PATHOGENIC ADVANCES AND THERAPEUTIC PERSPECTIVES FOR EOSINOPHILIC INFLAMMATION

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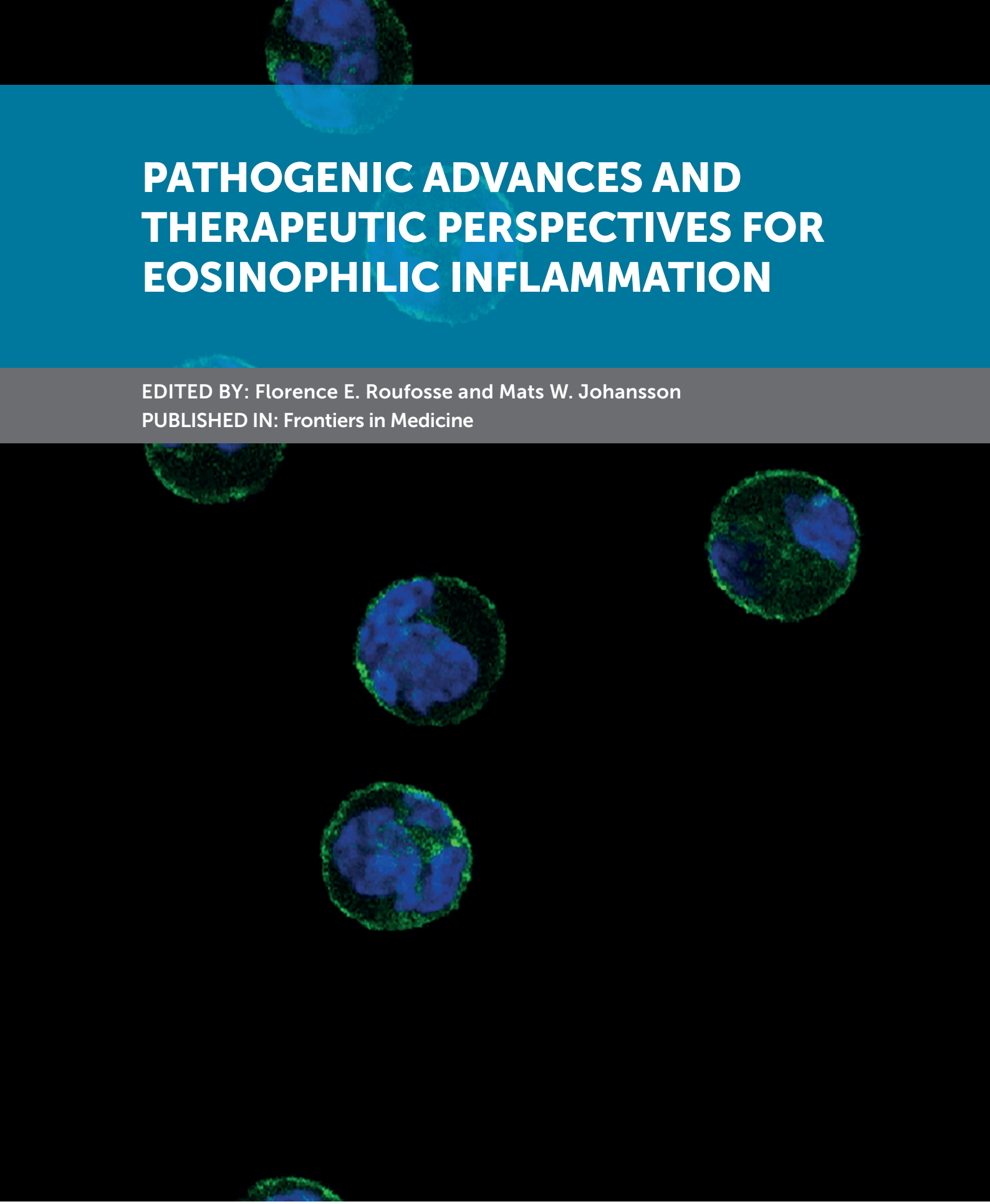

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PATHOGENIC ADVANCES AND THERAPEUTIC PERSPECTIVES FOR EOSINOPHILIC INFLAMMATION

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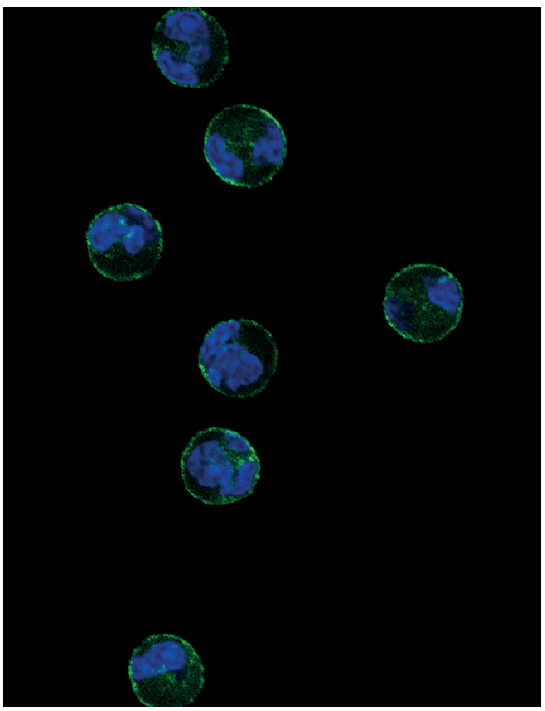

Immunofluorescence confocal microscopy image of cytospun purified blood eosinophils, stained for PSGL-1 (P-selectin glycoprotein ligand-1, green) and nuclei stained with DAPI (blue). Image: Mats W. Johansson.

With the recent approval of the first eosinophil-depleting therapeutic agents targeting the IL-5 pathway for treatment of severe eosinophilic asthma, eosinophils and eosinophilic disorders are in the limelight. Indeed, setbacks during clinical development of these compounds have revealed how much remains to be known about eosinophil biology in vivo, and have nurtured profuse research both on basic eosinophil biology and on pathogenic disease mechanisms, in order to better delineate the most meaningful targets for innovative therapeutic strategies. On one hand, variable degrees of eosinophil depletion observed in some compartments during IL-5-targeted treatment indicate that certain eosinophil subsets may not rely on this cytokine and/or that other important pro-eosinophilic mediators and signaling pathways are operative in vivo. On the other hand, it is increasingly clear that disorders involving eosinophils such as asthma are the final outcome of complex interactions between diverse cell types and mediators, beyond eosinophils and IL-5.

These include type 2 helper T (Th2) cells and innate lymphoid cells, mast cells, and a variety of factors that either activate eosinophils or are released by them. Although a considerable amount of research has focused on asthma because it is a common condition and because management of severe asthma remains a major challenge, several rare eosinophilic disorders with more homogenous features have proven to be extremely useful models to reach a better understanding of the involvement of eosinophils in tissue damage and dysfunction, and of the micro-environmental interactions operating within the complex network of eosinophilic inflammation. Unraveling this interplay has resulted in advances in the development of molecular tools to detect disease subsets and to monitor therapeutic responses, and in identification of promising new therapeutic targets.

This Research Topic dedicated to eosinophilic conditions covers aspects of the biology of eosinophils and closely related cells of particular relevance for drug development, reports on translational research investigating pathogenic mechanisms of specific eosinophilic disorders in humans that will likely result in significant changes in the way patients are managed, and presents an overview of the current advancement of targeted drug development for these conditions, with a special focus on asthma.

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EDITORIAL
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Editorial: Pathogenic Advances and Therapeutic Perspectives for Eosinophilic Inflammation

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Keywords: eosinophils, interleukin-5, asthma, hypereosinophilic syndromes, eosinophilic esophagitis, mastocytosis

Editorial on the Research Topic

Pathogenic Advances and Therapeutic Perspectives for Eosinophilic Inflammation

With the recent approval of the first eosinophil-depleting therapeutic agents targeting the Interleukin-5 (IL-5) pathway for treatment of severe eosinophilic asthma, eosinophils, and eosinophilic disorders are in the limelight. Setbacks during clinical development of these compounds have revealed how much remains to be known about eosinophil biology *in vivo*, and have nurtured profuse research both on basic eosinophil biology and on pathogenic disease mechanisms, in order to better delineate the most meaningful targets for innovative therapeutic strategies. On one hand, variable degrees of eosinophil depletion observed in some compartments during IL-5-targeted treatment indicate that certain eosinophil subsets may not rely on this cytokine and/or that other important pro-eosinophilic mediators and signaling pathways are operative *in vivo*. On the other hand, it is increasingly clear that disorders involving eosinophils such as asthma are the final outcome of complex interactions between diverse cell types and mediators, beyond eosinophils and IL-5. These include type 2 helper T (Th2) cells and innate lymphoid cells, mast cells, and a variety of factors that either activate eosinophils or are released by them. Although a considerable amount of research has focused on asthma because it is a common condition and because management of severe asthma remains a major challenge, several rare eosinophilic disorders with more homogenous features have proven to be extremely useful models to reach a better understanding of the involvement of eosinophils in tissue damage and dysfunction, and of the micro-environmental interactions operating within the complex network of eosinophilic inflammation. Unraveling this interplay has resulted in advances in the development of molecular tools to detect disease subsets and to monitor therapeutic responses, and in identification of promising new therapeutic targets. Precision medicine for management of eosinophilic disorders has now become a realistic endeavor.

This Research Topic dedicated to eosinophilic conditions comprises 26 articles, including reviews, mini-reviews, and perspective as well as hypothesis and theory articles. Their scope ranges from basic immunological research to clinically oriented topics, all chosen to stimulate curiosity and offer a wider and more comprehensive understanding of the numerous actors involved in these disorders. Translational aspects of research in the field of eosinophilic inflammation are highlighted throughout the Topic. Recent progress in the understanding of eosinophil biology and heterogeneity is reviewed, as well as insights into the contributions to and regulation of eosinophil trafficking and recruitment in asthma and other eosinophilic or allergic diseases by various families

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of mediators and their receptors, and by interactions with platelets. Eosinophil interactions with other lineages, including Th2 cells and mast cells, in inflammatory disorders are also addressed. Importantly, the Topic covers aspects of particular relevance for drug development, reporting on translational research investigating pathogenic mechanisms of specific eosinophilic disorders in humans, including asthma, that have greatly implemented modern classification of these disorders, and will likely result in significant changes in the way patients are managed through a more personalized approach to prognostication, prediction of treatment responses, and targeted therapy.

The first collection of papers presents the state-of-the-art in selected fields of basic eosinophil biology where significant progress has been made, including development, translation, and proteomics, offering a broad perspective on potential eosinophil-expressed targets. Fulkerson reviews insights into the mechanisms of gene regulation during eosinophil lineage commitment, differentiation, and maturation. She describes a model according to which classes of transcription factors (including GATAs, C/EBPs, PU.1, and XBP1) cooperate to direct eosinophil development and discusses the potential for therapeutic intervention. Accumulating evidence, reviewed by Johnston and Bryce, indicates that IL-33, usually considered an epithelial-derived cytokine that orchestrates allergic inflammation and contributes to type 2 immunity, together with its receptor ST2 also have roles in regulating eosinophil development. In addition, IL-33 is a potent activator of mature eosinophils. These advances should impact our understanding of how therapeutic targeting of this pathway may modulate disease. Esnault et al. provide an overview on protein translation within eosinophils, and its regulation by intracellular signaling. They address mRNA post-transcriptional regulation and focus on the role of IL-3, which, unlike the other IL-5 family cytokines IL-5 and GM-CSF, drives sustained signaling in eosinophils and increased translation of a subset of mRNAs, including semaphorin 7A and Fcγ receptor II (CD32), which may have clinical relevance in terms of eosinophil priming/activation *in vivo*. Further, they discuss mechanisms regulating mRNA-binding protein activity in eosinophils and the potential therapeutic targeting of these signaling pathways. As they indicate, mRNA and protein levels do not always correlate. Proteomic analysis of human eosinophils, using liquid chromatography coupled to tandem mass spectrometry, has recently identified and quantified >7,000 proteins and is reviewed by Mosher et al. They give examples of the power of such analysis to provide novel information on isoforms of proteins, including eosinophil STATs. Further, they describe how isobaric labeling has identified 220 phosphosites that change significantly upon acute eosinophil activation with IL-5. Finally, and importantly for this Research Topic, they discuss how these methods may prove valuable to address whether certain eosinophil proteins are altered or predict therapeutic outcomes in patients with eosinophilic diseases.

