular interest is a double mutant protein, pitrakinra (formerly BAY-16-9996 or AER001), where tyrosine at position 124 is replaced by aspartic acid, which also substitutes for arginine at position 121. Pitrakinra inhibits airway inflammation and AHR in a primate model of allergen challenge [50].

2.7.3. IL-13

While IL-4 is thought to have a greater role than IL-13 in promoting eosinophilia, it is IL-13 that is associated with AHR [19]. In mice, the intratracheal instillation of IL-13, or the transgenic over-expression of the cytokine in airway epithelial cells, produces AHR and airway eosinophilia [4,19]. Blockade of IL-13 with a neutralizing monoclonal antibody (mAb), or the intratracheal instillation of soluble IL-13 receptor, or genetic deletion of IL-13 or its receptor (IL-13Rα1) reduces AHR and mucus production following antigen challenge, but has variable effects on the inflammatory component [4,19]. Targeting the common α-chain of the IL-4 and IL-13 receptors appears to overcome the limitations associated with inhibiting the individual cytokines.

2.7.4. IL-5

IL-5 is noted for its limited spectrum of activity, where in human its sole function is to regulate eosinophil production, differentiation, maturation, activation and survival [4]. Administration of exogenous IL-5 causes eosinophilia in animal models but not asthmatic subjects [51]. Transgenic mice, in which IL-5 is constitutively expressed in T cells, show a marked and life-long eosinophilia [4]. IL-5$^{-/-}$ mice have decreased circulating eosinophils while antigen challenge results in a diminished airway eosinophilia compared to wild-type animals, although the effects on AHR are variable [4,48]. Interruption of the activities of IL-5, using monoclonal antibodies to either IL-5 [4,48] or IL-5R [52], combinations of the two, or antisense oligonucleotides to block mRNA and protein expression of IL-5 or IL-5R [19,53] uniformly reduce antigen-induced airway eosinophilia across species, but with variable effects on AHR that have not been reconciled.

2.7.5. IL-3/IL-5/GM-CSF

IL-5-induced promotion of eosinophil survival is an activity shared by IL-3 and GM-CSF. It is suggested that more effective eosinophil depletion from the airways of animal models can be achieved by inhibiting all three cytokines that share a common β-chain of their receptors, and in combination with inhibition of the chemokine receptor CCR3. ASM8 is a combination of two antisense oligonucleotides targeting the mRNA for both the common β-chain and CCR3. In rat models ASM8 decreases airway inflammation after antigen challenge, reducing eosinophils, lymphocytes and macrophages, and blunting antigen-induced AHR to LTD4 [53,54].

2.7.6. IL-9

The lung-specific over-expression of IL-9 produces AHR and other morphological changes similar to asthma, including airway inflammation and a marked mast cell hyperplasia, in the absence of any antigen [55]. While IL-9 neutralizing antibodies suppress the development of allergen-induced airway inflammation and AHR, IL-9-deficient mice show little difference compared to wild-type mice with regard to airway eosinophilia or T cell development and differentiation in a pulmonary granuloma model [19,56]. Moreover, some of the pro-inflammatory actions of IL-9 can be attributed to induction of IL-5 and IL-13 from non-T cell sources [19]. IL-9 may have importance in promoting airway mast cells and migration of mast cell precursors, as this is an activity not shared by other Th2 cytokines [19,56].

There are several alternative cell sources of IL-9, including mast cells, eosinophils, and Th17 cells. A discrete population of IL-9 producing CD4+ T cells has been described, which require expression of the transcription factor PU.1 and are regulated by TGFβ (see [21,56] for review). However, it remains to be demonstrated that these Th9 cells actually exist in man, or play any substantive role in airway inflammation.

2.8. Other T cell populations

2.8.1. TH1 cells and associated cytokines

Th1 cells are present in the BALF of asthmatic patients, and increased levels of the Th1 archetypal cytokine, IFN-γ, and its mRNA are found in the sputum of asthmatic patients, with higher levels of message in moderate to severe asthmatics when compared to those with mild disease [57,58]. According to the traditional Th1/Th2 paradigm, these Th1 cells and their cytokines would be expected to serve an inhibitory role on Th2 activity. Deletion of the master Th1 transcription factor, T-bet, results in the *spontaneous* development of AHR and eosinophilia in mice [17], without the need to invoke a Th2 response. Allergen challenge in wild-type mice is accompanied by an influx of Th1 cells into the lungs. In contrast to the results with T-bet deletion, a positive cooperation between Th1 and Th2 cells has been described, that results in a more robust, eosinophil-rich inflammatory response attributed to TNFα derived from the Th1 cells [59,60].

IFN-γ prevents the development of antigen-induced airway eosinophilia and AHR in mice [61], while IFN-γ-receptor-deficient

mice show an enhanced response to allergen [62]. These findings are consistent with the study of T bet deletion [17], and the concept of a balance between Th1 and Th2 cells. However, transgenic expression of IFN-γ in mouse lung increases IL-5 and IL-13 production and airway eosinophilia [63], while adoptive transfer of eosinophils to SCID mice produces an IFN-γ-dependent AHR [64]. One proposal for these seemingly discrepant results is that low levels of IFN-γ promote, while high levels inhibit, the airway inflammatory response to antigen [19].

IL-2, a member of a family of cytokines that includes IL-4, IL-7, IL-9, IL-15 and IL-21, influences the differentiation and survival of cytotoxic T cells and regulatory T (Treg) cells. TCR stimulation by antigen provokes the secretion of IL-2 and expression of IL-2 receptors (IL-2R). IL-2 levels are elevated in the airways of symptomatic asthma patients when compared to those without symptoms [65], while IL-2R+ T cells are increased in asthmatics and correlate with the peripheral eosinophilia [66]. The IL-2R complex comprises an α-chain (also known as CD25), which binds IL-2; a β-chain that is common to the IL-15 receptor; and a γ-chain common to this cytokine family. Blocking either the α- or β-chains reduces airway inflammation in a murine model of allergic asthma, while antagonism of only the β-chain suppresses AHR [67].

2.8.2. Suppressive cytokines – IL-12 and IL-10

IL-12 plays an important role in Th1 differentiation, and its expression is decreased in bronchial biopsy samples from asthmatic patients [68]. Administration of IL-12 to mice uniformly inhibits allergen-induced Th2 responses, airway inflammation and AHR [69,70], mediated in part by increased levels of IFN-γ.

Like IL-12, the levels of the anti-inflammatory IL-10 are diminished in BALF of asthmatic patients [71], while correcting or raising the IL-10 concentration could be expected to help resolve the inflammation. Administration of IL-10, or an IL-10 producing plasmid vector inhibits ovalbumin-induced airway inflammation, but with variable effects on AHR [72,73]. Anti-IL-10 or anti-IL-10R mAbs, or genetic deletion of IL-10, enhances airway inflammation [19], while the suppression of AHR by Treg cells is considered dependent on IL-10 [74].

2.8.3. Th17 cells and IL-17

Th17 cells have been identified as a distinct subset of CD4+ T helper cells that are differentiated by the cytokines IL-6, IL-1β, TGF-β, and IL-23, and regulated by the transcription factor retinoic acid orphan receptor γt [75]. Th17 cells produce IL-17 (also known as IL-17A), IL-17F, IL-22 and IL-21. Increased numbers of Th17 cells are found in the airways of asthmatic patients [76], while enhanced IL-17 expression has been reported in the sputum, BALF and lung cells of asthmatic patients [21], where IL-17 levels increase with asthma severity. Upon allergen sensitization, Th17 cells home to the lung, although other cell-types in asthmatic airways, including CD8+ T cells, γδ T cells, natural killer T cells and alveolar macrophages, can also produce IL-17 [21].

The intratracheal instillation of IL-17 prompts a neutrophil influx into the airways of rats [19], so this cytokine is often implicated in a neutrophilic asthma that is resistant to inhaled steroids for no other reason than both are associated with airway neutrophilia. IL-17 acting on its receptor, IL17RA, activates various pro-inflammatory downstream pathways including nuclear factor-kB (NF-kB) and mitogen activated protein kinase (MAPK) to stimulate genes involved in inflammation, including those encoding various chemokines for neutrophils and eosinophils, cytokines such as IL-6, adhesion molecules including ICAM-1 and other inflammatory genes such as cyclooxygenase-2 [21,75]. Despite these many pro-inflammatory actions, in the murine ovalbumin challenge model, IL-17 deficient mice react in the same way as IL-17 sufficient animals, while IL-17R deficient mice exhibit a reduced airway eosinophilia and Th2 cytokine production [19,77]. Exogenous IL-17, given in conjunction with the antigen, can either blunt [77] or enhance [19] the eosinophilia and AHR, while neutralizing IL-17 antibodies decrease airway neutrophilia but augment eosinophilia [77]. Differences between low levels of endogenous IL-17 and the high levels achieved with exogenous administration have been proposed as one reason for these discrepancies [77], as have differences in the strain of mice used [19]. Regardless, this serves as another example of the anomalies inherent in basic research in asthma, and the confusion regarding the significance of IL-17 or Th17 cells. Nonetheless, a mAb targeting IL-17R is in clinical development for asthma (Section 5.4.1).