The next set of articles deals with the roles of eosinophils in asthma, regulation of eosinophil recruitment by multiple agonists and receptors, and eosinophil effector functions. McBrien and Menzies-Gow give a general overview of the eosinophil and of the current understanding of the (possible) roles of eosinophils in key asthma processes. These include evidence of eosinophil contributions to exacerbations and airway remodeling as well as mechanisms by which eosinophils may promote airway hyperresponsiveness and mucus secretion. In addition, the immunomodulatory roles of eosinophils are discussed. In this line, it is now well established that eosinophils are heterogeneous, with varying membrane-expressed receptors and secreted products. The characterization of homeostatic versus inflammatory eosinophils proposed by Marichal et al. in this Topic represents a major advance in understanding eosinophil heterogeneity and accounts for some of the intriguing findings made previously in the field of eosinophil research. Variable expression of the IL-5 receptor by certain homeostatic eosinophils is particularly relevant for treatment responses in anti-IL-5(R) treated patients. Larose et al. review eosinophil chemoattractants with an emphasis on eotaxins, other chemokines, and their receptors. Chemoattractants for type 2 innate lymphoid cells (ILC2s) are also evoked, emphasizing however that mechanisms for recruitment of these cells are relatively poorly defined so far. Although historically the focus has been on protein-protein interactions in biological systems, protein-carbohydrate interactions has recently received greater recognition. O'Sullivan et al. describe how lectin-glycan interactions can modulate eosinophil functions, including recruitment, survival, and inflammation. Their primary focus is on Siglec-8, expressed on human eosinophils, but selectins and their ligands, and other siglecs are also discussed. Finally, they consider potential therapeutic exploitation of these interactions in eosinophilic diseases, e.g., the Siglec-8 pathway in inducing cell death. Galectins, one of the lectin families, are expressed by various cells including eosinophils. Rao et al. review galectins that regulate eosinophil recruitment, activation, and apoptosis in allergic asthma, and are pro- (e.g., galectin-3, by interacting with α_4 integrin) or anti-inflammatory (galectin-1). They discuss their potential utility as therapeutic targets. In addition, human but not murine eosinophils contain galectin-10 (Charcot-Leyden crystal protein), a potential biomarker for eosinophilic inflammation. Prostaglandins and leukotrienes are other families of molecules that can be pro- or anti-inflammatory. Peinhaupt et al. focus on prostaglandins (PG) D_2 and E_2, prostacyclin I_2, and their receptors on eosinophils. PGD_2 activates eosinophils, whereas PGE_2 and I_2 suppress activation. They summarize potential drug interventions, including antagonists of the PGD_2 receptor DP2 (CRTH2). Such antagonists have produced improvements in lung function in subsets of asthmatic patients and some improvement in eosinophilic esophagitis (EoE). Thompson-Souza et al. review the cysteinyl leukotrienes LTC4, D4, and E4, which have various activities on eosinophils, in light of development of therapeutic compounds targeting their receptors. They discuss the two cysteinyl leukotriene receptors expressed by eosinophils pointing out that it has recently been recognized that they are also present and functional in the membrane of eosinophil free granules, raising the question whether free granules may be therapeutic targets beyond intact eosinophils. Related to eosinophil activation and recruitment,

the activation status of eosinophils, as assessed by eosinophil surface proteins that are potential biomarkers, is described by Johansson. Circulating eosinophils may be non-activated or pre-activated (sensitized or "primed") and their β_1 integrin activation is associated with aspects of disease in non-severe asthma. β_2 integrins on blood, but not airway, eosinophils, respond to intervention with anti-IL-5 mepolizumab. A model of eosinophil activation status in the circulation and the airway in asthma is presented; however the potential relevance of these biomarkers in eosinophilic diseases other than asthma requires future exploration. Tissue remodeling is a key feature of eosinophilic inflammation in a number of type 2 immune diseases. Nhu and Aceves review data and concepts on the pathogenesis of remodeling and fibrosis, primarily in EoE, including cytokines, eosinophils, and other immune cells, with relevant parallels in asthma. Additionally, they focus on how emerging therapies may reduce remodeling in a subset of patients.

Dealing with complex eosinophilic conditions is not achievable without considering the factors that interact with and/or are associated with eosinophilia. The following group of papers emphasizes some of the recent data and concepts relating to such factors. Like eosinophils, Th2 cells are heterogeneous, with pathogenic effector Th2 cells (peTh2) representing the most terminally differentiated subset, showing an exacerbated capacity to produce IL-5 in addition to IL-4 and IL-13, both produced earlier in the maturation process of Th2 cells. The phenotypic and functional characteristics of these cells are reviewed by Mitson-Salazar and Prussin, who argue that the critical role shown to be played by these cells in eosinophilic gastroenteritis may be operative in other eosinophilic conditions. Specific membrane-expressed molecules on these upstream inducers of eosinophilic inflammation may prove to be interesting targets for future therapeutic intervention. IL-13 is a cytokine involved in the pathogenesis of asthma and other type 2 immune conditions, eliciting mechanisms that promote eosinophil trafficking. Doran et al. provide a perspective on these roles of IL-13 in asthma and other eosinophilic disorders. They depict the IL-13 (and IL-4) receptors and antibodies blocking IL-13 or IL-13/IL-4 receptors, and describe the ongoing clinical trials with these antibodies. Another component interacting with eosinophils, e.g., in asthma, is the platelet. Although the importance of platelet activation during hemostasis is well understood, it is now also recognized that platelets can be activated and function in a distinct manner during inflammation; evidence indicates that they are critical in the pathogenesis of allergic diseases. Shah et al. explore non-thrombotic platelet activation in the context of allergy and the association of platelets with eosinophils, including conclusions drawn from platelet depletion experiments in animal models, as well as how these phenomena may yield novel therapeutic targets. The important interactions between eosinophils and mast cells, which are almost invariably present together in inflamed tissue (composing the "allergic effector unit"), are described in detail by Galdiero et al. in this Topic. In addition to the numerous direct interactions between these cells, possible Th2 cell-dependent indirect interactions may be relevant in eosinophilic disorders, all contributing to certain aspects of treatment responses. This paper closes the biological part of this Research Topic on eosinophilic conditions.

The final series of papers is clinically focused, and is meant to illustrate how translational research can contribute to improved understanding not only of disease mechanisms, but also of eosinophil functions and interactions. We begin with recent disease classifications incorporating molecularly or immunologically defined disease variants. The first two papers illustrate the complex and constantly evolving interplay between advances in pathogenic understanding and refinement of classification schemes. Current definitions and categories of asthma and hypereosinophilic syndromes are summarized by Svenningsen and Nair and Kahn et al., respectively, showing how they have been implemented in clinical practice and improved patient management considerably. However, their limitations are highlighted and perspectives for further amelioration are discussed. These limitations are mainly related to numerous gaps in our understanding of underlying pathogenic mechanisms, namely at the molecular level, with rigorous but empirical clinical observations supporting most current definitions. EoE has been chosen for this Topic as a model to show how rigorous application of disease-defining criteria has resulted in the constitution of a large and fairly homogenous collection of patients, paving the way to pathogenic breakthroughs that are likely to translate into major therapeutic advances in the coming years. Collins et al. describe the slow but rewarding process that has led to consensual determination of thresholds for tissue "hyper"-eosinophilia in different compartments of the digestive system. At the level of the esophagus, this has allowed for selection of patients for large-scale pathogenic studies. Elucidation of the transcriptome of EoE has delineated novel candidate targets for future drug development and has led to the development of the "EoE diagnostic panel" (EDP), the first application of a molecular approach to diagnosis in the setting of an allergic disorder. The EDP, described by Wen and Rothenberg herein, is now available for clinical use as a commercialized test. Its widespread use has revealed further heterogeneity within EoE, and it is hoped that this tool will allow for a personalized approach to future therapeutic decision-making, on the basis of specific molecular signatures in individual patients and the availability of an increasing number of targeted treatment options. Furthermore, genes whose transcription levels change with effective therapy may prove useful as future biomarkers of disease activity in eosinophilic disorders, for which there is much need. Khoury et al. provide an overview of the currently available biomarkers used to assess hypereosinophilic disorders, showing how improved understanding of pathogenesis (e.g., in EoE) has delivered the few robust markers that have been validated so far. For the majority of disorders, biomarkers enabling diagnosis of disease variants, and/or predicting disease severity and monitoring disease activity are lacking.

There is increasing interest for investigation of biomarkers in the setting of clinical trials with targeted therapy, in hopes of improving future selection of patients for tailored treatment. Two papers in this Topic review the use of monoclonal antibodies directed against IL-5 or its receptor and against IgE in

eosinophilic disorders. Interestingly, elevated eosinophil counts are predictive of treatment response to both compounds in asthmatic subjects. This appears logical in that the cytokines responsible for induction of eosinophils and IgE production (i.e., IL-5 and IL-4/IL-13, respectively) are generally produced together by type 2 lymphocytes. Moreover, both eosinophils and IgE contribute jointly to inflammation and disease manifestations, and eosinophils express IgE receptors, so it is legitimate to explore how these factors interact *in vivo* in treated subjects. Roufosse reviews clinical trials targeting the IL-5 pathway and summarizes transversal data across these studies on biomarkers that may predict treatment responses, and on how other mediators and cell types, namely mast cells, are impacted by treatment. This paper also describes how the slow but determined development of anti-IL-5 antibodies, with careful *post-hoc* assessment of data collected during clinical trials, has resulted in a better understanding of pathogenic mechanisms underlying specific aspects of diseases under study, leading to improved design of subsequent trials, and ultimately, approval of new first-in-class drugs. Stokes reviews the studies evaluating efficacy of anti-IgE treatment, showing that eosinophilia decreases during treatment through mechanisms that remain largely unexplored. Some of the favorable effects of anti-IgE treatment on allergic disease may therefore actually be related to indirect effects on eosinophils, either through their depletion or their decreased priming *in vivo*. The article by Vaes et al. approaches mast cell targeted therapy and has been included in this Topic to provide insight on the different levels at which therapeutic intervention is possible in a hematological disorder where, like in hypereosinophilic syndromes, cell-mediated toxicity is often more of a concern than tumor burden. Drug development in mastocytosis and related disorders is very broad, including molecular targets, signaling machinery, mediator interception, and apoptosis, and may inspire new avenues of thought for predominantly eosinophilic diseases. Finally, the interesting pathogenic complexity of a secondary hypereosinophilic disorder, drug-reaction-with-eosinophilia-and-systemic-symptoms (DRESS), is reviewed by Musette and Janela. The combined existence of genetic predisposition, environmental exposure to an offending agent (a drug), and viral reactivation concurs toward the rapid development of marked blood and tissue eosinophilia, contributing to vital organ damage and death in some cases. The uncontrolled immune activation involves not only eosinophils but also cytotoxic CD8 T cells whose pathogenic contribution to other eosinophilic conditions has barely been explored and may be largely underestimated.

To conclude, we are very grateful to our colleagues including researchers, physicians, and clinical investigators who have contributed to this Research Topic. We believe that these articles offer an interesting and translational perspective on basic eosinophil biology, with clinical applications in diagnosis and treatment of eosinophilic conditions. They illustrate particularly well how combined experimental and clinical efforts to break down heterogeneous human diseases into mechanistic subsets can be rewarding and can translate into major improvements in patient management and outcome. We hope the contents of this Topic will further stimulate transversal thinking in the exciting field covered by this e-book.

AUTHOR CONTRIBUTIONS

FR and MJ designed and drafted this manuscript, revised it, and approved the final version.

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MINI REVIEW
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Transcription Factors in Eosinophil Development and As Therapeutic Targets

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Dynamic gene expression is a major regulatory mechanism that directs hematopoietic cell fate and differentiation, including eosinophil lineage commitment and eosinophil differentiation. Though GATA-1 is well established as a critical transcription factor (TF) for eosinophil development, delineating the transcriptional networks that regulate eosinophil development at homeostasis and in inflammatory states is not complete. Yet, recent advances in molecular experimental tools using purified eosinophil developmental stages have led to identifying new regulators of gene expression during eosinophil development. Herein, recent studies that have provided new insight into the mechanisms of gene regulation during eosinophil lineage commitment and eosinophil differentiation are reviewed. A model is described wherein distinct classes of TFs work together *via* collaborative and hierarchical interactions to direct eosinophil development. In addition, the therapeutic potential for targeting TFs to regulate eosinophil production is discussed. Understanding how specific signals direct distinct patterns of gene expression required for the specialized functions of eosinophils will likely lead to new targets for therapeutic intervention.

Keywords: hematopoiesis, eosinophilopoiesis, transcriptional regulation, eosinophil development, eosinophil lineage commitment

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INTRODUCTION

Eosinophils differentiate in the bone marrow from an eosinophil lineage-committed progenitor (EoP) that is derived from the granulocyte/macrophage progenitor (GMP) in mice and the common myeloid progenitor or an upstream multipotent progenitor in humans (1, 2). Cell fate choices, including lineage commitment, are specified by the action of primary, or lineage-determining, transcription factors (TFs) and then reinforced by induction of secondary TFs that orchestrate gene expression and lineage commitment and differentiation. TF concentrations can be important, as lineage-determining TFs can antagonize each other's activity (3, 4). We have recently shown that markedly more transcriptome changes (1,199 genes) are associated with eosinophil maturation from the EoP than with eosinophil lineage commitment (EoP from GMP, 490 genes), highlighting the greater transcriptional investment necessary for terminal differentiation (5). These dynamic changes in gene expression during eosinophil development included a repertoire of TFs, many of which had never previously been associated with eosinophil development (5). New information from genome-wide and single-cell RNA sequencing (scRNA-seq) studies have built upon well-established models of transcriptional regulation of eosinophilopoiesis. The molecular regulatory network that yields functional, mature eosinophils from EoPs is slowly being delineated.

Defining how eosinophil production is regulated is critical to understanding how dysfunction of the immune response results in eosinophil overproduction and will likely lead to new eosinophil-targeting therapeutics.

EOSINOPHIL LINEAGE COMMITMENT

The first stage in eosinophil development is commitment to the eosinophil lineage by a myeloid multipotent progenitor to generate an EoP (**Figure 1**). The EoP is identified *via* surface expression of CD34, interleukin 5 (IL-5) receptor alpha (IL-5Rα, a.k.a. CD125), and low levels of c-KIT (CD117) in murine bone marrow (1). In humans, EoPs are identified by surface expression of CD34, CD38, and CD125 (2). EoPs reside in small numbers primarily in the bone marrow (~0.05% of lineage-negative $CD34^+$ cells), with even lower levels found in peripheral blood and in human umbilical cord blood (2). Targeting the EoP and the steps determining eosinophil lineage fate for treatment purposes is an attractive strategy, as it would prevent the production of mature eosinophils and all of their immune-activating contents; thus, delineating the factors that are essential for eosinophil lineage commitment will likely be clinically relevant.