2.8.4. CD8+ T cells

Cytotoxic T cells, also known as CD8+ T cells since they express the CD8 glycoprotein on their cell surface, primarily serve to destroy virally infected cells and tumor cells by secreting IFNγ and cytolytic factors. A subset of CD8+ T cells called Tc2 cells, produce the "Th2 cytokines" namely IL-4, IL-5 and IL-13, and are found in bronchial biopsies and BALF of asthma patients [21,78]. However, as with Th1 cells, Th17 cells, NKT and γδ cells, animal models provide conflicting information as to whether these cells are protective or deleterious [79]. While CD8+ T cells can suppress allergic airway inflammation via IFNγ production and stimulation of IL-12 formation, mice deficient in CD8+ T cells also have a reduced airway inflammation and AHR response [79]. CD8+ T cells exhibit functional and phenotypic plasticity depending on their microenvironment [80] that could contribute to difficulties in establishing their role.

One area in which CD8+ T cells may be important in asthma is the association between viral infections and acute asthma exacerbations [81]. Rhinovirus, the common cold virus, is the most frequent cause of exacerbations. Epithelial cell production of IFN-β and IFN-λ is decreased in asthmatic patients, which may predispose these patients to an enhanced response to the virus since these interferons represent the first line of defense against the respiratory viruses. In the presence of Th2 cells, virus-specific CD8+ T cells switch from making IFN-γ to producing IL-5 and augmenting airway eosinophilia, indicative of the functional plasticity of these cells [21,80].

2.8.5. Regulatory T (Treg) cells

Treg cells, formerly called suppressor T cells, are important in immunological tolerance and limiting T cell mediated immune responses. There are several Treg subsets including the naturally occurring CD4+CD25+FoxP3+ Treg cells and inducible Treg cells stimulated by antigen, all identified by their dependence on the transcription factor FoxP3. Treg cells control the immune response through the production of anti-inflammatory cytokines such as IL-10 and TGFβ, expression of inhibitory molecules like CTLA4, and down-regulation of MHC class II and the co-stimulatory molecules CD80 and CD86 on APCs (see [23]).

Treg cells are found in the airways of asthmatic patients [82], show a positive correlation with FEV1, and increase in number following treatment with corticosteroids [83]. Transfer of CD4+CD25+ Treg cells suppresses airway inflammation and AHR in animal models, and prevents antigen-induced activation of airway DCs (see [23]). TNF receptor superfamily member 25 (TNFRSF25) is constitutively expressed on CD4+CD25+Treg cells. Using a mAb to activate the receptor, Treg cells proliferate to protect against antigen-induced airway inflammation and decrease Th2 cytokine production [84]. Moreover, Treg cells express TLRs, and activation of these TLRs can increase or decrease the immunosuppressive activity, providing another link between innate and adaptive immune responses [85].

The suppressive effect of Treg cells can decline if they are not continuously stimulated by chronic local antigen exposure. A waxing and waning of the Treg cell response due to intermittent allergen exposure is suggested to contribute to the episodic nature of the asthmatic disorder [23].

While stimulation of Treg cells as a means to modulate the airway inflammatory response is attractive, it is sobering to recall the experience with the CD28-specific superagonist mAb, TGN1412, designed to do just that in treating patients with rheumatoid arthritis, multiple sclerosis and leukemia, by targeting the co-stimulatory molecule on T cells. Despite compelling animal data, administration of the mAb to healthy volunteers in a Phase 1 study resulted in a "cytokine storm", and multi-organ failure requiring intensive care and hospitalization for more than 3 months in some cases [86]. One patient developed necrosis necessitating amputation of all their toes and several fingertips.

2.8.6. Innate-like T cells: NKT and γδ cells

NKT cells express an invariant TCR that recognizes glycolipids such as α-galactosyl ceramide (αGC) presented by CD1d rather than peptide antigens presented via MHC II, and this invariant receptor behaves like a pattern-recognition receptor. Once activated, these cells perform functions similar to both helper and cytotoxic T cells producing cytokines including IL-4, IL-13 and IFNγ [87]. NKT cells are present in BALF of asthmatics [87,88], and their numbers increase following allergen challenge [89]. However, mouse models of asthma have yielded contradictory information on the role of NKT cells in the allergic response (see [21,87]), and since no NKT-cell-specific therapeutics have advanced into clinical trials in asthma to date, they will not be discussed further.

γδ T cells are a small subset of T cells and a local population resides in the airways in close association with the airway epithelium to subserve an immunosurveillance role. Asthma patients have more IL-4 secreting γδ T cells in their BALF than healthy subjects [90]. Mice lacking γδ T cells develop AHR in the absence of antigen sensitization and challenge [91], while CD8+γδ T cell-derived IFNγ inhibits airway eosinophilia and the late airway response in rats, associated with decreases in cysLT levels in BALF [92]. Pulmonary γδ T cells are divided into a pro-inflammatory subset that expresses Vγ1+ TCR and an anti-inflammatory subset expressing Vγ4+ TCR. Vγ1+ TCR γδ T cells produce IL-5 and IL-13 and have been implicated together with NKT cells in provoking AHR, while the suppressive effects of Vγ4+ TCR γδ T cells are mediated, at least in part, by secretion of IL-17 [21].

2.8.7. Pro-inflammatory cytokines

Also found in asthmatic airways are the pro-inflammatory cytokines that include IL-1, IL-6 and TNFα.

IL-1α and IL-1β are the products of distinct genes, have only 25% sequence homology at the amino acid level, but bind to the same receptors, designated IL-1R1 and IL-1R2, albeit with different affinities. IL-1R1 is considered the functional receptor, while IL-1R2 might be a decoy receptor. There is also a naturally occurring IL-1 receptor antagonist, IL-1Ra, that binds to the receptors without triggering downstream signal transduction pathways, and blocks the effects of IL-1α and IL-1β (see [93]). The expression of IL-1β and/or IL-1Ra is increased in the bronchial epithelium, macrophages and BALF of asthmatics [94], and IL-1Ra levels increase following allergen challenge which are not affected by ICS [94].

While there is not complete concordance between preclinical studies investigating the importance of IL-1 in antigen-induced asthma [95], overall mice deficient in both IL-1α and IL-1β show a reduced AHR, T cell proliferation and production of IL-4 and IL-5 following challenge with ovalbumin, while mice deficient in IL-1Ra develop an augmented response [95]. Administration of a recombinant adenovirus expressing human IL-1Ra reduces airway inflammation and AHR elicited by ovalbumin in sensitized mice, accompanied by decreased IL-5 and eotaxin production with increased IFNγ levels in BALF [95]. These studies support the notion that IL-1 contributes to the allergic airway response in the mouse model.

IL-6, together with TNFα and IL-1β, is traditionally regarded as a pro-inflammatory mediator rather than a regulatory cytokine orchestrating the immune response. However, it now appears that IL-6 can promote differentiation of CD4+ T cells to the Th2 phenotype, while suppressing Th1 formation [96]. IL-6 produced by DCs also inhibits the suppressive function of Treg cells and controls their survival [97], thereby further enhancing the Th2-mediated response. IL-6 is produced by lung epithelial cells, and in greater quantities in those from asthmatic subjects. IL-6 levels are also elevated in BALF and induced sputum of asthmatics, where the levels correlated inversely with lung function in subjects with mild to moderate asthma [98]. Assigning a role for IL-6 in animal models has been confounded by the finding that IL-6-deficient mice are protected against allergen-induce airway inflammation, while studies with neutralizing antibodies indicate IL-6 promotes the airway response [99].

TNFα is a pro-inflammatory cytokine whose levels are elevated in bronchial biopsy specimens and BALF of asthmatic patients [100], particularly those with severe asthma [101], where bronchial biopsies show a 30-fold increase in TNFα mRNA expression localized predominantly to mast cells. Inhalation of TNFα in healthy subjects produces AHR together with an increase in sputum neutrophils [102]. Although TNFα is normally associated with neutrophils, TNF antibodies suppress airway inflammation, including eosinophilia, together with IL-5 production in the murine antigen challenge model [103].

2.9. Chemokines

The link between cytokine-mediated leukocyte activation, proliferation and their eventual recruitment into the airways is mediated by the chemokines. Chemokines comprise a group of almost 50 chemotactic proteins of 8–15 kDa that vary with respect to sequence homology, but share similar three-dimensional structure. They act on G-protein-coupled receptors, and while some of these receptors are selective, many respond to multiple chemokines. For a detailed review of chemokines and their role in allergy and asthma, the reader is referred to [20,24]. The function of the chemokines is to serve as a chemotactic signal to promote cell accumulation; activate a group of adhesion molecules, the integrins, which allow the leukocytes to adhere to the endothelium and diapedese into the airways; and also direct leukocyte trafficking into different compartments of the tissue.