Eosinophil Lineage Instruction by GATA-1 and GATA-2

It is well established that myeloid progenitor expression of the TF GATA-1 is essential for eosinophil lineage commitment (6–9). The findings of these earlier studies were supported recently by global gene expression profiling of single murine multipotent progenitor cells revealing that the commitment to the eosinophil lineage segregated with *Gata1* expression (10). In addition, scRNA-seq of murine GMPs ($Lin^-CD34^+c\text{-}KIT^+CD16/32^{hi}$) revealed a rare GMP subset with eosinophil lineage potential and that maintained expression of *Gata1* (11).

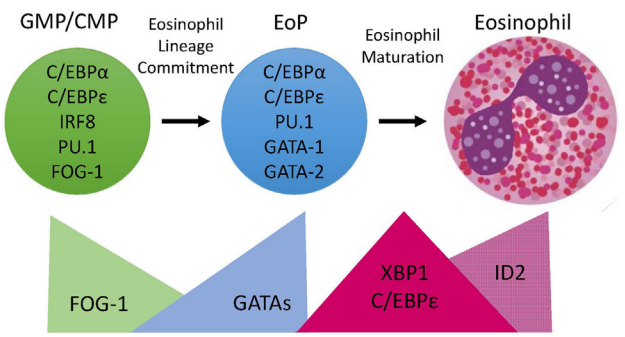

FIGURE 1 | Transcription Factor (TF) expression during eosinophil development. Eosinophils differentiate in the bone marrow from an eosinophil lineage-committed progenitor (EoP) that is derived from the granulocyte/macrophage progenitor (GMP) in mice and the common myeloid progenitor (CMP) in humans. For eosinophil lineage commitment to occur, the myeloid progenitor (GMP or CMP) must express C/EBPα, C/EBPε, interferon regulatory factor 8 (IRF8), and PU.1. Expression of friend of GATA-1 (FOG-1) declines, allowing for increasing expression and activity of GATA TFs, which is necessary for EoP production. Following lineage commitment, eosinophil granule protein gene expression is markedly increased with the collaborative interaction between C/EBPε, PU.1, and GATA-1. To assist with the elevated granule protein synthesis in the EoP and eosinophil precursors, XBP1 expression is increased and promotes survival during the demanding maturation process. Expression of activator isoforms of C/EBPε peaks during eosinophil maturation and then declines during the final stages. Expression of ID2 increases during eosinophil maturation and enhances the rate of maturation.

Two nuclear factors, friend of GATA-1 (FOG-1; *Zfpm1*) and interferon regulatory factor 8 (IRF8; *Irf8* or *Icsbp*), have been shown to be important for regulating *Gata1* expression and/or function in myeloid progenitors and, consequently, to affect eosinophil production. FOG-1 is a transcriptional cofactor that facilitates binding of GATA factors to DNA and recruits chromatin remodeling complexes (12–14). FOG-1 is highly expressed by multipotent progenitors, antagonizes GATA-1 transcriptional activity, and must be downregulated to allow for eosinophil lineage commitment (15, 16). Loss of FOG-1 expression in mice is early embryonic lethal from severe anemia due to the requirement for FOG-1 for the formation of erythroid-lineage progenitors (17). FOG-1 deficiency in hematopoietic stem cells results in increased commitment along the myeloid lineages and aberrant expression of myeloid-related genes in megakaryocytic and erythroid cells (18), highlighting the role for FOG-1 in suppressing myeloid cell development. In contrast, loss of *Irf8* expression in mice resulted in reduced EoP (and eosinophil) frequency in the bone marrow and lower *Gata1* expression in the EoPs that were produced (19), suggesting that the TF IRF8 is critical for upregulating and/or maintaining GATA-1 expression in myeloid progenitors for eosinophil lineage commitment. Notably, murine GMPs with eosinophil lineage potential and that maintained *Gata1* expression also expressed intermediate levels of *Irf8* (11).

Murine EoPs express both GATA-1 and GATA-2, whereas GMPs express no GATA-1 and low to no level of GATA-2 (5, 20). Ectopic expression of GATA-2 in murine GMPs and human $CD34^+$ hematopoietic progenitors was sufficient to instruct commitment to the eosinophil lineage (7, 20) and induce expression of GATA-1 (20). GATA-1 and GATA-2 have identical DNA sequence binding preferences, but their target genes and transcriptional responsibilities can be cell specific and/or overlapping, likely *via* a multitude of coregulators (e.g., FOG-1) (21). Targeted deletion of GATA-1 or GATA-2 has revealed that they control distinct biological processes that affect multiple hematopoietic lineages (21). Taken together, these studies emphasize the essential and instructive role for GATA TFs in eosinophil development; yet, targeting GATA-1 or GATA-2 therapeutically is likely to have significant and unacceptable effects on other hematopoietic lineages.

C/EBPα Co-Expression with GATA-1 or GATA-2

In addition to expressing GATA-1 and GATA-2, EoPs express relatively high levels of the TF CCAAT/enhancer-binding protein alpha (C/EBPα) (20). C/EBPα is necessary for eosinophil development, as C/EBPα-deficient mice lack eosinophils (and neutrophils) (22). The level of C/EBPα expression is important for eosinophil- vs neutrophil-lineage commitment, as elevated expression of C/EBPα in GMPs due to an impaired protein degradation pathway results in increased neutrophil differentiation

at the expense of eosinophils (23). In addition, the order of expression of GATA factors and C/EBPα is critical for eosinophil lineage commitment (8, 20, 24). Enforced expression of GATA-1 or GATA-2 in a C/EBPα-expressing progenitor results in eosinophil lineage commitment (20). In contrast, ectopic expression of GATA-2 prior to C/EBPα expression leads to basophil-lineage commitment (20). It is believed that C/EBPα is at least partially responsible for the downregulation of FOG-1 expression in myeloid progenitors promoting eosinophil development (15).

C/EBPε Promotes Eosinophil Cell Fate

Multiple isoforms of the TF C/EBPε with distinct transcriptional functions (e.g., activators and repressors) are expressed during eosinophil maturation, and expression levels of the varying isoforms change with developmental stage (25, 26), reinforcing that ratios of TFs with combinatorial and even antagonistic activities are highlights of the eosinophil developmental program. Low levels of the activator C/EBPε isoforms are expressed in $CD34^+$ hematopoietic progenitors, and all isoforms increase in expression during IL-5-mediated differentiation, with the repressor isoforms predominating during later stages of maturation (25). Mice deficient in C/EBPε fail to generate mature eosinophils or normal neutrophils (27), supporting a critical role for C/EBPε in a common upstream myeloid progenitor. Notably, ectopic expression of the activator isoforms of C/EBPε in umbilical cord blood $CD34^+$ progenitors resulted in markedly increased commitment to the eosinophil lineage (25). In contrast, expression of the repressor isoforms decreased eosinophil cell fate, but not other myeloid lineages (25), suggesting that inducing expression of repressor isoforms in early myeloid progenitors may specifically inhibit eosinophil production. Expression of the four isoforms of C/EBPε results from differential splicing and alternative use of promoters (26, 28), but the critical transcriptional regulators that orchestrate the expression of the different isoforms is not known.

Unclear Roles for PU.1

The TF PU.1 is a member of the ETS family of DNA-binding proteins with an essential function in both myeloid and lymphoid development (29, 30). Though the PU.1 expression level in myeloid progenitors has been shown to be important in regulating macrophage and neutrophil cell fates (3, 31), a definitive early role for PU.1 in eosinophil lineage commitment has not been defined. Gene expression analysis of PU.1-deficient fetal liver cells revealed expression of eosinophil peroxidase and major basic protein (*Prg2*), but little to no *Il5ra* (32), suggesting that PU.1 is not essential for eosinophil lineage commitment, but studies with a specific focus on the eosinophil lineage potential of hematopoietic cells deficient in PU.1 are needed.

Summary of Eosinophil Lineage Commitment

In summary, eosinophil lineage commitment occurs in a myeloid multipotent progenitor that expresses C/EBPα, C/EBPε, and IRF8 followed by concomitant declining FOG-1 expression and increasing GATA-1 and GATA-2 expression (**Figure 1**). This hierarchical combination of TFs has been shown to be necessary for eosinophil lineage commitment.

EOSINOPHIL MATURATION

Human eosinophils have characteristic morphologic features, including a bilobed nucleus and cytoplasmic granules filled with cationic proteins that are packaged in a specific manner (**Figure 1**). Eosinophils are terminally differentiated and do not proliferate once they leave the bone marrow. We noted that mature eosinophils share expression of 60 TFs with EoPs and express an additional 35 TFs that EoPs do not (5), suggesting that it requires a greater number of TFs to produce a more complex and differentiated cell. Identifying the critical TFs for specific eosinophil functional responses will provide potential new therapeutic targets.

PU.1 Priming for Transcription

Recent studies in macrophages have revealed a collaborative interaction between PU.1 and other lineage-determining TFs, such as C/EBPα, to open chromatin and "prime" genes for transcription (33, 34). Consistent with this role as a "pioneer" TF, PU.1 has been shown to cooperatively regulate the expression of eosinophil granule protein genes (35–37), including *PRG2* (major basic protein) and *RNS2* (eosinophil-derived neurotoxin), highlighting an important role for PU.1 in eosinophil maturation. Future studies are needed to determine how the distribution of PU.1 across the genome differs between granulocytes (eosinophils, neutrophils, basophils, and mast cells) and what partnerships are critical for terminal differentiation of the distinct cell types.

C/EBPε Interaction with PU.1

One of the PU.1 collaborators in regulating gene expression during eosinophil maturation is the TF C/EBPε. The peripheral blood and bone marrow of adult mice deficient in C/EBPε have a pronounced increase in immature myeloid precursors, indicating a blockade in terminal granulocyte differentiation in the absence of C/EBPε (27). In addition, ectopic expression of C/EBPε in $CD34^+$ hematopoietic progenitors increased the rate of eosinophil maturation (25). C/EBPε is important for the expression of secondary granules in both neutrophils and eosinophils (36, 37), and C/EBPε deficiency results in impaired functional responses for neutrophils (27). Individuals with mutations that abolish C/EBPε expression produce abnormal neutrophils and eosinophils that lack specific granules; thus, these individuals suffer from early and frequent bacterial infections (26, 38, 39), providing clinically relevant support for a critical role for C/EBPε in terminal differentiation of granulocytes. Interestingly, peripheral blood eosinophils predominantly express one of the repressor isoforms of C/EBPε (36), suggesting that C/EBPε's repressive activity is more important during late-stage eosinophil maturation.

XBP1 Is Required for EoP Survival

Murine EoPs have been shown to contain nascent granules (1, 5) and express granule protein mRNAs at a higher level than mature eosinophils (5); thus, early EoP differentiation likely represents

a developmentally restricted period during eosinophilopoiesis when protein production and endoplasmic reticulum (ER) demand peaks. XBP1 (*Xbp1*) is a TF that is involved in the unfolded protein response triggered by ER stress (40). In response to ER stress, *Xbp1* mRNA is spliced by the endoribonuclease IRE1α followed by translation of the active TF XBP1. Accumulation of the spliced *Xbp1* mRNA was higher in GMPs and EoPs than eosinophil precursors, and no spliced *Xbp1* mRNA was noted in mature eosinophils, which is consistent with activation of the ER stress pathway during high protein synthetic demands through eosinophil maturation (41). Notably, loss of *Xbp1* expression in hematopoietic cells resulted in a compete loss of mature eosinophils (41). EoPs were present in the bone marrow but at a lower frequency in *Xbp1*-deficient than *Xbp1*-sufficient mice, likely due to poor survival (41); thus, *Xbp1* is essential for eosinophil maturation but not lineage commitment.

ID2 Enhances Terminal Differentiation

Inhibitor of DNA-binding (ID) proteins is a family of negative transcriptional regulators that heterodimerizes with basic helix-loop-helix TFs and prevents binding to the DNA (42). Expression of ID2 was upregulated during eosinophil maturation, and ectopic expression of ID2 in human $CD34^+$ hematopoietic progenitors resulted in increased mature eosinophils, with no change in frequency of the earlier precursors (43), suggesting that ID2 enhances terminal differentiation. In contrast, expression of ID1 declines during eosinophil maturation and inhibits terminal differentiation (43).

EOSINOPHIL FUNCTION

In addition to orchestrating eosinophil production, TFs also participate in eosinophil functional responses and survival. Glucocorticoids are the first-line therapy for eosinophil-associated disorders, such as allergy, asthma, eosinophilic gastrointestinal disorders and hypereosinophilic syndrome (44, 45); yet, there are a subset of individuals with severe asthma with eosinophilia despite high doses of glucocorticoids (46–48) and patients with hypereosinophilic syndrome often become glucocorticoid refractory (49, 50). The TF NFIL3 has recently been shown to be induced by IL-5 stimulation in eosinophils and to protect against glucocorticoid-induced apoptosis (51), suggesting that targeting NFIL3 in patients may restore glucocorticoid sensitivity. STAT6 is another TF that has been shown to regulate eosinophil functional responses, specifically in experimental asthma. Sensitized mice with STAT6-deficient eosinophils were protected against mucus overproduction and airway hyperresponsiveness following allergen challenge (52), highlighting an important role for STAT6 signaling in eosinophils in allergic asthma. Yet, eosinophil-intrinsic STAT6 was not required for eosinophil recruitment into tissues in response to parasitic infection (53), highlighting the need for further investigations to delineate the impact of environmental signals on gene regulatory programs. Together, these studies suggest that targeting TFs in specific clinical settings may impact eosinophil function and survival.