The phenotypic transformation of T cells into Th2 cells produces dramatic changes in chemokine receptors with expression of CCR3, CCR4 and CCR8 [20,24]. Although attempts to identify specific chemokines and chemokine receptors using Th2 cells from human lung have yielded contradictory information, attention has focused on the potential roles of CCR3 and CCR4.

IL-5, together with IL-4 and IL-13, regulates the trafficking of eosinophils to sites of inflammation [104]. These cytokines induce chemokine expression by airway epithelial cells, and directly upregulate the adhesion molecules VLA-4 and P-selectin on the eosinophil cell surface [104]. Eosinophils also express CCR3, the ligands for which are CCL11 (eotaxin), CCL24 (eotaxin-2), and CCL26 (eotaxin-3), which are all upregulated in asthmatic airways in a STAT6 dependent manner [20,24]. Bronchial biopsies and BALF from asthma patients show increased mRNA expression and protein levels for CCR3, CCL11 (eotaxin) and CCL5 (RANTES), while the number of CCR3 mRNA-positive cells in the bronchial mucosa correlate with airway eosinophilia and AHR [104]. In mice,

blockade of CCR3 with small molecule antagonists attenuates the pulmonary influx of eosinophils, but not lymphocytes or macrophages following ovalbumin challenge, and shows evidence of reduced airway remodeling [105,106].

CCR4 is expressed on Th2 cells. In asthmatic subjects there is a correlation between peripheral blood CCR4+ T cells and asthma severity, while CCR4+ T cells accumulate preferentially in asthmatic airways, and it is these cells, rather than CCR4− T cells that produce Th2 cytokines [107]. Administration of a CCR4 blocking antibody abolishes airway eosinophilia and AHR in a mouse model that uses peripheral blood mononuclear cells from allergic asthma patients sensitive to house dust mite [108]. A small molecule CCR4 antagonist, K327, attenuates the recruitment of CCR4+CD4+ T cells and eosinophils into the airways of mice challenged with ovalbumin [109].

2.10. Leukotriene B4

Various leukocyte populations including neutrophils, mast cells and macrophages synthesize leukotriene (LT) B4. While LTB4 was originally considered as a chemotactic factor for neutrophils, it has been implicated as an important chemotactic factor for eosinophils, and effector CD8+ T cells during anaphylaxis [110]. Increased levels of LTB4 and T cells expressing the high affinity LTB4 receptor (BLT1) are found in the airways of asthmatic patients [111]. BLT1 expression on effector CD8+ T cells is considered important for antigen-induced airway eosinophilia and AHR [112]. A BLT1 antagonist inhibits ovalbumin-induced neutrophilia, eosinophilia and AHR in mice subjected to a second challenge 6 weeks after the primary challenge, as a model of established disease [113]. Deletion of the LTB4 receptor in mice results in reduced T cell accumulation in the airways following ovalbumin challenge [110].

2.11. Adhesion molecules

The process of leukocyte recruitment to the airways involves a series of adhesion molecules on the leukocyte and counter-receptors on the vascular endothelium to capture, arrest and then allow the leukocyte to squeeze through gaps between endothelial cells and enter the airways. Eosinophils express seven integrin receptors involved in cellular adhesion, of which the most important is VLA-4 ($\alpha4\beta1$ heterodimer) since it mediates cell rolling and adhesion at physiological shear rates (see [114]). The counter receptor to VLA-4 is endothelial VCAM-1, which is induced by mediators released from Th2 cells, and upregulated in the asthmatic lung [115].

Antigen challenge in asthma patients results in eosinophils adopting a phenotype with increased expression of $\beta1$ and $\beta2$ integrins, which correlate with BALF eosinophilia [116]. The role of VLA-4 in mediating eosinophil influx into asthmatic airways prompted a number of companies to develop antagonists to the integrin. These antagonists are uniformly efficacious in ameliorating airway inflammation and functional responses following allergen challenge protocols in mouse, rat, guinea pig, rabbit and sheep [114].

Another group of adhesion molecules, the selectins, has also been implicated in leukocyte recruitment in airways of asthmatics. The selectins contribute to the "rolling" of leukocytes along the vascular endothelium through an interaction with their counter-receptors, thereby allowing the integrins to effect the capture and firm adhesion. There are 3 selectins, named after their original source – E-selectin (endothelial), P-selectin (platelet) and L-selectin (leukocyte). The selectins can be constitutively expressed or upregulated by inflammatory mediators such as TNF, IL-1 or lipopolysaccharide (LPS). The counter-receptors to the selectins contain the oligosaccharide Lewis-x or Lewis-a, and there can be some promiscuity between ligands. For example, P-selectin glycoprotein ligand-1 (PSGL-1) expressed on the surface of leukocytes binds all of the selectins [117]. Notably, all three selectins have been implicated in airway inflammation in animal models of asthma [118].

3. Eosinophils

Classically, eosinophils are regarded as the terminal effector cells of airway inflammation that link the immune system to the functional sequelae of asthma. Although their role has expanded in recent years to include antigen presentation, cytokine production and participation in the development of airway inflammation (see [119]), they are still regarded as an important source of mediators that modulate bronchomotor tone. While murine asthma models involving antigen sensitization and challenge show no evidence for bronchoconstriction, they do develop a hypersensitivity to spasmogens that cause bronchoconstriction. Consequently, AHR is the functional endpoint equated with evidence for an effect on bronchomotor tone.

3.1. Eosinophil-derived mediators of enhanced bronchial smooth muscle activity

A debate has raged for years over the importance of airway inflammation in general, and eosinophils in particular, to AHR (see [120]). Particularly surprising is the paucity of studies defining which eosinophil-derived mediator(s) is responsible for changes in bronchial smooth muscle sensitivity. Various mediators and cytotoxic products released by eosinophils have been implicated, based largely on these factors being present and having the *potential* to elicit some effect, but direct links establishing cause and effect are rare.

Initial studies suggested the toxic cationic proteins, in particular major basic protein, were involved in the development of AHR [121,122]. Since this action could occur without evidence for cell denudation, but rather required an intact epithelium, it was attributed to release of other bronchoactive mediators by cells within the airway wall. It was concluded that the cationic proteins were not directly responsible for AHR, but served as an intermediary between the eosinophil and the ultimate response.

A role for cytokines in AHR has also been proposed. Incubation of human bronchus muscle preparations with recombinant human IL-5 for 24 h results in increased sensitivity to the spasmogenic effects of acetylcholine [123]. Transgenic mice, in which only smooth muscle cells either express or lack IL-4Rα, treated with IL-4, IL-13 or allergen implicate this cytokine receptor as one means to develop AHR [124]. IFNγ is a cytokine normally associated with Th1 cells, but IFNγ derived from eosinophils has been implicated in AHR [125], where the adoptive transfer of IFNγ-deficient eosinophils, or eosinophils treated with anti-IFNγ antibody, fails to induce AHR in IFNγ-deficient recipients. However, in these studies IFNγ also augments airway inflammation, and it is necessary to divorce effects on AHR from changes in local inflammation that could influence reactivity by some other mechanism. A direct effect of the cytokine on bronchial smooth muscle has not been demonstrated. IFNγ does induce cysLTR2 expression and enhance cysLT-induced inflammatory responses [126], so could act indirectly via cysLTs.

The ability of activated eosinophils to produce cysLTs that are potent bronchoconstrictors, has focused attention on these mediators as the link between eosinophils and changes in bronchomotor tone. Since these are recognized direct acting bronchial spasmogens, they will be considered in more detail.

3.2. Leukotrienes and bronchial smooth muscle tone

The actions of the cysLTs – LTC4, LTD4 and LTE4 – are mediated through specific G-protein-coupled receptors. Two were identified originally and designated cysLT1 and cysLT2. Attention focused on the importance of cysLT1 since it is upregulated in the bronchial mucosa of asthmatics [127], has the highest affinity for LTD4, and is thought to mediate many of the effects of the cysLTs in asthma [110]. The identification of cysLT1 antagonists such as zafirlukast and montelukast, coupled with their efficacy in preclinical models of asthma to reduce bronchomotor tone [128], and AHR [129,130], further directed attention primarily towards this receptor. However, none of these studies define the cellular source of the cysLTs or provide a link between airway eosinophils and bronchomotor tone. Indeed, in these studies [128–130] cysLT1 antagonism is associated with decreased airway inflammation and eosinophilia, which voids attempts to link improvements in airway function solely to blockade of a bronchoconstrictor agent. Clinically, cysLT1 antagonists like montelukast improve FEV1, but have little effect on AHR [131].

A convincing argument has been made that the importance of LTE4 in bronchoconstriction and AHR may have been overlooked [132,133]. In 1984 it was noted that LTE4 was more potent than LTC4 or LTD4 at contracting strips of tracheal muscle *in vitro*, and uniquely among the cysLTs, only LTE4 enhanced sensitivity to histamine thereby prompting the suggestion that there are three cysLT receptors [134]. The recent recognition of two receptors with high specificity for LTE4 over LTC4 or LTD4, the cysLTE receptor and the purinergic $P2Y_{12}$ receptor [132,135], provide new targets to address the potential importance of this LT in bronchiolar reactivity.

Evaluating the source and role of eicosanoids in asthma is complicated by the phenomenon of transcellular synthesis, where biosynthetic intermediates can be transferred during cell–cell interactions to form either increased amounts of products, or mediators not synthesized by either cell alone [136]. For example, alveolar macrophages can take up LTA4 to produce LTB4, while mast cells convert neutrophil-derived LTA4 to LTC4 in addition to producing their own cysLTs [136].

The promiscuity of many of the proposed mediators of bronchial smooth muscle sensitization – with both multiple sources of production and action – coupled with the difficulty in divorcing effects on bronchial smooth muscle from influences on airway inflammation, make it hard to pinpoint any eosinophil product as *the* link between airway inflammation and functional derangements. In murine models of asthma there is a difference between strains in the development of AHR [137–139], while contamination of antigen preparations with LPS that can itself promote AHR through activation of Toll-like receptor 4 (TLR4) and release of mediators such as TNFα [140], all contribute to the variability in experimental results reported.

3.3. Airway hyperreactivity: clinical symptom versus research phenomenon

AHR, a cardinal feature of asthma, is not specific to this disorder. It is also observed in chronic obstructive pulmonary disease (COPD), cystic fibrosis, pulmonary tuberculosis, and primary Sjögren's syndrome, but not eosinophilic bronchitis. The degree of hyper-responsiveness is variable between these conditions, and can be enhanced by viral infections, allergens and other noxious stimuli. For example, mild asthmatics with sensitivity to a particular allergen show evidence of basal AHR that can be enhanced further when challenged with the antigen [141].

Clinically AHR is intriguing since the hyper-responsiveness is not non-specific [141], although it does occur to a variety of diverse agents that include autacoids such as histamine, serotonin, PGF2α, and cholinergic agonists like methacholine, and to a lesser extent to LTD4 (together broadly termed *direct* agents); but also cold air, sulfur dioxide, hyperosmotic saline and mannitol (called *indirect* agents since they do not stimulate bronchial smooth muscle themselves) [120]. Tests using indirect rather than direct agents are considered more specific, but suffer by being less sensitive [120]. Studies suggest that the indirect agents act through inflammatory cells to elicit bronchoconstriction. Mannitol-induced bronchoconstriction is accompanied by evidence of mast cell activation with increased levels of histamine, cysLTs and prostaglandins, and is attenuated by drugs such as cromoglycate [142,143]. Since cromoglycate also blocks the initial bronchoconstriction induced by allergen challenge, before any evidence for an inflammatory cell influx [144], it is likely that the bronchoconstrictor mediators are derived in large part from cells resident in the airways, such as mast cells and alveolar macrophages, rather than infiltrating eosinophils or lymphocytes. Despite the association of indirect agents with airway inflammation, they have not been used to assess AHR and its relationship to aspects of airway inflammation in animal models of asthma.

Most studies in the mouse just use a cholinergic agonist, often carbachol, to define AHR. A recent study [145] reproduced AHR to methacholine in the mouse antigen challenge model *in vivo*, then removed the trachea, confirmed increased sensitivity to methacholine *in vitro*, but found no such super-sensitivity to histamine, serotonin or adenosine. The methacholine effect *ex vivo* was still evident when the trachea was removed 72 h after antigen challenge, and implies a specific change in the transduction pathway between muscarinic M3 receptor activation and smooth muscle contraction, even when tissues are removed from the inflammatory milieu. Studies by Morley and co-workers [146,147] using the guinea pig model of antigen challenge provide the most comprehensive analysis of AHR to different spasmogens, defining the rank order as acetylcholine, serotonin, LTE4, bradykinin, PGF2α, histamine and LTC4.

The profile of agonist sensitivity is relevant to ensure the AHR being studied in basic research models as the basis for defining expectations in clinical studies, is actually the same AHR as described clinically in asthma. In guinea pigs, an infusion of platelet-activating factor evokes an AHR with a different sensitivity profile to various spasmogens than that elicited by allergen challenge [146,147]. In animals, AHR can also be elicited by LPS, ozone, infectious agents, proteolytic enzymes and cigarette smoke, so is a common response to injury of the airways, and not specific to models of asthma. Clearly the pattern and localization of any accompanying inflammatory response can vary widely between these different conditions: for example the neutrophilic influx elicited by LPS or ozone, versus the role of alveolar macrophages in the response to cigarette smoke, and the eosinophilia associated with antigen challenge. As a result, it would be inappropriate to regard AHR as a singular, non-specific phenomenon. Absent detailed analysis of the response, caution must be exercised in extrapolating basic research results to the clinical scene.

4. Allergen challenge studies in mild asthmatic subjects: an early Phase 2 bridge between basic research and asthma clinical trials

Clinical allergen challenge studies are frequently used to provide early clinical proof-of-concept data [144], so recognition of the main features of this clinical evaluation model will aid interpretation of the clinical results discussed in the next section. Performed in mild asthmatic subjects with a known sensitivity to a particular allergen, this model offers a number of advantages. These include an ability to examine a range of endpoints such as bronchoconstriction, AHR and airway inflammation through

sampling sputum or BALF (thereby reproducing the key features of the preclinical models); and the need for only a small number of patients, since the response to challenge is standardized and each patient can act as their own control in a crossover study design. A more detailed analysis of this model's merits and limitations is to be found in the accompanying article [2].

In brief, inhalation of the allergen results in an immediate bronchoconstrictor response termed the "early airway response" (EAR), which subsides over the ensuing 1–3 h. This is followed by a second phase of bronchoconstriction that develops over hours 3–12, is often more severe than the early response and is persistent. Associated with an influx of inflammatory cells and mediators this "late airway response" (LAR) is generally used as the primary endpoint in these studies [144]. AHR is usually measured 24 h after allergen administration, sometimes along with the cellular content of BALF samples.

5. Clinical asthma studies based on targets identified from the immune/Th2 paradigm developed in basic research

As indicated in Fig. 1, there are multiple sites in the immune response cascade to intercede with therapeutic strategies. A host of drugs targeting these sites have been developed and advanced into clinical studies. The following section seeks to review the current status of these different approaches. Numbers with the prefix NCT represent clinical trial identification numbers on the web-site www.clinicaltrials.gov. An overview of these results is provided in Tables 1 and 2.

5.1. Co-stimulatory molecules

At least two agents that block co-stimulatory molecules are under clinical investigation in asthma. One is a CTLA4-Ig fusion protein (abatacept) to block the stimulation of B7 by CD28, and the other a mAb directed at OX40L.

Abatacept has been developed for the treatment of rheumatoid arthritis refractory to anti-TNF drugs [34]. Abatacept binds to B7 (CD80 and CD86) on APCs with a higher affinity than CD28, thereby competitively blocking CD28 binding and its ability to activate T cells. Abatacept is currently undergoing a Phase 2 evaluation in a segmental allergen challenge setting in mild asthmatics to assess its effects on eosinophils in BAL. This study (NCT00983658) has an anticipated completion date of December 2010, but no results have yet been announced.

Table 1
Synopsis of clinical results in completed asthma trials with therapies targeted at different aspects of the immune response.

Target	Drug	Clinical benefit +/−	Comments
Th2 cytokines			
IL-4	IL-4 mAb, pascoluzimab	−	Discontinued
	sIL-4R, altrakincept	−	Discontinued
IL-5	IL-5 mAb, SCH55700	−	Discontinued
	IL-5 mAb, reslizumab	+	Severe eosinophilic asthma
	IL-5 mAb, mepoluzimab	+	Severe eosinophilic asthma
IL-4/IL-13	IL-4Rα mAb, AMG317	−	Discontinued
	IL-4Rα mutein, pitrakinra	+	Severe eosinophilic asthma
	IL-4Rα antisense, AIR654	−	Discontinued
	mab, GSK 679586	−	Discontinued
IL-13	IL-13 mAb, IMA-026	−	Discontinued
	IL-13 mAb, IMA-638	− (?)	Study completed 2008; discontinued?
	IL-13 mAb, CAT-354	?	Study completed 2010
	IL-13 mAb, lebrikizumab	?	Study completed 2009
IL-9	IL-9 mAb, MEDI-528	−	Larger P2 study ongoing
Suppressive cytokines			
IFN-γ	hrIFN-γ	−	Discontinued
IL-10	hrIL-10	−	Discontinued
IL-12	hrIL-12	−	Discontinued
Inflammatory cytokines			
TNFα	TNFα mAb, golimumab	−	Risk/benefit unacceptable
	TNFα mAb, infliximab	−	Discontinued
	Soluble TNFR, etanercept	+/−	Discontinued
IL-1	IL-1ra, anakinra	−	Discontinued
Other cytokines			
IL-2	IL-2Rα mAb, daclizumab	+	Some side effects
Dual cytokine/chemokine			
IL-5Rβ + CCR3	Dual antisense, ASM8	+	Allergen challenge
Chemotactic factor			
LTB4	LTB4 R antag, LY239111	−	Discontinued
Adhesion molecules			
VLA-4	VLA-4 antags, various	−	7 discontinued
CD11a	CD11a mAb, efaluzimab	−	Discontinued
Selectins	pan antag, bimosiamose	+	Now in COPD trials
Immunosuppressants			
CD4	CD4 mAb, keliximab	−	Discontinued
Various	Methotrexate	−	Discontinued
	Cyclosporine	−	Discontinued
	Azathioprine	−	Discontinued
	IVIG	−	Discontinued
	Auranofin	−	Discontinued