CONCLUSION AND FUTURE DIRECTIONS

As there have been no described TFs that are specific to the eosinophil lineage, targeting eosinophil production currently has been achieved primarily *via* indirect means. A wealth of evidence support a critical role for the cytokine IL-5 in mediating disease-associated eosinophilia, and neutralizing IL-5 indirectly suppresses eosinophil maturation (54). IL-5 is produced by type 2 helper T (Th2) cells and the TF GATA-3 has been shown to control expression of IL-5 in Th2 cells (55). In addition, group 2 innate lymphoid cells (ILC2s) produce large amounts of IL-5 upon activation by epithelial-derived cytokines (56, 57) and GATA-3 is essential for ILC2 development (58); thus, GATA-3 is an attractive therapeutic target to prevent IL-5 expression. Notably, treatment with a DNA enzyme that cleaved *GATA3* mRNA resulted in reduced airway eosinophilia and plasma levels of IL-5 in individuals with asthma (59, 60), highlighting the feasibility of targeting TFs in patients with eosinophil disorders. With emerging technology and public databases of information available to investigators around the world, the future for research in eosinophil development is bright. Many new questions have arisen as our knowledge expands. Recently, a new regulatory eosinophil subset has been described in the murine lung and with a transcriptome that differed from that of inflammatory eosinophils (61). In addition, thymus-resident eosinophils have a distinct phenotype from other tissue-resident eosinophils (62). Together, these studies indicate that extrinsic signals from the local environment likely affect gene expression *via* changes in the regulatory program or that these eosinophil subsets are produced *via* a differential developmental program. Understanding how specific signals direct distinct patterns of gene expression required for the specialized functions of tissue-resident eosinophils will likely lead to new targets for therapeutic intervention.

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MINI REVIEW
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Understanding Interleukin 33 and Its Roles in Eosinophil Development

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Over the last decade, significant interest in the contribution of three "epithelial-derived cytokines," such as thymic stromal lymphopoietin, interleukin 25, and interleukin 33 (IL-33), has developed. These cytokines have been strongly linked to the early events that occur during allergen exposures and how they contribute to the subsequent type 2 immune response. Of these three cytokines, IL-33 has proven particularly interesting because of the strong associations found between both it and its receptor, ST2, in several genome-wide association studies of allergic diseases. Further work has demonstrated clear mechanisms through which this cytokine might orchestrate allergic inflammation, including activation of several key effector cells that possess high ST2 levels, including mast cells, basophils, innate lymphoid cells, and eosinophils. Despite this, controversies surrounding IL-33 seem to suggest the biology of this cytokine might not be as simple as current dogmas suggest including: the relevant cellular sources of IL-33, with significant evidence for inducible expression in some hematopoietic cells; the mechanistic contributions of nuclear localization vs secretion; secretion and processing mechanisms; and the biological consequences of IL-33 exposure on different cell types. In this review, we will address the evidence for IL-33 and ST2 regulation over eosinophils and how this may contribute to allergic diseases. In particular, we focus on the accumulating evidence for a role of IL-33 in regulating hematopoiesis and how this relates to eosinophils as well as how this may provide new concepts for how the progression of allergy is regulated.

Keywords: interleukin 33, eosinophils, stem cells, ST2/ST2L, asthma, allergy and immunology

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INTRODUCTION

Allergic diseases are increasing worldwide, and the mechanisms of both allergic sensitization and the subsequent effector responses following reexposure, including by eosinophils, are still not fully understood. Significant focus has recently centered on three cytokines as regulating type 2 immunity in allergic individuals: thymic stromal lymphopoietin (TSLP), interleukin 25, and interleukin 33 (IL-33). Evidence shows that these cytokines influence allergic mechanisms that include activating type 2 innate lymphoid cells (ILC2s), the development of T helper type 2 (Th2) cells, and several other effector cells, including eosinophils. Development of antibodies targeting these epithelial-derived cytokines in allergic disease is underway: an antibody against TSLP is currently showing beneficial effects in patients (1), and anti-IL-33 has entered phase two in clinical trials (2). IL-33 in particular seems important for eosinophil biology, both in homeostatic development and activation during disease. As an example, a recent loss-of-function mutation in *Il33* was identified in patients

and associated with reduced blood eosinophils and protection from asthma (3). In this review, we will discuss not only how IL-33 contributes to eosinophil biology but also recent evidence for roles of IL-33 in eosinophil development, which challenge the accepted view of IL-33 as regulating mainly local tissue responses.

CONTROVERSIES IN IL-33 BIOLOGY

Interleukin 33 was originally found as a nuclear factor of high endothelial venules and termed NF-HEV (4). Interest was reignited when computational predictions showed a characteristic β-trefoil domain similar to the IL-1 family of cytokines (i.e., IL-1α, IL-1β, and IL-18), thus becoming the 11th family member known as IL-33 (5). Notably, Schmitz et al. also identified IL-33 as the ligand for the previously orphan receptor suppression of tumorigenicity 2 (T1/ST2) (also called interleukin 1 receptor-like 1), which had already been associated with allergic disease; indeed, IL-33 injection into mice led to increased spleen weight, IgE, type 2 cytokines, mucus production by epithelial cells, and significant eosinophilia. Thus, much of the early research on the IL-33/ST2 axis defined clear roles in type 2 immune-mediated responses.

Despite this, controversy has surrounded IL-33 with unanswered questions related to its cellular sources, subcellular location, and release mechanisms. While many have assigned IL-33 as an epithelial-derived cytokine, we and others have established that immune cells also express IL-33 upon activation, including macrophages, dendritic cells (DCs), eosinophils, B cells, monocytes, and mast cells (6–11). While IL-33 has been shown to be present in surface epithelial cells from human biopsies (12), studies of gene expression using a reporter mouse demonstrate that type 2 pneumocytes are the dominant cell expressing IL-33 within the lung (6) and that Clara cells, ciliated epithelial cells of the bronchiolar system, do not express IL-33 unless inflammation is induced (13). A point of contention is the question of functional contributions of structural- vs immune cell-derived IL-33. In mouse studies addressing this question, IL-33 from macrophages (14), DCs (8), and monocytes (7) are sufficient to support the development of Th2 responses and eosinophilia. In contrast, one study showed that transferring IL-33 knockout (KO) bone marrow into irradiated mice had no effect on allergic inflammation (15). Further studies are needed, especially given the significant caveat that several of these immune cells are highly radiation resistant. Taken together, while current evidence shows clear roles for immune cell-derived IL-33, the relative importance of structural- vs immune cell-derived IL-33 remains to be determined.

The mechanism of how cells release IL-33 is also subject to debate. IL-33 has been described as residing exclusively in the nucleus of structural cells (16), yet evidence now suggests this conclusion is likely influenced by alterations in the IL-33 protein upon fusion with fluorescent tags used to track the protein; a more careful assessment of native IL-33 revealed both nuclear and cytoplasmic presence (17) in endothelial cells and fibroblasts—indeed, we demonstrated cytoplasmic location within mast cells (18). Unlike many other IL-1 family members, IL-33 does not utilize the inflammasome pathway (19). Although its release upon necrotic cell death gave rise to the concept of IL-33 as an "alarmin" (20), mechanical stress could also induce IL-33 release from fibroblasts in the absence of necrosis (17). Relevant to allergy, IL-33 release was shown in response to the established adjuvant aluminum hydroxide (alum) (21). Allergens interact with mucosal tissue surfaces in many ways including through toll-like receptors 2 and 4 (TLR2 and TLR4), dectin-1, and protease-activated receptor-2 (PAR-2) (22), wherein dectin-1 and PAR-2 are necessary for allergen-induced increases in IL-33 in lung tissues (23, 24). The source of such increases in IL-33 remains poorly defined: while several studies have described IL-33 secretion from structural cells (17, 25–27), mast cells (28), DCs (9), and human monocytes (29) can also express and release IL-33. Since alveolar macrophages serve as a front line of allergen exposure in the airway and TLR ligands being shown to stimulate IL-33 in macrophage (30), immune cells as a source of IL-33 might occur both within the airspace and tissues.

THE EFFECTS OF IL-33 ON MATURE AND DEVELOPING EOSINOPHILS

Eosinophil expansion is a hallmark of most allergic disease, but the underlying mechanisms are not fully understood. Several scenarios may explain this expansion: proliferation of resident eosinophils, increased trafficking of blood eosinophils into tissues, increased output from the bone marrow, increased survival, and local maturation of progenitors in tissues. Mature eosinophils, which respond to IL-33, do not seem to possess a robust proliferative capacity, and so focus has been on developmental processes. The current knowledge of the ways in which IL-33 influences eosinophil biology during homeostasis and disease is discussed below.

IL-33 on Mature Eosinophils

Cellular responses to IL-33 have been extensively studied. The ST2 receptor is highly expressed on several "allergy-associated" immune cells, including eosinophils, Th2 cells, ILC2s, mast cells, and basophils, as well as structural cells like epithelial and endothelial cells (31). IL-33 induces the production of type 2-associated cytokines from many of these cell types. Consequently, IL-33 had been presumed to affect eosinophilic inflammation through induction of IL-5—a cytokine known to activate eosinophils. For example, early work concluded that IL-33-induced eosinophilia was dependent on IL-5, but this conclusion was based largely on a neutralizing antibody approach and limited markers for defining eosinophils (32). As outlined in this review, IL-33 is now understood to act directly on eosinophils and regulate their biology, including survival, activation, and adhesion (**Figure 1B**).

Administration of IL-33 is sufficient to drive *in vivo* eosinophilia in various tissues (5). While IL-33 does not act as an eosinophil chemoattractant (33), several studies show that IL-33 regulates eosinophil survival. For example, transferring ST2 KO eosinophils into recipient mice led to significantly fewer lung eosinophils after allergen challenge than wild-type (WT) eosinophils despite normal migratory functions, implying impaired survival (34). IL-33 also induces GM-CSF that acts in an

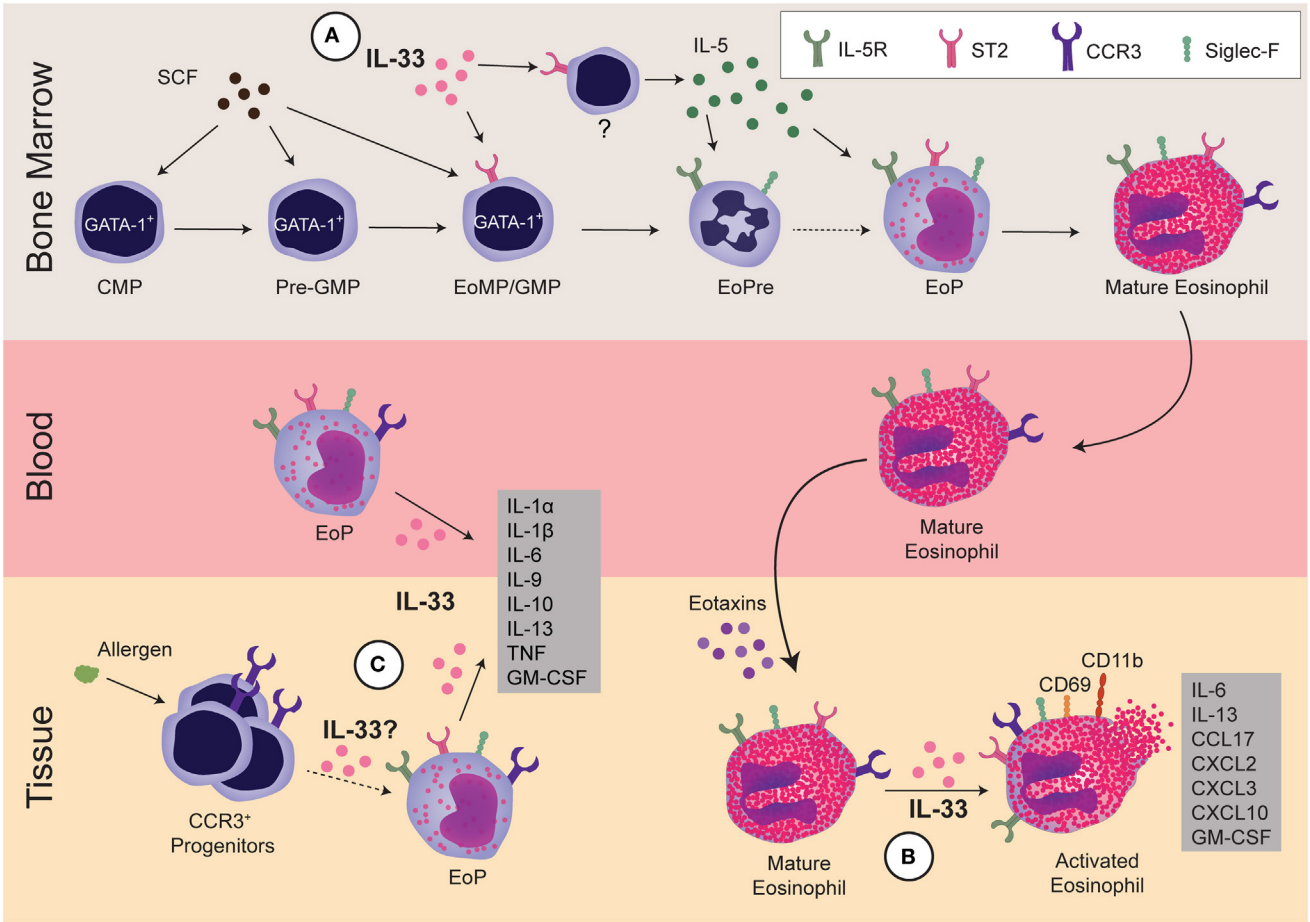

FIGURE 1 | Regulation of eosinophils by interleukin 33 (IL-33). IL-33 regulates eosinophils during three stages: development within the bone marrow, activation of mature cells, and development and/or activation of progenitors within the tissue. **(A)** In the bone marrow, GATA-1$^+$ common myeloid progenitor (CMP) differentiates into GATA-1$^+$-pre-granulocyte macrophage progenitor (Pre-GMP) (Lin$^-$Sca-1$^-$c-kit$^+$CD41$^-$CD16/32$^-$CD105$^-$CD150$^-$GATA-1$^+$), then to GATA-1$^+$-granulocyte and macrophage progenitor (GMP), also known as eosinophil/mast cell progenitors (EoMP, Lin$^-$Sca-1$^-$c-kit$^+$CD41$^+$CD16/32$^+$GATA-1$^+$). At this early stage, IL-33 regulates the expansion of eosinophil precursor (EoPre) through differentiation of the EoMP/GMP. Since IL-33 also upregulates IL-5Rα on EoPre, it regulates commitment to the eosinophil lineage. Simultaneously, IL-33 induces another currently unidentified cell within the bone marrow to make IL-5, which promotes final eosinophil maturation. **(B)** In the tissue, IL-33 can activate mature eosinophils, leading to cytokine production and upregulation of CCR3, CD69, and CD11b. Notably, IL-33-driven production of GM-CSF and IL-13 promote eosinophil survival and differentiation of alternatively activated macrophages, respectively. **(C)** Finally, IL-33 can regulate EoP outside of the bone marrow. IL-33 increases the number of EoP in blood as well as activates EoP to produce many cytokines. Although allergens increase CCR3$^+$ progenitors in tissue, it is unclear if increases in EoP in asthma patients are due to EoP leaving the bone marrow or extramedullary eosinophilopoiesis. It has yet to be determined if IL-33 also regulates eosinophilopoiesis within the tissue.

autocrine fashion to promote survival by inducing the antiapoptotic protein Bcl-x_L (35), a response that is negatively regulated by dual-specificity phosphatase 5 (36). Beyond these positive effects of IL-33 on eosinophil survival, IL-33-primed human eosinophils are more susceptible to Siglec-8-induced death; while this priming effect is less effective than IL-5, the two cytokines may function synergistically (37). Thus, the effects of IL-33 on eosinophil survival support a role on both survival and death, most likely in a context-specific fashion.