N.B. Public companies are required to report on events that could have a significant impact on their market valuation. Early stage (Phase 2) negative clinical studies often go unreported, particularly by large pharmaceutical/biotechnology companies, since they are part of doing business, and do not impact valuations. A positive Phase 2 study could be reported to garner attention to a healthy portfolio, or kept secret in the hopes of maintaining a competitive edge. For smaller biotechnology companies a Phase 2 outcome can have a substantial effect on company valuations. Consequently, there is a degree of interpretation of the status of some of these compounds. In general, if a compound was scheduled to complete a Phase 2 clinical study 1.5 years ago, and there has been no announcement and there is no subsequent study cited in the clinical trials database, or it is no longer listed on the company web-site, it is interpreted as "discontinued" for asthma.

Table 2
Products currently in Phase 2 asthma clinical trials with no results announced to date.

Target	Pharmaceutic	Clinical trials identifier
IL-5Rα	MEDI-563	NCT01238861
IL-13	QAX576	NCT01130064
IL-17R	AMG827	NCT01199289
OX40L	Anti-OX40L mAb	NCT00983658
B7	Abatacept	NCT00784459

An anti-OX40L mAb is currently under evaluation in a clinical Phase 2 allergen challenge study in mild asthmatics, where the LAR is the primary endpoint (NCT00983658). This study is scheduled for completion in April 2011. However, a recent study evaluating OX40/OX40L expression in asthma of different severities concluded that OX40/OX40L expression is increased in the bronchial submucosa of mild asthma, but not in the moderate to severe disease [148].

5.2. Th2 cytokines

Based on the substantial body of basic research studies, all the "Th2 cytokines" – IL-3, IL-4, IL-5, IL-13, IL-9, and GM-CSF – have been targeted in clinical studies.

5.2.1. IL-4

Two approaches to blocking IL-4 have been used – an anti-IL-4 mAb (pascolizumab), and the soluble extracellular domain of the α-chain of the IL-4R that binds IL-4, called altrakincept. While no detailed results have been published from the clinical trial(s) with pascolizumab, it was reported that the Phase 2 results in symptomatic steroid-naïve patients were unimpressive and the biologic has been discontinued [149].

Moderate atopic asthmatics taking daily ICS were given nebulized altrakincept, the recombinant human soluble IL-4 receptor, one day after stopping their steroid medication. Treatment of 9 subjects with 1500 μg altrakincept resulted in improved maintenance of asthma symptom scores, FEV1 measures and requirement for β2-agonist rescue over the ensuing 15 days compared to placebo or a 500 μg dose [150]. A follow-up 12-week study conducted in ICS-dependent moderate asthmatics who develop an exacerbation if their ICS therapy is reduced found that 3.0 mg altrakincept prevented the decline in FEV1 and asthma symptoms with ICS withdrawal, but not the incidence of exacerbations [151]. An anecdotal comment by Adcock et al. [152] suggests that a larger Phase 3 study failed to confirm these benefits, and the biologic has been discontinued.

5.2.2. IL-5

Due to the importance of IL-5 in eosinophil differentiation, proliferation, activation and survival, three different anti-IL-5 monoclonal antibodies – SCH55700, mepolizumab and reslizumab – have been advanced into clinical development, together with one mAb, MEDI-563, targeting the α-chain of the IL-5R. In addition, an antisense oligonucleotide called ASM8, targeting the β-chain of the IL-5R, has completed early Phase 2 clinical trials. Since ASM8 is a mixture of two antisense oligonucleotides, and the IL-5R β-chain is also common to the receptors for IL-3 and GM-CSF, it is discussed in Section 5.2.5.

A pilot study of SCH55700 in patients with severe, persistent asthma taking either oral or inhaled steroids, showed a dose-dependent reduction in circulating eosinophils, but no maintained benefits on any clinical indices of the disease [153], so has been discontinued. A Phase 2 study with reslizumab in patients with poorly controlled eosinophilic asthma (10% sputum cells) and followed for 15 weeks, reportedly showed improvements in lung function and decreased airway eosinophilia, with a trend for benefits in the asthma control questionnaire [154]. Phase 3 studies with reslizumab in eosinophilic asthmatics are underway (NCT01270464; NCT01285323; NCT01287039) using the incidence of asthma exacerbations and improvements in lung function as the primary endpoints.

The most advanced anti-IL-5 antibody is mepolizumab. When administered to mild asthmatics subjected to allergen challenge, there was a substantial decrease in blood and sputum eosinophils, but no effect on LAR or AHR [155]. In a more prolonged 20-week study in mild asthmatics mepolizumab again failed to improve clinical measures of asthma despite reductions in eosinophils, but not their complete ablation [156]. Similarly in patients with moderate asthma experiencing continued symptoms despite ICS therapy, mepolizumab produced no significant improvements in any measured clinical indices, but there was a trend ($p = 0.065$) for a ~50% decrease in exacerbation rates [157]. Subsequent studies in patients with severe eosinophilic (>6% sputum cells) asthma who experience a high incidence of exacerbations found that mepolizumab decreased the number of exacerbations [158,159], and patients were able to reduce their dependence on oral prednisone [159]. However, in these studies mepolizumab had no effect on other asthma outcomes, such as lung function, symptoms, and asthma control, and a marginal effect on the patients' quality of life scores was deemed not clinically meaningful in an accompanying editorial [160].

MEDI-563 binds to an epitope on IL-5Rα in close proximity to the IL-5 binding site, thereby disrupting its association with IL-5 [161]. A Phase 1 study reported a dose-dependent prolonged decrease in blood eosinophils. MEDI-563 has been advanced into two Phase 2 studies and is being explored by both intravenous and subcutaneous routes of administration, looking at exacerbation rates (NCT00768079) and measures of asthma control (NCT01238861). The former study was scheduled for completion March 2011, so the results could be available soon.

5.2.3. IL-4Rα

The α-chain of the IL-4 receptor is common to both IL-4R and IL-13R, and so is considered attractive as it targets both Th2 cytokines. Consequently, four drugs targeting this receptor component have completed early Phase 2 clinical trials, one of which, pitrakinra, has gone on to complete a larger clinical study. While the results to date have been mixed, the recent study with pitrakinra suggests that this target might be most relevant in the patients with severe eosinophilic asthma suffering exacerbations, analogous to the patient group for the anti-IL-5 antibodies.

An anti-IL-4Rα mAb, AMG317, completed a Phase 2 12-week study in moderate to severe atopic asthmatics evaluating its effect on ACQ. While the antibody had no significant effect in either the primary or any secondary endpoints, it was noted that the more severe patients tended to garner some benefit [162]. Review of the clinical trials database suggests currently there are no new studies ongoing with AMG317. An intravenous biologic targeting either IL-4Rα or IL-13 (the target has not been specified), GSK679586, has completed a clinical trial in moderate to severe asthmatics also using ACQ as the primary endpoint (NCT00843193). Although no results have been announced, it appears this drug has been discontinued [163].

Pitrakinra, an IL-4 protein with a double mutation so that it binds to IL-4Rα but acts as an antagonist, was studied in a Phase 2 proof-of-principle setting of allergen challenge in mild asthmatics. Pitrakinra blunted the LAR, but had no effect on AHR [164]. Administered as an inhaled dry powder in a large follow-on study in 534 patients with moderate to severe asthma taking ICS and β2-agonists, pitrakinra had no effect in the overall patient population.

However, at the highest dose (10 mg) pitrakinra significantly reduced the incidence of asthma exacerbations in eosinophilic asthmatics – a predefined sub-group [165]. This apparent disconnect between the allergen challenge observations and the ensuing profile in a "real world" asthma study parallels some other recent clinical findings. The anti-IL-5, mepolizumab had no effect in the allergen challenge setting [155], but reduced exacerbation rates in similar severe eosinophilic asthma patients [158,159]. AIR645, an antisense oligonucleotide targeting the mRNA for IL-4Rα, showed no activity in the same allergen challenge setting in which pitrakinra worked, causing the compound (and the company) to be discontinued.

5.2.4. IL-13

The lack of efficacy observed with anti-IL-4 strategies, coupled with the efficacy associated with interruption of IL-4Rα, could be interpreted as highlighting the importance of the other IL-4Rα ligand, namely IL-13. Five anti-IL-13 mAbs have advanced into clinical studies.