Interleukin 33 is a potent activator of eosinophils, even more so than IL-5 in terms of triggering degranulation and superoxide release from human eosinophils (38). In mice, IL-33 stimulation alters over 500 genes, many of which are immune related, including IL-6, IL-13, CCL17, CXCL2, CXCL3, and CXCL10 (39). IL-33 can also upregulate several cell-surface markers, including the adhesion molecule CD11b (33), the eotaxin receptor CCR3 (32), and the activation marker CD69 (36).

The functional nature of IL-33-activated eosinophils has been addressed. Transfer of eosinophils activated with GM-CSF, IL-4, and IL-33 into eosinophil-deficient asthmatic mice drove IL-13-dependent mucus production and accumulation of alternatively activated macrophages (40). In a complementary approach, increased IL-13 and alternatively activated macrophages were again observed after intranasal IL-33 administration to ST2 KO mice after adoptive transfer of WT eosinophils; recruitment of several cell types, including macrophages, neutrophils, lymphocytes, and the recipient's own eosinophils were also observed in this model (32). In the skin, IL-33 has been proposed to directly

act on eosinophils to promote fibrosis in an IL-4- and IL-13-dependent manner (41).

IL-33 on Eosinophilopoiesis

Typically, eosinophils develop in the bone marrow, enter the bloodstream as terminally differentiated cells, and become activated in tissues. However, $CD34^+$ progenitors can be detected in blood, and the idea that hematopoiesis can occur in tissues as well as the bone marrow is now accepted. IL-33 appears to have effects on these eosinophil precursors (EoPres) at both locations.

Histologically, eosinophil development was characterized into four classes (I–IV) based on nuclear morphology, granular morphology, and Wright–Giemsa staining. While Class I cells were described as granulocytic but not eosinophilic, Class II cells had small numbers of granules and appeared to have committed to the eosinophil lineage. Prior to terminal differentiation, Class III cells exhibited the characteristic donut-shaped nucleus. Class IV cells were the only eosin-positive cells and maintained the ring-shaped nucleus, which could twist into a figure 8-like structure (42). More recently, EoPres have been phenotyped using flow cytometry for cell-surface markers, including ST2 (**Table 1**). When the eosinophil lineage-committed progenitor (EoP) was initially identified in mice (43), it was proposed that eosinophils developed in four defined stages within the myeloid pathway. Originating from common myeloid progenitors (CMPs) that differentiate to granulocyte and macrophage progenitors (GMPs), a lineage decision into EoPs then occurs before terminal differentiation into eosinophils. Importantly, although EoP stains with eosin, eosin-negative precursors have been reported (42), suggesting a precursor stage prior to the granulation events occurring in EoP. We identified an EoPre that is eosin negative and exhibits the characteristic donut-shaped nuclei, which is driven by IL-33 exposure (44) (**Figure 1A**). Both EoP and EoPre are $IL\text{-}5R\alpha^+$, but EoPre is $Siglec\text{-}F^{lo}SSC^{lo}$ whereas EoP is $Siglec\text{-}F^+SSC^{hi}$.

TABLE 1 | Cell-surface markers of cells involved in murine eosinophilopoiesis.

	Common myeloid progenitor	Granulocyte and macrophage progenitor	Eosinophil precursor (EoPre)	EoP	Mature Eo
Lineage	–	–	high[a]	–	ND
Sca-1	–	–	–	–	–
c-Kit	+	high	–	low	–
CD34	+	+	–	+	–
FcγRII/III	low	high	ND	ND	ND
IL-5Rα	–	–	+	+	+
IL-3R	ND	+	ND	ND	+
IL-4Rα	ND	ND	ND	ND	+
GM-CSFR	ND	+	ND	ND	+
Siglec-F	ND	ND	low	+	+
CCR3	ND	ND	ND	ND	+
Granularity (SSC)	low	low	low	high	high
ST2	+/–	+/–	–	+	+

+ indicates expression; – indicates no expression; ND indicates expression not determined.

[a]*Unlike other studies, this study included CD11b in the lineage cocktail and demonstrated that the EoPre is $CD11b^{hi}$.*

From all of the markers defining eosinophils, three appear to be important for defining stages of eosinophil development: in mice, these are IL-5Rα, Siglec-F, and CCR3. IL-5Rα is an indicator of commitment to the eosinophil lineage, as it is a key differentiator between the EoP and earlier stages of development. Siglec-F, originally thought to only mark mature eosinophils and alveolar macrophages outside of the bone marrow, is expressed on EoP; moreover, colony forming assays comparing $Lin^-CD34^+CD117^{int}IL\text{-}5R\alpha^+$ (EoP–IL-5Rα) vs $Lin^-CD34^+CD117^{int}Siglec\text{-}F^+$ (EoP–Siglec-F) show that only EoP–IL-5Rα gave rise to pure eosinophils while EoP–Siglec-F cultures generate a mixture of eosinophils and macrophages (45). Thus, Siglec-F appears to mark eosinophil potential in the bone marrow, whereas IL-5Rα indicates commitment to the eosinophil lineage. Finally, CCR3 is a late marker, as it allows eosinophils to enter tissues in response to eotaxin (46).

In humans, the hEoP ($IL\text{-}5R\alpha^+CD34^+CD38^+IL\text{-}3R\alpha^+CD45RA^-$) differentiates directly from the hCMP ($Lin^-CD34^+CD38^+IL\text{-}3R\alpha^+CD45RA^-IL\text{-}5R\alpha^-$). Furthermore, the hGMP ($Lin^-CD34^+CD38^+IL\text{-}3R\alpha^+CD45RA^+$) is capable of generating neutrophils, monocytes, and basophils (47). Other stages of human eosinophil progenitors have yet to be determined. Although $IL\text{-}5R\alpha^+$ progenitors only generate eosinophils, IL-5Rα is expressed in blood on both mature eosinophils and mature basophils (47). Thus, it is unclear if IL-5Rα may be used to identify commitment to the eosinophil lineage as it does in mice. Furthermore, Siglec-8, the human functional paralog of Siglec-F, is expressed at late stages of development of eosinophils, mast cells, and basophils and does not mark eosinophil potential in progenitors the way Siglec-F does in mice (48, 49).

ST2 expression on these progenitors has been controversial. Two studies examining bone marrow stem cells showed opposing results: while Le et al. reported ST2 on $Lineage^-c\text{-}Kit^+Sca\text{-}1^+$ cells, CMP, GMP, megakaryocyte-erythroid progenitors (MEP), and common lymphocyte progenitors (50), Mager et al. found no evidence for ST2 on long-term or short-term hematopoietic stem cells, multipotent progenitors (MPP1, MPP2, and MPP3), MEP, CMP, or GMP (51). More recently, Tsuzuki et al. demonstrated that ST2 was expressed on CMP, MEP, and EoP, but not GMP (52). We described a GMP-like cell ($Lin^-Sca1^-Siglec\text{-}F^+IL\text{-}5R\alpha^-SSC^{lo}c\text{-}Kit^{hi}CD34^-$) that was $ST2^+$, but EoPre was $ST2^-$, although this finding came from IL-33-treated mice or *in vitro* cultures (44). These differences in ST2 expression may be partially resolved by new research that redefines the early stages in eosinophil development (53). Using single-cell RNA sequencing of pre-granulocyte macrophage progenitors (Pre-GMP, $Lin^-c\text{-}Kit^+Sca\text{-}1^-CD41^-CD16/32^-CD105^-CD150^-$), Pre-GMP clustered into two groups: $GATA\text{-}1^+Flt3^-$ and $GATA\text{-}1^-Flt3^+$. By sorting cells from a GATA-1–EGFP reporter and culturing them in eosinophil-promoting conditions, $GATA\text{-}1^+$ Pre-GMPs generate eosinophils, whereas $GATA\text{-}1^-$ Pre-GMPs generate neutrophils and monocytes. Drissen et al. proposed that $GATA\text{-}1^+$-GMPs be renamed eosinophil/mast cell progenitors, and $GATA\text{-}1^-$ GMPs retain their name. Thus, instead of the classical model (CMP, GMP, EoP, and mature eosinophil), the EoP population can develop independently of the GMP (**Figure 1A**). This aligns with the description of the hEoP arising from the hCMP and not the

hGMP (47). Notably, gene expression of ST2 differentiated the GATA-1$^+$ Pre-GMP and GATA-1$^-$ Pre-GMP populations (53). Thus, despite continuing debate over ST2 on CMPs and GMPs, eosinophils likely arise from ST2-expressing progenitors.

GATA-1 is a member of the GATA family of transcription factors and known to be critical for eosinophil development. In agreement with the potential of GATA-1$^+$ Pre-GMPs to produce eosinophils, human CD34$^+$ stem cells transduced to express GATA-1 developed into eosinophils while disruption of GATA-1 expression in mice ablated eosinophils (54). GATA-1 was also one of transcription factors identified as defining the eosinophil lineage through transcriptome analysis comparing GMPs, EoPs, and mature eosinophils; 56 transcription factors were identified including GATA-1, C/EBPε, NFκB, NFAT2, STAT1, STAT3, STAT6, IRF1, IRF2, Helios, and Aiolos (45). However, if and how all of these transcription factors play a role in eosinophil development has yet to be determined, and how IL-33 and ST2 might impact these transcription factors is unclear. ST2 signaling is known to lead to NFκB activation in mature cells (5), and GATA-1 and GATA-2 can regulate ST2 expression through two GATA binding sites upstream of the ST2 promoter (55), supporting a likely interplay at this level.

Several cytokines are important for eosinophil differentiation and maturation from bone marrow. Notably, IL-5 is the hallmark eosinophil-associated cytokine (56). IL-5-overexpressing transgenic mice (NJ.1638) have an excessive number of eosinophils in blood, bone marrow, and tissues with significantly more Class III and Class IV cells in the bone marrow, which, in conjunction with the fact that IL-5Rα marks eosinophil lineage commitment, indicates that IL-5 acts on later stages of development (42). Indeed, IL-5 promotes terminal eosinophil differentiation by upregulating CCR3 (57). IL-5 also upregulates its own receptor on human CD34$^+$ cells, but whether this occurs *in vivo* and affects eosinophil development is unclear (58).

The IL-5 receptor shares a β-chain (CD131, CSF2RB) with receptors for IL-3 and GM-CSF. While IL-3 and GM-CSF were initially thought to be important for eosinophil development, we now know that they promote the development of many myeloid cells (56). IL-3 drives mast cell and basophil development and affects mature eosinophils (59). While GM-CSF promotes survival of mature eosinophils, it appears to antagonize eosinophil development *in vitro* (60), although the mechanism has yet to be determined.

Current protocols for developing eosinophils from bone marrow also typically utilize stem cell factor (SCF) and Flt3 ligand (Flt3L) for 3–4 days before IL-5 treatment (61). Flt3L does not seem to be required for eosinophil development (53, 62) while the SCF receptor, c-Kit, is expressed on many stem cells and then lost in the later stages of eosinophil development. Interestingly, we demonstrated that culturing bone marrow cells with SCF and Flt3L for 3 days promoted the expansion of GMP-like cells and mature eosinophils but not the EoPre pool (44).

Recently, we reported that IL-33 may be the missing signal that directs stem cells to commit to the eosinophil lineage. IL-33 treatment significantly expanded the EoPre pool and led to a significant upregulation of IL-5Rα on EoPre, enhancing their responsiveness to IL-5; simultaneously, IL-33 induced IL-5 and mature eosinophil development (44) (**Figure 1A**). We also demonstrated that NJ.1638 mice had diminished eosinophils in the absence of ST2, indicating that IL-33 regulated the capacity of IL-5 to drive eosinophilia. In agreement with our data, IL-33 treatment of cultured c-Kit$^+$ bone marrow cells induced mature eosinophils in an IL-5-dependent manner (32). Interestingly, *in vitro* ST2 KO cells cultured with IL-5 do produce eosinophils, while ST2 KO and IL-33 KO mice have significantly reduced—not absent—eosinophils (44), demonstrating that IL-5-driven eosinophilopoiesis can occur in the absence of IL-33. Unlike GATA-1 that is absolutely required for eosinophil development, the absence of IL-5 may be compensated by other cytokines or factors since IL-5 KO mice also develop basal eosinophil populations (63). Perhaps deleting ST2 and IL-5 or CD131 would be required to ablate eosinophil development.

IL-33 and Alternative Eosinophilopoiesis Mechanisms within Tissues

There is increasing evidence that progenitors can circulate in the blood and that local hematopoiesis may occur in tissues [reviewed here (64); **Figure 1C**]. Eosinophil progenitors are increased in the blood and sputum of asthmatic patients (65, 66), but their role in disease not fully understood. Intravenous IL-5 increased not only circulating eosinophil progenitors but also CCR3 expression on CD34$^+$ progenitors (67). Similarly, IL-33 increased peripheral blood EoP (52). In response to allergen, CD34$^+$CCR3$^+$ and Sca-1$^+$CCR3$^+$ cells proliferated within the lung tissue, demonstrating expansion of local eosinophil lineage-committed stem cells (68). Whether these lung stem cells express ST2 and how IL-33 may affect these cells is unclear. *In vitro*, IL-33 activated EoP to produce chemokines, Th2 cytokines, and pro-inflammatory cytokines, with more IL-9, IL-10, IL-13, IL-1α, IL-1β, IL-6, TNFα, and GM-CSF than mature eosinophils (52); thus, these data implicate EoP as potential regulators over inflammation. Further research is certainly required to determine how eosinophil progenitors contribute to tissue eosinophilia in disease and if IL-33 serves to initiate their responses.

CONCLUSION

The biology of IL-33 continues to be a topic of significant discovery and controversy. By focusing on eosinophils, our understanding of this cytokine has begun to be elucidated and shows a complex regulation that extends into homeostasis and disease. While much of this challenges some established views of IL-33 as a local epithelial-derived cytokine, these understandings should significantly impact the interpretations and predictions for using new therapeutics that target this pathway in human health.

AUTHOR CONTRIBUTIONS

PB contributed to the overall design and content, as well as edited the final document. LJ contributed to the overall design and content, wrote initial drafts, and designed figures and tables.

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REVIEW
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Protein Translation and Signaling in Human Eosinophils

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We have recently reported that, unlike IL-5 and GM-CSF, IL-3 induces increased translation of a subset of mRNAs. In addition, we have demonstrated that Pin1 controls the activity of mRNA binding proteins, leading to enhanced mRNA stability, GM-CSF protein production and prolonged eosinophil (EOS) survival. In this review, discussion will include an overview of cap-dependent protein translation and its regulation by intracellular signaling pathways. We will address the more general process of mRNA post-transcriptional regulation, especially regarding mRNA binding proteins, which are critical effectors of protein translation. Furthermore, we will focus on (1) the roles of IL-3-driven sustained signaling on enhanced protein translation in EOS, (2) the mechanisms regulating mRNA binding proteins activity in EOS, and (3) the potential targeting of IL-3 signaling and the signaling leading to mRNA binding activity changes to identify therapeutic targets to treat EOS-associated diseases.

Keywords: eosinophils, protein translation, ribosomal S6 protein, Pin-1, IL-3, intracellular signaling

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INTRODUCTION

Control of protein production is critical for the maintenance of cell and tissue homeostasis. Excessive protein production may lead to hypertrophy and an unnecessary use of energy and other resources. However, inadequate protein synthesis antagonizes cell growth, proliferation, adaptation to environmental changes, and the implementation of new cell functions. Overproduction of transcription factors or cytokines contributes to or causes transformation and cancer. Thus, a carefully controlled balance within metabolic constraints but responsive to environmental and signaling cues is essential for optimal cellular function.

Circulating eosinophils (EOS) are differentiated, non-proliferative cells, which become apoptotic within 2–3 days if lacking contact with pro-survival cytokines, such as IL-5, GM-CSF, and IL-3 (1). Therefore, resting EOS have modest needs for new protein production. Protein production is dependent on (1) the level of coding mRNA, which in turn depends on the amount

Abbreviations: aa, aminoacyl; AMPK, AMP-activated protein kinase; ARE, adenosine-uridine (AU)-rich element; AUBP, ARE binding protein; AUF1 or hnRNP D, heterogeneous nuclear ribonucleoprotein D; CDK1, cyclin-dependent kinase 1; CSNK2A1, casein kinase 2; EDN, eosinophil-derived neurotoxin; eEF, eukaryotic elongation factor; eIF, eukaryotic translation initiation factor; EOS, eosinophil; FCGRII (CD32), receptor for Fc fragment of IgG, low affinity II; GEF, guanine-nucleotide-exchange factor; GTP, guanosine triphosphate; HA-IgG, heat-aggregated-IgG; hnRNP C, heterogeneous nuclear ribonucleoprotein C; La or SSB, Sjögren syndrome type B antigen; MAPK, mitogen-activated protein kinase; mRNP, messenger ribonucleoprotein; mTOR, mammalian target of rapamycin; mTORC1, rapamycin complex 1; p90S6K (RSK), 90-KDa ribosomal S6 kinase; PA, phosphatidic acid; PABP, poly-A binding protein; PIC, 43S preinitiation complex; RBP, RNA-binding protein; RIPP1, ribosomal-associated inhibitor of phosphatase 1; RPS6, ribosomal protein S6; TIA-1, cytotoxic granule-associated RNA binding protein; TIAR, cytotoxic granule-associated RNA binding protein like 1; TOP, terminal oligopyrimidine 5′-UTR, 5′ untranslated region; YB-1, Y-Box Binding Protein-1.

of mRNA transcribed and spliced excluding the amount degraded, and (2) the translation rate of the transcripts, which is governed by ribosomal content, activity, and associated ribosomal and mRNA binding proteins. Extracellular inputs *via* cell surface and intracellular receptors leading to the propagation of intracellular signals control each of these steps [reviewed in Ref. (2)].

Eosinophils have the ability to differentially regulate translation. As shown in **Figure 1**, the presence of high levels of a specific mRNA may or may not lead to protein translation, making inference of protein expression from mRNA quantification tenuous. Cell stimulation can trigger (1) the transcription and translation of mRNA expressed at very low level under basal conditions, (2) the stabilization of mRNA contributing to its accumulation and translation, (3) the translation of mRNA constitutively present but translationally quiescent in resting cells, and (4) an increase in the activity of the machinery, contributing to increased, global protein synthesis. As these topics are far too large to be covered adequately, here we will focus on how changes of both the translation machinery activity and the content of mRNA binding proteins affect the translatability of a subset of mRNA. We will start with an overview of protein translation and its control by intracellular signaling. During this overview, we will use previously published proteomic and phospho-proteomic data from peripheral blood EOS (3) to generalize these known protein translation mechanisms in EOS. Then, we will discuss how changes in mRNA binding proteins and the IL-3-dependent translation of a group of mRNA influence the production of the pro-survival cytokine, GM-CSF, and EOS function, respectively. Finally, the last section, titled "Regulation of translation and potential therapeutic targets," describes potential molecular drug targets that are implicated in protein translation in EOS in addition to EOS survival and activity. This review may help identify targets that are upstream of GM-CSF and downstream of IL-3 to supplement anti-IL-5 therapies, which despite their efficacy, have not totally controlled eosinophilia and EOS-related pathology. Of note, unless indicated, the observations discussed in this manuscript were obtained using human EOS.

GENERAL MECHANISMS CONTROLLING PROTEIN SYNTHESIS

In eukaryotic cells, initiation, elongation, and termination are the three fundamental steps of protein translation. Some of the main proteins/mRNA interactions involved in the initiation and elongation of translation are shown in **Figure 2**. During translation, *initiation* begins with the binding of eukaryotic translation initiation factor 4E (eIF4E) to the mRNA 5′-cap. Next, eIF4E binds to eIF4G, which interacts with the other eIF4 proteins, eIF4A and eIF4B. The helicase activity of eIF4A is amplified by eIF4B, and most likely unwinds secondary GC-rich structures of the 5′-UTR, thus facilitating initiation of mRNAs possessing these structures. The interaction of eIF4G with the poly-A binding protein (PABP), which circularizes the mRNA, also increases mRNA translatability. The binding of eIF4B and eIF4G to the 43S preinitiation complex (PIC) *via* eIF3 links the mRNA to the ribosome. The 43S PIC is composed of the ribosomal 40S subunit, eIF3, eIF5 eIF1, eIF1A, and the complex eIF2/Met-tRNA. EIF2 binds Met-tRNA in its GTP-bound state (eIF2-GTP). The complex Met-tRNA/eIF2-GTP along with the initiation factors/40S complex scans the 5′UTR until the start codon (AUG) is recognized by complementarity with the anticodon of Met-tRNA (4). Once the start codon is reached, protein translation becomes initiated by the eIF5B-catalyzed hydrolysis of eIF2-GTP into eIF2-GDP, which frees the ribosomal 40S from eIF2 (5). The release of eIF2-GDP and other initiation factors from the 40S complex is followed by the recruitment of the 60S ribosome subunit. The newly formed 80S ribosomal complex is now ready to start elongation (6). *Elongation* is predominantly controlled by eukaryotic elongation factor 1 (eEF1) and eEF2. Next, eEF1A-GTP recruits the second aminoacyl (aa)-tRNA complementary to the adjacent, C-terminal codon (A-site). After the peptide bound formation between Met

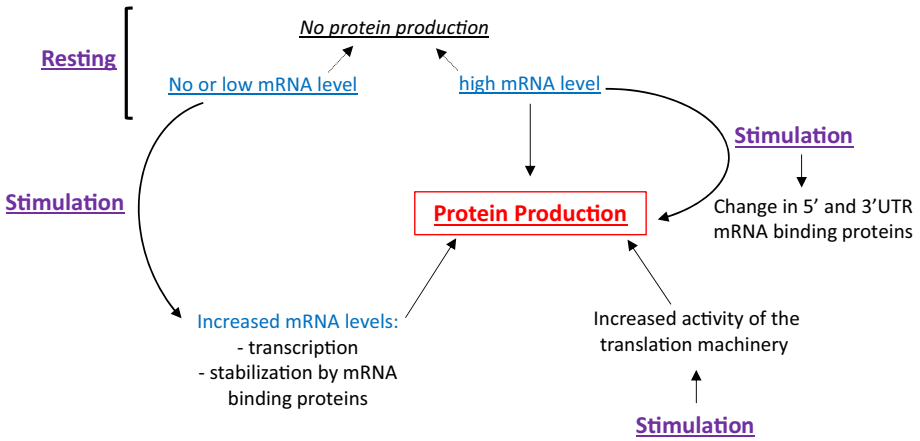

FIGURE 1 | Protein production is a function of cellular stimulation state, mRNA expression level and RNA-binding protein functionality. In resting eosinophils (EOS), protein synthesis can be suppressed irrespective of mRNA content. Cell stimulation can trigger protein production through increased transcription, mRNA stabilization and increased translation, typically regulated by changes in RNABP function.