IMA-638 and IMA-026 are humanized IgG1 antibodies that bind to different epitopes on IL-13 to neutralize the cytokine. Both antibodies were studied in the allergen challenge setting. IMA-638, but not IMA-026, blunted both the EAR and LAR, while neither antibody affected AHR or sputum eosinophils [166]. IMA-638 was then advanced into a 12-week study in persistent asthmatics on ICS, using a measure of lung function (morning PEFR) as the primary endpoint (NCT00425061). This study was scheduled for completion in 2008, but since no results have been announced, it would appear that these antibodies are no longer being pursued for the treatment of asthma.

CAT-354 is being investigated in a Phase 2a study in subjects with moderate to severe disease not adequately controlled with existing therapy, using ACQ as the primary endpoint (NCT00873860). This study was scheduled for completion in August 2010, and is cited as completed, although no results have been announced to date. A similar trial of poorly controlled asthmatics is ongoing with another antibody, QAX576, also using ACQ as the primary endpoint and asthma exacerbations as a secondary measure (NCT01130064).

Another anti-IL-13 antibody, lebrikizumab, completed a Phase 2 study in allergen challenge over a year ago, but no results have been announced. The clinical trials database indicates an ongoing study of lebrikizumab in stable patients not taking ICS, using FEV1 as the primary endpoint (NCT00971035), which is a major departure from the more severe, poorly controlled asthmatics being studied in all other Th2 cytokine trials.

5.2.5. IL-3/IL-5/GM-CSF

ASM8 contains an antisense oligonucleotide targeting expression of the common β-chain to the receptors for IL-5, IL-3 and GM-CSF, together with an antisense oligonucleotide strand to mRNA for CCR3 expression. In the clinical allergen challenge model, ASM8 reduced EAR, LAR and sputum eosinophilia in a dose-dependent manner, with a maximal reduction in LAR and eosinophils of ~50% at 8 mg given once a day by inhalation [167]. Clearly the contribution of each component antisense is unknown, but contrasts with the lack of effect of anti-IL-5 strategies in this setting. A small Phase 2 follow-on study in 16 patients with moderate to severe asthma treated for 14 days in a crossover design was initiated in December 2010.

5.2.6. IL-9

MEDI-528, a humanized IgG1 antibody, is currently the only therapeutic in clinical trials for asthma that targets the IL-9-dependent pathway. This pathway is of interest, in part because it might represent a non-eosinophilic approach. MEDI-528 has completed two early Phase 2 trials. The first, conducted in patients with mild asthma subjected to allergen challenge (NCT00394654) was completed over 2 years ago. Although little information has been reported, it is suggested that this small study shows modest effects [168]. The second study of exercise-induced bronchoconstriction showed signs of improvements with MEDI-528, but the study recruited only 2 placebo subjects and 7 drug-treated before it was terminated, so statistical analysis could not be performed [169]. A Phase 2b study in 329 moderate to severe asthmatics who were poorly controlled with existing therapy is ongoing (NCT00968669) using the ACQ score as the primary endpoint.

5.3. Inhibitory cytokines

IFN-γ is the quintessential Th1 cytokine thought to redress the Th1/Th2 imbalance, and has been studied in patients with severe disease. Additionally Treg cell-derived cytokines, such as IL-10, also limit Th2 activity, as does IL-12 produced by stimulated macrophages and dendritic cells. Consequently, these cytokines have been employed in clinical studies to determine their direct effects on asthma.

A study of subcutaneously administered rIFN-γ given daily for 90 days to patients with severe asthma requiring oral steroids showed no effect on FEV1, PEFR or dose of prednisone required, despite reducing systemic eosinophils by 31% [170]. A follow-up study using nebulized rIFN-γ to increase the effective concentration in the airways also failed to show any clinical benefit [171].

IL-12 was studied in an allergen challenge setting with weekly s.c. injections for 4 weeks prior to allergen administration [172]. Most subjects developed an influenza-like syndrome and 4/19 subjects treated with IL-12 withdrew from the study – one with liver toxicity, two with cardiac arrhythmias and one with severe flu-like symptoms. IL-12 decreased blood and sputum eosinophils ~80 and 60%, respectively. Despite the obvious pharmacological activities, there was no effect on LAR or AHR.

Although lacking in detail, it has been reported that IL-10 also had no effect in asthma patients [18]. Thus none of the three cytokines capable of suppressing Th2 cell proliferation and activities showed any effect in clinical asthma, despite decreasing lung eosinophils.

5.4. Other cytokines

5.4.1. IL-17 and TSLP

AMG 827 is a mAb targeting the IL-17 receptor. Subcutaneously administered AMG 827 is under investigation in a Phase 2, 300 patient, 12-week study of asthmatics inadequately controlled by ICS, using changes in ACQ scores as the primary endpoint (NCT01199289).

Amgen also has a mAb, AMG 157 that blocks the interaction of TSLP with its receptor that has completed a multi-dose Phase 1 study (NCT00972179).

5.4.2. IL-2

Binding of antigen to the TCR stimulates secretion of IL-2 and expression of IL-2 receptors (CD25). BALF of patients with symptomatic asthma show increased levels of IL-2 and IL-2R, which correlate with the percent eosinophils and inversely with FEV1 [173]. Daclizumab, a mAb originally approved for preventing renal transplant rejection, binds to the α-chain of IL-2R, thereby preventing the biological activity of the cytokine. In patients with moderate to severe asthma taking ICS, daclizumab improved FEV1, while decreasing daytime symptom scores and β2-agonist use, and prolonging the time to an exacerbation [174]. Six of 88 patients treated with daclizumab had serious adverse events compared to 1

of the 27 placebo-treated subjects. However, the success of this therapy clearly warrants further study.

5.5. Chemokines

CCR3 is expressed predominantly on eosinophils, but also Th2 cells and lung mast cells [20,24]. GW766944 is an orally active competitive antagonist of CCR3. It is currently in a Phase 2 clinical trial in patients with mild to moderate asthma that have high sputum eosinophils, reduction of which is the primary endpoint (NCT01160224). Secondary endpoints include measures of lung function and AHR to methacholine, and while the study was scheduled for completion in March 2011, at the time of writing it is still ongoing.

CCR4 is also expressed on Th2 cells. AMG 761 (mogamulizumab), is a mAb against CCR4 that targets CCR4 positive T cells, leading to their depletion, and is currently in several early clinical trials. The biologic was licensed from Kyowa Hakko Kirin Co., Ltd. in 2008, where it was under investigation for T cell lymphomas.

5.6. Other chemotactic factors – LTB4

LTB4 is a potent chemoattractant for a number of leukocyte populations including neutrophils, Th1 and Th2 cells, and eosinophils. An orally active LTB4 antagonist, LY239111, was studied in the clinical allergen challenge setting where it had no effect on EAR, LAR or AHR to histamine, despite reducing the number of neutrophils (but not eosinophils or lymphocytes) in BALF [175]. The study of this LTB4 antagonist as a treatment for asthma has been discontinued.

5.7. Adhesion molecule antagonists

5.7.1. Integrins

The VLA-4 receptor (also known as $\alpha4\beta1$) has been the primary focus of attempts to suppress Th2 and eosinophil accumulation in asthmatic airways. At least seven different VLA-4 antagonists have entered clinical trials for asthma, however, none have shown efficacy. The Biogen/Merck compound, BIO-1211, Ranbaxy's RBx-7796 (Clafrinast), and two orally active antagonists, R411 (Roche) and TR14035 (Tanabe/GSK), have all disappeared from their respective company's pipelines.

The effects of three of the antagonists have been reported – HMR1031 (sanofi), IVL745 (sanofi) and GW559090X (Leiden University). Following administration by inhalation to mild atopic asthmatics in clinical allergen challenge studies, uniformly they had no effect on LAR, AHR or other markers of the asthmatic response [176–178]. These compounds also had minimal effect on sputum eosinophils, questioning the importance of VLA-4 in the pulmonary cellular infiltration.

Other integrins implicated in eosinophil recruitment [114] have also been targeted – so far without success. Efaluzimab, an anti-CD11a mAb developed to treat psoriasis, but subsequently withdrawn from the market, also had no significant effect on LAR in the clinical allergen challenge setting [179]. Attention has now shifted to another integrin, $\alpha M\beta2$ (CD11b/18), alone or in combination with VLA-4, as the primary instigator of lung eosinophilia associated with asthma [114]. However, the lack of concordance between animal models and human studies with the VLA-4 or CD11a antagonists makes extrapolating results with $\alpha M\beta2$ antagonists from animal models rather fraught.