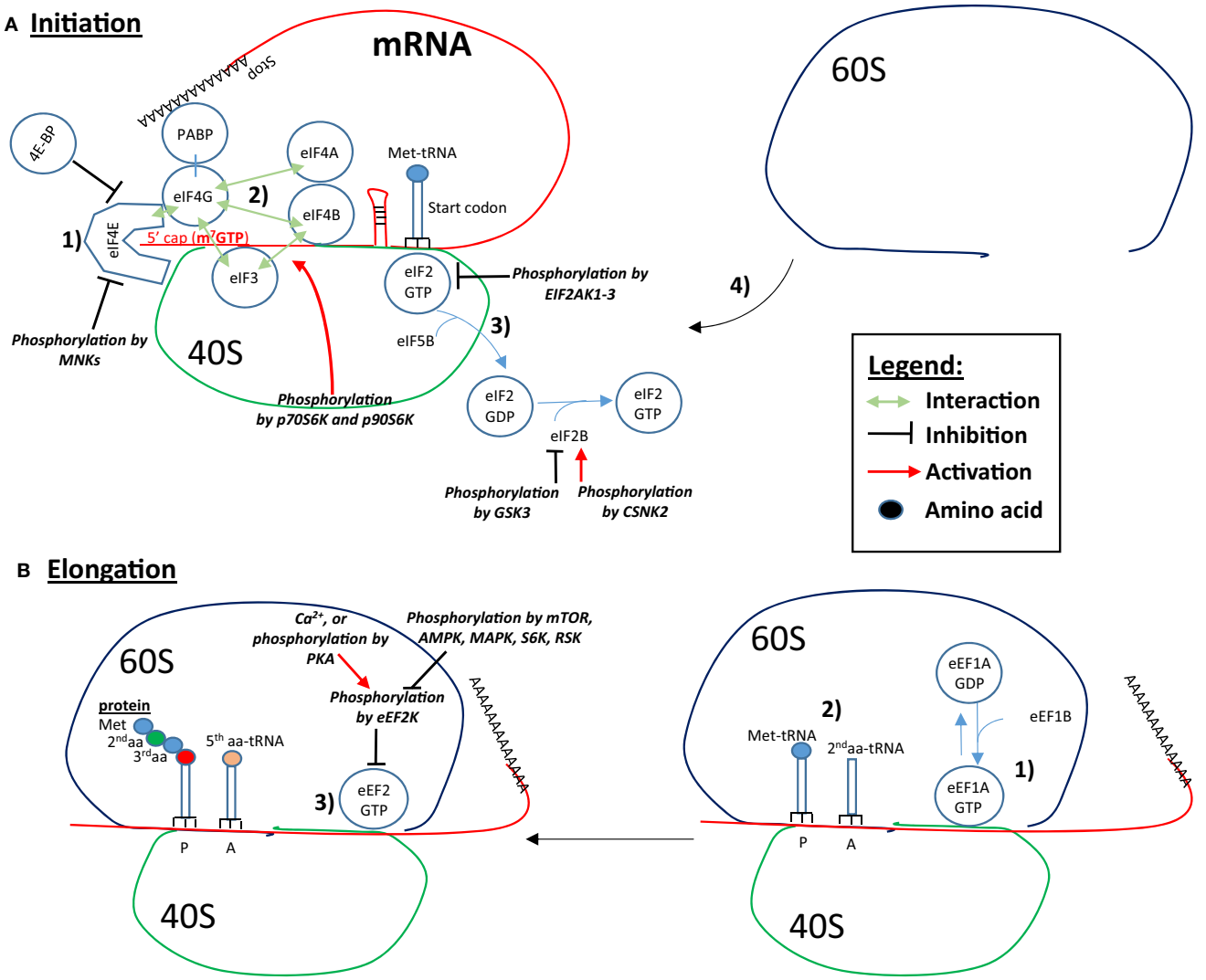

FIGURE 2 | Controls of initiation and elongation of protein translation. **(A)** *Initiation*: (1) eukaryotic translation initiation factor 4E (eIF4E) binds to the 5′-cap structure of the mRNA and eIF4G, which interacts with eIF4A and eIF4B. eIF4G also interacts with the poly-A binding protein (PABP), which circularizes the mRNA and increases the translation rate. (2) eIF4B and eIF4G binds to eIF3, which is associated with the ribosomal 40S subunit, which forms the link between the mRNA, the ribosome and the complex eIF2-GTP/Met-tRNA. In addition, the initiation factors/40S complex scan the 5′UTR until the recognition of the start codon. (3) Protein translation is initiated by the eIF5B-catalyzed eIF2-GTP hydrolysis into GDP, which results in the freeing of the ribosomal 40S from eIF2 and other initiation factors. (4) The recruitment of the 60S ribosomal subunit forms the ribosome by binding to the 40S subunit. eIF4B interaction with eIF3 is increased by p70S6K- and p90S6K-mediated phosphorylation. Binding of eIF4E to eIF4G and to the 5′ cap can be inhibited by 4E-BP and by Mnk-mediated phosphorylation. Phosphorylation of eIF2 by the EIF2AKs inhibits eIF2 recycling. eIF2B is phosphorylated and inhibited by glycogen synthase kinase 3 (GSK3), while phosphorylation of eIF2B by CSNK2 increases its activity toward eIF2 recycling. **(B)** *Elongation*: (1) eEF1A-GTP recruits the second aminoacyl (aa)-tRNA on the A-site. (2) A peptide bond forms between Met and the second aa. (3) eEF2-GTP pushes the mRNA, Met-tRNA is removed from the P-site and is replaced by the next aa-tRNA previously on the A-site. In addition, a third aa-tRNA is placed in the now empty A-site. Eukaryotic elongation factor 2 (eEF2) is inhibited by eEF2K-mediated phosphorylation. EEF2K is inhibited by mTOR, AMP-activated protein kinase (AMPK), mitogen-activated protein kinase (MAPK), S6K, and RSK. Conversely, Ca^{2+} and PKA phosphorylation leads to eEF2K phosphorylation and activation, and inhibition of the elongation.

and the second aa at the P-site, eEF2-GTP pushes (translocates) the mRNA and allows the third aa-tRNA to become positioned on the third codon at the A-site. Simultaneously, the first Met-tRNA is removed from the P-site and is replaced by the second aa-tRNA previously on the A-site. When the ribosome reaches a stop codon, no complementary tRNA exists to fill the A-site. At that point, the release factor ERF1 (*ETF1*) takes position in the A-site, and along with ERF3A-B (*GSPT1–2*) hydrolyzes the peptide chain (protein) attached to the last t-RNA to *terminate* translation.

Regulation of Protein Translation

In general, in eukaryotic cells, initiation can be controlled at multiple levels. The eIF4BP proteins (4E-BP) interact with eIF4E, preventing its interaction with eIF4G and, therefore, inhibiting translation initiation (7). 4E-BP are regulated at

multiple phosphorylation sites, often by the mammalian target of rapamycin (mTOR), which reduces 4E-BP interactions with eIF4E and enhances translation initiation (8). In addition, the cyclin-dependent kinase 2 (CDK2) phosphorylates 4E-BP on Thr70 leading to its release from eIF4E (9).

Eukaryotic translation initiation factor 4E (eIF4E) can be phosphorylated on Ser209, which decreases its affinity for the 5′-cap structure and, therefore, inhibits translation (10, 11). eIF4E is phosphorylated by the mitogen-activated protein kinase (MAPK) signal-integrating kinases Mnk1 and Mnk2 (*MKNK1* and *2*), which are downstream targets of the MAPK (ERK and p38) (10, 11). Also among the eIF4 family, eIF4B is phosphorylated by p70S6K and p90S6K (RSK) at Ser422, which increases its interaction with eIF3, enhancing translation initiation (12).

When the inactive form of eIF2, eIF2-GDP, leaves the 40S during initiation, it must be recharged with GTP for continuous translation initiation. Then, eIF2-GDP is converted to eIF2-GTP by eIF2B, which is a guanine-nucleotide-exchange factor (GEF). eIF2B is phosphorylated at multiple sites that includes two residues phosphorylated by casein kinase 2 (CSNK2A1) that are required for eIF2B/eIF2 interactions, eIF2 recycling and translation initiation (13). eIF2 is phosphorylated at Ser51 by as many as four kinases, all of which inhibit the eIF2–eIF2B interaction, demonstrating a critical role in protein synthesis (14).

The delivery of aa-tRNA required for *elongation* is driven by the hydrolysis of eEF1A-GTP to eEF1A-GDP. Thus, the GEF eEF1B acts on eEF1A-GDP as eIF2B does on eIF2-GDP. eEF1 is also targeted by a variety of kinases, including PKC, CSNK2, and cyclin-dependent kinase 1 (CDK1), but the role of the phosphorylation states of these elongation factors remains uncertain (15).

Phosphorylation by eEF2 kinase on Thr56 impairs eEF2's ability to bind to the 40S subunit of the ribosome (16). Thr56 phosphorylation is enhanced if Ser595 is previously phosphorylated (17). The eEF2 kinase activity is calcium/calmodulin-dependent. Its activation after Ca^{2+} flux leads to the attenuation of elongation. Of note, increased eEF2 kinase activity may provide mRNA with poor translation initiation efficiency a greater chance of being synthesized (18). eEF2 kinase is itself regulated at multiple phosphorylation sites, typically by the mammalian target of rapamycin complex 1 (mTORC1) that reduces its activity (19). AMP-activated protein kinase (AMPK) and the MAPK can also phosphorylate eEF2 kinase leading to translation enhancement (18). Conversely, cAMP/PKA signaling pathway phosphorylates Ser500 (20), rending eEF2 activity independent of Ca^{2+} ions and activating the kinase. **Figure 2** summarizes these different signaling events and control points.

General Translation in EOS

Recently, using two-dimensional liquid chromatography coupled with high-resolution mass spectrometry, 6,813 proteins were identified in unstimulated human blood EOS ((3), and *see article by Mosher* et al., *in this issue for more details*). In addition, 4,802 site-specific phosphorylation events were simultaneously identified (3). Furthermore, using RNA-Seq, ~7,981 protein-coding genes expressed by unstimulated human blood EOS were identified (21). The cellular content (mRNA and protein) and phosphorylation state of the main proteins involved in the initiation, elongation, and termination of protein translation have been extracted from the published proteome and transcriptome (shown in **Table 1**). Notably, **Table 1** shows examples of the disconnections between mRNA and protein levels, which suggests that production of certain proteins is tightly regulated at the translational level in EOS (i.e., EIF4G2, ETF1, etc.). For instance, while ratio of protein to mRNA expression generally reached ~1,000 and above, ratios for EIF4G2 and ETF1 were only 107 and 160, respectively (**Table 1**), suggesting marginal translation for these two transcripts in resting EOS. With the possible exception of the inhibitor of elongation, eEF2K, resting blood EOS possess all the essential proteins involved in protein translation. However, the identification of eEF2 phosphorylation on Thr56 (**Table 1**) suggests the existence of eEF2K activity, preventing the eEF2/40S interaction and blockade of translation elongation (16). In addition, the lack of phosphorylation of eIF2B (**Table 1**) suggests a possible lack of eIF2B/eIF2 interactions and reduced recycling of eIF2 into its active form (eIF2-GTP), which would dampen translation initiation (13). Conversely, in agreement with our previous report (22), 4E-BP is phosphorylated in resting EOS (**Table 1**). This indicates that 4E-BP does not act as a blocker of eIF4E binding to the 5′ cap in resting EOS and, therefore, other factors are responsible for restricting protein translation in resting EOS. Thus, the combined lack of eIF2B phosphorylation with the phosphorylation of eEF2 on Thr56 suggests attenuation of both initiation and elongation of protein translation in resting EOS (22).

IL-5, GM-CSF, and IL-3 are critical cytokines for EOS development and function. Each interacts with a specific α-chain receptor and a common, associated β-chain (24, 25). Not surprisingly, these receptors can generate both common and unique signals (22, 26–28). As indicated above, we have shown that 4E-BP is highly phosphorylated in resting EOS (22). After activation with IL-3, IL-5, or GM-CSF, 4E-BP phosphorylation state remains largely unaffected (22), suggesting that the increased translation induced by these cytokines is likely 4E-BP-independent. In addition to 4E-BP, we have unpublished observations indicating that EIF4B phosphorylation at Ser422 was unaffected by GM-CSF. Therefore, as for 4E-BP, eIF4B activity cannot explain the significant enhancement of translation in GM-CSF-activated cells (22). However, a slight but significant increase in the phosphorylation of eIF4B was observed in EOS activated by IL-3 for 20 h (unpublished data). This phosphorylation on Ser422 may account for the differences in translation seen in IL-3- versus GM-CSF-activated EOS (22). The signaling accounting for regulated translation in IL-3 or GM-CSF-activated EOS remains largely unstudied.

SIGNALING AND PROTEIN TRANSLATION

Two major intracellular signaling pathways regulate translation in eukaryotic cells: the phosphoinositide 3-kinase (PI3K)/Akt/mTOR and the MAPK pathways. These two pathways are generally triggered by extracellular stimuli *via* membrane receptors but also respond to intracellular ATP levels and amino acid availability.

PI3K/Akt/mTOR Signaling

Ligation of growth factors with tyrosine kinase or G-protein coupled (GPC) receptors typically leads to phosphorylation of the

TABLE 1 | Proteins involved in initiation, elongation, and termination, and present in fresh human blood EOS.

Protein/gene name	mRNA expression (RPKM)	Protein expression (iBAQ/10000)	Protein phosphorylated sites	Functional consequence of the phosphorylation state
Initiation factors				
EIF4E	4	28085	Not detected	eIF4E is functional?
EIF4EBP1 (4E-BP)	17	21457	T68	Allows eIF4E activity to initiate translation (9)
EIF4EBP2	46	65650	Not detected	
EIF4G1	19	12611	S1238, T1218, S1194	
EIF4G2	220	23543	Not detected	
EIF4A1	115	127510	Not detected	
EIF4B	42	37156	Y233, S406, S359, S459	
PABPC1	230	90073	Not detected	
EIF3A	20	19128	T574	
EIF5B	9	7267	S164	
EIF2A	Not detect.	7911	Not detected	
EIF2B1	22	17511	Not detected	No eIF2B/eIF2 interaction, *translation initiation is impaired* (13, 23)
CSNK2A1 (CK2)	20	24012		
CSNK2B (CK2)	7	27489		
Elongation factors				
EEF1A1	122	779580	Not detect.	
EEF1B2	10	170360	Not detect.	
EEF2	78	306930	T57 (Thr56), T59	Inhibits Ribosome binding, *elongation is impaired* (16)
EEF2K	2	2726	Not detected	
Termination factors				
ETF1	33	5269	Not detected	
GSPT1	7	42129	Not detected	
GSPT2	Not detected	204	Not detected	

For RNA Seq analysis, reads per kilobase per million mapped reads (RPKM) > 2.0 were used as positive mRNA expression by freshly purified blood EOS (5×10^6 cells) (21). 75 million EOS from 3 different donors were analyzed using two-dimensional liquid chromatography coupled with high-resolution mass spectrometry to generate a proteome and phospho-proteome (3). Protein intensity-based absolute quantification (iBAQ) and phosphorylated sites are shown.

membrane phospholipid, phosphatidylinositol-4,5-biphosphate (PI-4,5-P_2) into phosphatidylinositol-3,4,5-triphosphate (PIP_3), by the class I lipid kinase PI3K. This transformation into PIP3 is reversed by phosphatases such as the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and the SH2-domain containing inositol phosphatase (SHIP) (29). PIP3 drives the phosphorylation and activation of Akt (also called, PKB), *via* 3-phosphoinositol-dependent kinase 1 (PDK1) (30). Akt activity is also augmented by the mTORC2 complex, composed of mTOR and rictor (rapamycin-insensitive companion of mTOR) (29). Akt can in turn phosphorylate and inhibit the glycogen synthase kinase 3 (GSK3) leading to dephosphorylation and activation of eIF2B with translation initiation (31). In addition, Akt phosphorylates five sites leading to the inhibition of the GTPase activity of tuberous sclerosis complex 2 (TSC2), on the small GTPase Ras homolog enriched in brain (RHEB), which in its GTP form stimulates the kinase activity of mTORC1 (32, 33). Therefore, the activity of the mTORC1 complex, composed of mTOR, RHEB, the mTOR associated protein, LST8 (MLST8), and the regulatory-associated protein of mTOR (Raptor), is downregulated by unphosphorylated TSC2 that is derepressed by Akt kinase activity.