5.7.2. Selectins

Bimosiamose (TBC1269) is a small molecule pan-selectin antagonist active against all three selectins. Although a single dose given intravenously had no effect [180], when given twice a day for 4 days by inhalation to mild asthmatics in an allergen challenge study, bimosiamose decreased the LAR by 50%, but had no effect on AHR [181]. However, in this allergen challenge study, the magnitude of the LAR on placebo was smaller than generally observed – only 13% rather than a 20 – 35% decrease in FEV1 usually observed. Whether a milder response might be easier to block is not clear. Bimosiamose is now targeted towards COPD, where selectins are involved in the homing of neutrophils to the lung and are thought to contribute to the disorder. Also of potential concern is the observation that mice with specific gene deletions resulting in deficiencies in more than one selectin are susceptible to infections, which could promote asthma exacerbations.

5.8. Pro-inflammatory cytokines

5.8.1. TNFα

TNFα levels in BALF are elevated in severe asthmatics [100]. Etanercept is a soluble TNFα receptor-IgG1 fusion protein that binds TNFα and prevents it acting on its receptor. Etanercept has no effect on pulmonary eosinophilia or AHR to methacholine in segmental allergen challenge in mild to moderate asthmatics [182]. In contrast, etanercept improved lung function measures such as FEV1 and PEFR, while reducing symptom scores and AHR in a small, uncontrolled, open-label study in 17 subjects with severe asthma [100]. Consequently, TNFα antagonism has been studied predominantly in more severe forms of the disorder. In a small, randomized crossover study of 10 severe, corticosteroid-refractory asthmatics treated with etanercept, improvements in FEV1 and AHR were also noted, together with a benefit in asthma-related quality of life (AQLQ) [183]. In a larger, double-blind parallel group study in 39 patients with severe corticosteroid-refractory patients etanercept produced a small improvement in the ACQ score, but not lung function measures, AQLQ, AHR or exacerbation rates [184].

The anti-TNFα mAb, infliximab, was administered to 18 subjects with moderate asthma taking ICS in a double-blind, placebo-controlled, parallel group study [185]. Changes in lung function (morning PEF measures), symptom scores or need for β2-adrenoceptor agonists did not differ between treated and placebo groups, but there was a decrease in "moderate exacerbations" [185]. Another anti-TNFα mAb called golimumab was studied in a large multi-center trial in patients with severe, uncontrolled asthma despite high dose ICS and long-acting β2-agonists [186]. No improvements in lung function, ACQ, AQLQ or the incidence of severe exacerbations was observed; moreover, one death and eight malignancies occurred in treated patients. The authors concluded that the risk–benefit profile was unacceptable in the broader severe asthmatic patient population, but held open the possibility that this therapeutic strategy might still have benefits in particular sub-groups or severe phenotypes [186]. For example, it was noted that patients with a greater β2-agonist induced reversal of airway narrowing had a >50% reduction in severe exacerbations.

5.8.2. IL-1

A brief comment indicates that the use of human recombinant IL-1Ra (anakinra) was ineffective in the treatment of asthma [187], although published details of any study are lacking.

5.9. Immunosuppressants

A number of drugs generally classified as "immunosuppressants" have been studied as adjuncts to the treatment of asthma, largely from the late 1980s to mid-1990s, in small clinical trials. Although acting through a variety of different mechanisms, they have in common the ability to inhibit T cell proliferation and/or functions, and are used to treat autoimmune disorders including

organ transplant rejection, rheumatoid arthritis, Crohn's disease, psoriasis and severe atopic dermatitis. Since these drugs are associated with some significant side effects and are frequently used where steroids are ineffective, studies in asthma have generally targeted the severe asthmatic taking prednisone with a view to reducing their requirements for the oral steroid. Although some reduction of oral steroid needs are often observed, there are generally few improvements in lung function, and a Cochrane analysis for those agents studied most widely – methotrexate, cyclosporin, azathioprine and gold compounds – concluded that any changes were small and of questionable clinical significance [188–191]. To this list can be added intravenous immunoglobulin (IVIG), which showed some steroid-sparing effect in severe patients, but little other benefit [192].

Given the proposed central role of CD4+ lymphocytes in asthma, keliximab, the chimeric mAb to CD4, was investigated in severe, corticosteroid-dependent asthmatics. Drug treated patients showed no significant improvements in FEV1 or symptom scores when compared to placebo [193], despite a decrease in T cell proliferation and a reduction in CD4+, but not CD8+, T cells [194].

6. Clinical findings: a perspective

Analysis of results from asthma trials relies on information available in the public domain, and it is highly likely that other drugs targeting various facets of the immune response may also have failed, but the information was never released. Regardless, data is available on 39 pharmaceuticals studied in clinical asthma settings, directed at 23 separate targets within the immune cascade (Table 1), each backed by substantial basic research support of their potential efficacy, such that a valid interpretation of the translational success of the developed model can be established (Table 3). Various additional compounds, based on the same preclinical paradigm, are currently in Phase 2 (Table 2) and Phase 1 studies. Of the 39 drugs, there have been 6 qualified successes (two anti-IL-5 mAbs, mepolizumab and reslizumab; the anti-IL-4Rα protein, pitrakinra; the IL-2Rα mAb, daclizumab; the dual antisense compound ASM8 that targets expression of IL-5Rβ and CCR3, and the pan-selectin antagonist, bimosiamose) while the outcome for 3 others (two anti-IL-13 mAbs, CAT-354, and lebrikizumab, and the anti-IL-9 mAb, MEDI-528) is currently designated as "undecided", based on a lack of news despite completion of clinical studies. The other 30 products have been discontinued. Moreover, bimosiamose, which showed some activity in the clinical allergen challenge setting, is now directed towards COPD rather than asthma. Similarly, daclizumab is now in Phase 3 clinical studies for relapsing/remitting multiple sclerosis, and has not been pursued for asthma since 2006. The side-effect profile and requirement for intravenous administration might have contributed to this decision. That leaves 4 drugs out of the 39 that are known currently to be advancing for the treatment of asthma.

These successful new therapeutics are considered "qualified" because they show limited activity, and/or are at an early stage in their development. To date, ASM8 has only been tested in the clinical allergen challenge model, and requires validation in a "real world" asthma setting. Three of the successful pharmaceuticals (mepolizumab, reslizumab and pitrakinra), appear to target the same sub-group of severe asthmatics with high sputum eosinophils, and by decreasing the risk of exacerbations, not the underlying impairment and symptoms. Since severe asthmatics with high sputum eosinophils represent ~1–2% of the asthma population, there is concern that the basic research paradigm developed might identify new therapeutics for one particular, relatively uncommon, asthma phenotype. In contrast, IL-2 was not touted as a critical cytokine in the basic research Th2 model, so the success of daclizumab was rather unexpected. Daclizumab was originally developed and approved as an immunosuppressant to prevent kidney transplant rejection, and is the *sole* example of a drug that inhibits the immune system having effects on asthma symptoms and measures of lung function – indices of the ongoing impairment rather than the future risk of exacerbations. Analysis of the results with the successful compounds, combined with the disappointing outcome for the majority of products tested, indicates that despite the remarkable progress in the detailed mapping of the sequence of pathophysiological events leading to "asthma" in basic research (predominantly murine) models (Fig. 1), these immunological mechanisms are not critically important to most asthma sufferers.

This list of products pertains exclusively to those with a mechanism of action solely related to the immune response defined by the preclinical studies, and described in the foregoing section. Not included are products that have additional activities outside of this standard immunological paradigm, including selective phosphodiesterase inhibitors, PGD2 receptor antagonists, FLAP inhibitors, and iloprost, since a mechanism of benefit related exclusively to an anti-inflammatory effect cannot be discerned readily. Moreover, since the immune response is posited centrally as the cause of asthma, often the benefits of any compound are rationalized in terms of this immunological paradigm, when their true effects might lay elsewhere. Absent an effect on the established immunological mechanisms, these drugs may be rejected inappropriately.

Borish [195] insightfully defines the three phases of drug development for asthma as: first, an intellectual argument that a given mediator is central to asthma pathogenesis; followed by the murine phase that unambiguously confirms the singular importance of that mediator; and then the human phase in which the same intervention fails to produce any therapeutic benefit. Indeed, when reviewing the preclinical (largely murine) literature, it is remarkable how many cellular mediators and products are deemed "essential," "critical," "crucial," or "obligatory." When contrasted with the clinical results, such hyperbole is rendered meaningless. While a debate over the value of research asthma models has been ongoing for decades, it is legitimate to ask how many clinical failures are required before the scientific paradigm of asthma as a Th2-mediated disorder is rejected?

Karl Popper, widely regarded as one of the greatest philosophers of science of the 20th century, argued that a scientific theory cannot be confirmed by positive outcomes, but one single counter-example is logically decisive, and shows that the theory is false and requires revision [196]. The report card for the translational success of the asthma paradigm developed over the last three decades must show a failing grade at this time.

While most asthma researchers recognize that the original concept of asthma being solely a Th2-mediated disorder is an oversimplification, nonetheless, it remains the underlying tenet, with added refinements and embellishments [18,19,21]. Some limitations of asthma models are also acknowledged, and attempts have been made to improve the models [19,21]. Differences in response between strains of mice, sensitization protocols and antigens used have been noted, although they all beg the question of which modifications, if any, bring the model any closer to the human condition, and require persuasion that they are not akin to merely re-arranging the deckchairs on the Titanic. This is not exclusively a mouse issue (or even unique to asthma models) – studies in rats, guinea pigs and even primates have been interpreted along similar immunological lines as they all use artificial sensitization techniques. It should be recalled that these are the same models that led to clinical trials with antagonists for histamine, platelet-activating factor, substance P and neurokinins, inhibitors of thromboxane synthase and various cathepsins, as well

Table 3
Compounds/biologics targeted at different aspects of the immune response paradigm developed in basic research are compared for activities in animal models of asthma with clinical outcomes in asthma patients. Note the lack of any association between beneficial effects in animal vs. clinical studies.

Antagonism of	Animal models			Clinical setting	
	Airway inflamm^{n}	AHR	Refs	Clinical event (1° endpoint)	Refs
Initiation/co-stimul^{n}					
TSLP	Decrease	Decrease	[28,30]	Ongoing	
CTLA-4	Decrease	Decrease	[35]	Ongoing	
OX40/OX40L	Decrease	?	[38]	Ongoing	
"Th2" cytokines					
IL-4	Decrease	No change	[4,45]	No effect	[149–152]
IL-4R	Decrease	Decrease	[4,50]	+[a]	[165]
IL-13	Variable	Decrease	[4,19]	No effect?	[163,166]
IL-5	Decrease	Variable	[48]	+[a]	[158,159]
IL-5Rα	Decrease	Variable	[52]	Ongoing	[161]
IL-9	Variable	Variable	[19,56]	No effect/ongoing	[168,169]
IL-3/5/GMCSF Rβ-chain[d]	Decrease	Decrease	[53,54]	+[b]	[167]
Other cytokines					
IL-2	Decrease	Decrease	[67]	+	[174]
IL-17	Variable	Variable	[19,77]	Ongoing	
Inflammatory cytokines					
IL-1	Decrease	Decrease	[95]	No effect	[187]
IL-6	Variable	Variable	[99]		
TNF	Decrease	No change	[103]	No effect	[185,186]
Adhesion/chemotaxis					
VLA-4	Decrease	Decrease	[114]	No effect	[176–178]
CD11a	Decrease	?	[201]	No effect	[179]
Selectin	Decrease	Decrease	[118]	+[c]	[181]
CCR3	Decrease	Decrease	[105,106]	Ongoing	
CCR4	Decrease	Decrease	[108,109]	Ongoing	
LTB4	Decrease	?	[110,113]	No effect	[175]
Administration of suppressive cytokines					
IFN	Decrease	?	[61]	No effect	[170,171]
IL-10	Decrease	Decrease	[72,73]	No effect	[18]
IL-12	Decrease	Decrease	[69,70]	No effect	[172]

Animal studies: a decrease in airway inflammation (inflamm^{n}) or AHR is regarded as a positive outcome. Clinical studies: + is a positive outcome.
[a] Biologics effective only in severe asthmatics with airway eosinophilia, where they reduce the incidence of asthma exacerbations.
[b] ASM8 has only been studied in an allergen challenge setting in mild asthmatics to date.
[c] Bimosiamose has only been studied in an allergen challenge setting in mild asthmatics to date.
[d] ASM8 is a combination of antisense oligonucleotides to the IL-5Rβ *and* CCR3.

as prostaglandin analogs, to name but a few. The translational history does not inspire confidence.

Asthma is not a single disease, but a variety of disorders, each with a breathing difficulty as the principal clinical sign. Diverse asthma phenotypes are being described, that respond variably to different treatments. This is exemplified by the ability of anti-IL-5 mAbs and pitrakinra to decrease asthma exacerbations only in a severe eosinophilic phenotype. Contrast the different clinical phenotypes with the singular, reductive, unifying mechanism developed from the basic research studies, and it is not surprising that this paradigm does not fit many asthma patients. This is a fundamental disconnect between basic research and clinical understanding that must be resolved for research and drug discovery to be more effective.

While one interpretation of the poor translational aspects of basic research models to the clinical condition is that inflammation is not the critical underlying event in asthma, such a conclusion might be premature. The results with mepoluzimab, reslizumab and pitrakinra indicate that there is a subset of patients where the risk of exacerbations is linked to sputum eosinophils, which was an endpoint selected, in part, from observations that treating similar patients with ICS, specifically dosed to reduce sputum eosinophilia, likewise decreased the incidence of exacerbations [197,198]. While these more severe, eosinophilic patients might be a small sub-group (representing only a fraction of all asthma patients), they may characterize one extreme of a larger group. The identification of a "Th2 high" phenotype with elevated eosinophil counts in BALF, which represents a significant proportion (~50%) of asthmatic subjects [199], and the finding that these patients rather than the "Th2 low" phenotype benefit from treatment with ICS, suggests that Th2-driven inflammation is relatively common, but responsive to current treatment. In clinical trials new therapeutic agents are generally given on the background of "standard of care" – in this case including ICS. There may be no additional gain from adding another drug targeting the same Th2-eosinophil nexus except in a small segment of patients who respond relatively poorly to ICS, perhaps because of superimposed pharmacogenomic differences, or who represent an extreme end of the spectrum of more severe eosinophilic subjects that require enhanced treatment. Notably, basic research studies evaluating new potential therapeutics are not performed in the presence of ICS, so any mutual overlap of activities would be missed. Sadly for drug discovery efforts, if this interpretation has any validity, then it would be apt to conclude that the predominant finding from the last 30 years of basic research is to reaffirm the value of ICS. Profiling the "Th2 low" phenotype, which represents the other 50% of subjects in the study [199], and determining why they are poorly responsive to ICS, could be more valuable.

Additional reasons why it might be premature to disregard airway inflammation as a key event in asthma is that clinical events could be linked to an inflammatory cell or mechanism that has yet to be targeted, or the inflammation underlies an aspect of the disorder not addressed in clinical trials. For example, there are several lines of evidence implicating airway inflammation in long-term remodeling and progressive loss of airway function [200]. Such remodeling occurs over years, so is not a basis for demonstrating the activity of a drug in early clinical studies. However, despite these qualifications, the uniform lack of effect of all the therapeutic modalities targeting

the immune/inflammatory component, apart from the anti-IL-2Rα mAb, daclizumab, on asthma symptoms, lung function and need for β2-agonists, indicates that the contribution of airway inflammation to these clinical events merits revising. The separation of effects on exacerbation rate (future risk) versus daily symptoms and lung function (current impairment) seen with mepolizumab, reslizumab and pitrakinra, is intriguing, and indicates these biologics would be useful additions to, but not replacements for, existing therapies.

Certain aspects of clinical research compound the problem of poor translation of science into therapeutics. It is clear that in asthma there are a host of mediators produced, and multiple cell-types that make their way to the lungs, or, already resident in the airways, undergo dramatic changes. As a result, it is not surprising when the latest cell/mediator *du jour* is found to be present in tissue/BAL/sputum. As this commentary has attempted to highlight, all of the pharmaceutical products that entered clinical trials did so not only based on data from basic research models, but also evidence for the target (e.g. cytokine) being increased in the airways of asthmatics. But these events cannot be measured in isolation. At a very basic level, an increase in only one mediator might be meaningful, but if it is one of a hundred mediators that are increased, and it is increased to a lesser extent than the other 99, then clearly that could merit a completely different interpretation. But quantity alone can be misleading too. Localization, potency, as well as genetic polymorphisms and epigenetic factors that influence responses to mediators and drugs are a few of the multiple confounding factors that need to be considered. The trend in asthma research has been reductionistic, to look at each component in isolation, in a setting that is far from isolated pathophysiologically.

7. Conclusions

Experimentalists have been remarkably successful in mapping the pathway of events that follow antigen sensitization and challenge in exquisite detail. From the model that has been developed critical sites of regulation can be identified involving recognition receptors, co-stimulatory molecules, key transcription factors, cytokines, chemokines, adhesion molecules, and other mediators. A comprehensive, detailed, unifying model of the events that translate into asthma has been developed. Based on this compelling model, a number of these key targets have been selected and therapeutic modalities developed, with excellent rationale and evidence of efficacy in animal models. Unfortunately, the clinical results have failed to match expectations. The translational success of the basic research model must be accorded a failing grade at this time.

The focus of asthma research for the last 30 years has been on the mechanisms of airway inflammation and the identification of key cells, cytokines and pathways. To date, the result of all this effort is the identification of three compounds directed at 2 targets that decrease the risk of exacerbations in a small subset of asthmatics – those with the more severe disorder accompanied by airway hyper-eosinophilia; and two promising compounds. One of the latter, daclizumab, used to treat a broader moderate to severe asthmatic patient group and alleviate several features of the daily asthma condition, has not been continued; while the second, ASM8, is at an early stage of development and its target population or clinical profile have yet to be identified. For the bulk of asthma sufferers, the last 30 years of work has yet to translate into anything meaningful or tangible.

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