Downstream mTORC1, the TOS (target of rapamycin signaling)-containing 4E-BP and p70S6K are phosphorylated. As seen above, phosphorylated 4E-BP is inactive and allows eIF4E to bind eIF4G to initiate translation. In dividing cells, mTOR phosphorylates p70S6K at Thr389, which in turn can phosphorylate ribosomal S6 protein (RPS6), eIF4B and programmed cell death 4 (PDCD4). While the function of phosphorylated RPS6 remains largely unknown, eIF4B and PDCD4 are positive and negative regulators, respectively, of the RNA helicase, eIF4A (34). mTORC1 also downregulates the activity of the eEF2 Kinase, which then subsequently enhances the elongation step of translation by eEF2. The general protein translational capacity is also enhanced by mTOR via increased transcription (more mRNA), and stimulation of the translation of mRNAs containing a string of 5′–pyrimidines (5′TOP mRNA) (35). In addition to its activation by growth factors, mTOR also senses cellular nutrient, oxygen, and energy level (36). As its name implies, most of mTOR effects are neutralized by rapamycin. The FKBP12–Rapamycin complex quickly binds close to the kinase domain (37), leading to mTOR conformational changes, dissociation from Raptor (38, 39) and inhibition of some of mTORC1 functions (40). By binding newly produced mTOR, FKBP12–rapamycin complex also inhibits the assembly of mTORC2 (41). Rapamycin also inhibits the binding of phosphatidic acid (PA) to mTOR, reducing the stabilization of the mTORC1 and mTORC2 complexes (42). PA is synthetized during membrane phospholipid biogenesis (43), and its intracellular level modulates the amount of rapamycin required to inhibit mTOR (44). Interestingly, low doses of rapamycin inhibit mTOR-induced p70S6K phosphorylation while much higher doses are required to block mTOR-induced 4E-BP Thr37/Thr46 phosphorylation (45). As a result, other compounds that are stronger inhibitors of mTORC1 and C2 than rapamycin, such as PP242 and AZD8055, were developed.

mTOR Signaling in EOS

Surprisingly, mTOR has not been studied in EOS, although its inhibitor, rapamycin has shown effects on EOS *in vitro* and *in vivo*. As shown in **Table 2**, resting human blood EOS express relatively little mTOR, but very high amount of FKBP12. FKBP12 is bound by both rapamycin and FK506 and is required for these drugs to exert their inhibitory effects in cells. Interestingly, nanomolar doses of FK506 strongly inhibit calcium ionophore-induced cytokine (GM-CSF) production in EOS, while micromolar doses of rapamycin does not (46). Due to the competition between rapamycin and FK506 on FKBP12, high amount of rapamycin antagonizes the FK506-mediated inhibition of cytokine production in EOS (46). However, rapamycin is more potent than FK506 in inhibiting IL-5-induced prolonged EOS survival (46). The divergence between FK506 and rapamycin has also been described in T lymphocyte and mast cells, where rapamycin modulates proliferation rather that gene expression (47, 48). Another study (49) confirmed that rapamycin reduces IL-5-induced pro-survival signaling in EOS but the effect was modest and required high doses of drug for at least 72 h. In the same study, rapamycin also partially inhibited IL-5-induced eosinophil cationic protein (ECP) release from EOS (49). In addition, mTOR has important functions during EOS differentiation as rapamycin inhibited mouse EOS differentiation downstream of IL-5 (50). This is in agreement with the dependence of T cell proliferation and differentiation on mTOR (51). Remarkably, rapamycin has no inhibitory effect on mouse EOS recruitment into the BALF after exposure to dust-mite allergen in chronic allergic models (52), suggesting that the role of mTOR signaling is confined to development and possibly survival but not cell migration.

In both human and mouse EOS, PI3K is required for a variety of functions. These include chemokine-induced EOS granule proteins release (53), platelet-activating factor (PAF)-induced chemotaxis but not LTC4 release (54). The PI3K/Akt pathway is also essential for IL-5-induced β2-integrin adhesion to bovine serum albumin (BSA) (55), and IL-5-induced guinea pig EOS mobilization from the bone marrow (56). In EOS, the PI3K/Akt pathway can be activated by fMLP or RANTES after priming with IL-5 or IL-3 (57). Prostaglandin E_2 (PGE_2) *via* EP4 induces PI3K/PDK1-dependent increase in Akt phosphorylation, which consequently inhibits eotaxin-induced EOS shape changes and chemotaxis (30). Therefore, the PI3K/PDK1/Akt pathway is important in EOS and regulates a variety of functions depending on its activator.

TABLE 2 | Proteins present in human blood eosinophil (EOS) and involved in the phosphoinositide 3-kinase/mammalian target of rapamycin pathway.

Protein/gene name	Protein expression (iBAQ/10000)	Phosphorylated sites
AKT1 (PKB)	6656	Not detected
AKT2 (PKB)	2282	S478
FKBP1A (FKBP12)	1997100	Not detected
GSK3A	9069	Not detected
GSK3B	12297	S9
INPP5D (SHIP)	53856	S243, S971, S1039
MLST8	3323	Not detected
MTOR	1503	Not detected
PDCD4	41522	T90, S94
PDPK1	21325	Not detected
PIK3CA	94	Not detected
PIK3CB	2190	Not detected
PIK3CD	8867	Not detected
PIK3CG	9815	Not detected
PTEN	9587	Not detected
RHEB	10969	Not detected
RICTOR	1251	Not detected
RPS6	143160	S235, S326
RPS6KB1 (p70S6K)	970	Not detected
RPS6KB2 (p70S6Kb)	1869	Not detected
RPTOR	710	S863
TSC1	3037	Not detected
TSC2	2707	S1420

75 million EOS from 3 different donors were analyzed using two-dimensional liquid chromatography coupled with high-resolution mass spectrometry to generate a proteome and phospho-proteome in resting EOS (3). Intensity-based absolute quantification (iBAQ) and phosphosites are shown.

MAPK Signaling

The MAPK (ERK and p38) signaling pathways are involved in most of cellular functions, including differentiation and proliferation. ERK1, ERK2, p38α, and p38β are coded by four different genes (MAPK3, MAPK1, MAPK14, and MAPK11). Following intracellular or extracellular activation, the MAP kinase kinase kinases (MEKK) are activated, leading to phosphorylation of MAP kinase kinases (MEK) and, finally, MAPK are phosphorylated (58). ERK1/2 alone possess more than 150 substrates involved in a large variety of cell functions, including transcription, cell death, autophagy metabolism, and translation (59). Among the kinases activated by ERK or p38 are kinases involved in protein translation, including p90S6K (RSK), the MAPK-interacting kinases (Mnk), and the MAPK-activated protein kinase 2 (MK2) (2). The latter has an important role in 3′UTR directed, mRNA binding protein-dependent translation. P90S6K are activated by ERK signaling that can then phosphorylate TSC2 at Ser1798, activating mTORC1 and protein synthesis (60, 61). Of note, ERK may also directly phosphorylate and inhibit TSC2, leading to increased mTORC1 activity (62). Like p70S6K, p90S6K also phosphorylates both eIF4B and eEF2 kinase, which enhances eIF4B/eIF3 interactions and eEF2 function and, consequently, protein initiation and elongation (12, 23). While Mnk2 activity is thought to be constitutive, Mnk1 phosphorylation and activation can be triggered downstream ERK and p38 leading to eIF4E phosphorylation at Ser209 (63). Although this phosphorylation inhibits eIF4E binding to the 5′-cap, it may also control the translation of specific mRNAs (63).

MAPK Signaling in EOS

Mitogen-activated protein kinases have important roles in many critical events, including EOS survival, migration, adhesion, production of inflammatory mediators, and degranulation. In EOS, ERK and p38 are phosphorylated and active following stimulation with a variety of mediators, including the β-chain cytokines (IL-3, IL-5, and GM-CSF), chemokines, fMLP, the PAF, and matrix proteins (26, 28, 53, 57, 64–71). **Table 3** shows the

TABLE 3 | Proteins present in human eosinophil and involved in the mitogen-activated protein kinase signaling upstream of protein translation.

Protein/gene name	Protein expression (iBAQ/10000)	Phosphorylated sites
MAPK3 (ERK1)	57843	Not detected
MAPK1 (ERK2)	119320	Not detected
MAPK14 (p38α)	22843	Not detected
MAPK11 (p38β)	Not detected	Not detected
RPS6KA1 (p90S6K, RSK1)	75911	T393, S389, S372
RPS6KA2 (p90S6K, RSK3)	21015	T595, S402
RPS6KA3 (p90S6K, RSK2)	26924	T577, S227, S386, T231, S369
MKNK1 (Mnk1)	3601	S221
MKNK2 (Mnk2)	112	Not detected
MAPKAPK2 (MK2)	44194	Not detected
MAP2K2 (MEK2, upstream ERK)	153200	S226, T394
MAP2K4 (MEK4, upstream p38)	14813	S91, T89
MAP3K3 (MEKK3, upstream ERK)	5375	S178, S270, S281

75 million EOS from 3 different donors were analyzed using two-dimensional liquid chromatography coupled with high-resolution mass spectrometry to generate a proteome and phospho-proteome in resting EOS (3). Intensity-based absolute quantification (iBAQ) and phosphosites are shown.

expression levels of ERK, and their downstream targets, RSK1–3 (p90S6K), all of which are phosphorylated at a detectable level in resting cells. However, EOS contain little Mnk1/2 (**Table 3**), suggesting that the MAPK activation likely does not regulate protein translation *via* eIF4E phosphorylation (**Figure 2**); and despite its phosphorylation, the low level of Mnk2 probably have little effect on eIF4E phosphorylation. Consistent with MAPK activation, upstream MEK and MEKK were also phosphorylated at multiple sites in circulating EOS (**Table 3**). These data suggest that such cells are not truly resting but have been partially activated or primed either *in vivo* or during isolation.

MESSENGER RNA-SPECIFIC PROTEIN TRANSLATION

mRNA translation is clearly not an all or nothing event. Agonists may increase or decrease ribosomal mobilization of all, the majority or subsets of mRNAs. This may occur through a slowdown of global elongation by phosphorylated eEF2 allowing poorly translated mRNAs to enter initiation and to be translated when elongation becomes derepressed (18). Alternatively, increased eIF4A helicase activity may preferentially facilitate the translation of mRNAs possessing secondary structures in their 5′-UTR that require unwinding prior to initiation.

Selective regulation requires the recognition of unique cis-elements within the mRNA by sequence-specific mRNA binding proteins. In this way, subsets of mRNAs can be selectively identified and regulated for differential translation and mRNA decay. One well-studied example is the pyrimidine-rich domain termed terminal oligopyrimidine (TOP). mRNA containing TOP usually code for elongation factors and ribosomal proteins (72) and their translation is preferably induced by the mTOR pathway (73). We will discuss additional examples below.

IL-3 Induces Translation of Semaphorin-7A mRNA in EOS

Semaphorin-7A mRNA level is relatively high in resting cells and changes only slightly in activated blood EOS. However, its translation remains almost undetectable despite GM-CSF activation (22). Surprisingly, despite similar mRNA levels, the translation rate for semaphorin-7A is more than 10-fold higher in IL-3- versus GM-CSF-activated EOS (22). Consistent with increased translation, semaphorin-7A mRNA was enriched in polyribosome fractions following IL-3 compared to GM-CSF (22). Of note, TOP mRNAs (EEF1A1 and PABP) were not enriched in the polyribosome fraction by IL-3, suggesting unique and highly selective signaling from IL-3 receptor to the translational machinery.

Freshly purified blood EOSs possess surface semaphorin-7A, which tends to decrease overtime during the first 20 h of cell culture (unpublished data). Activation with IL-5 or GM-CSF maintains or slightly increases surface semaphorin-7A over this same time span (27). On the other hand, over a broad range of doses, IL-3 significantly increased surface semaphorin-7A expression (27). Interestingly, IL-3-induced semaphorin-7A translation occurred more than 6 h after activation (unpublished data), suggesting that considerable signaling and possibly the translation of accessory proteins precedes semaphorin-7A translation initiation.

ERK/p90S6K/RPS6 Signaling Downstream from the β-Chain Cytokines in EOS

Along with RL13A (74), RPS6 is one of the rare ribosomal proteins that is phosphorylated following cellular stimulation in eukaryotic cells (75, 76). In stromal cells, RPS6 phosphorylation is directly controlled by the kinases p70S6K1 and p70S6K2, downstream of mTOR (77). In knock-in mice, genetically modified at RPS6 phospho-sites, aggregate protein synthesis was decreased in liver and embryonic fibroblasts (78). Other studies have suggested that RPS6 phosphorylation facilitated more efficient 40S ribosomal subunit assembly (79). This idea is supported by structural and biochemical data demonstrating that phosphorylated RPS6 is located at the interface between the small and the large ribosomal subunits near the tRNA-binding sites (80), and is enriched in polyribosomes (75). The correlation of RPS6 phosphorylation with cell division during mitogenic activation suggests that RPS6 participates in translation control in dividing cells (81). However, the role of phosphorylated RPS6 in non-dividing cells, such as EOS, remains unexplored.

We found that all β-chain cytokines strongly induced RPS6 phosphorylation at Ser235 and Ser236. However, while RPS6 phosphorylation persisted for only 1–4 h in EOS culture with IL-5 or GM-CSF, IL-3 induced continuous RPS6 phosphorylation for as long as IL-3 remained present in the culture medium (22). Of note, this unique feature of IL-3 to prolong RPS6 phosphorylation has also been observed in basophils (82, 83). Anti-IL-3 neutralization rapidly reversed RPS6 phosphorylation indicating that constant presence of IL-3 was required and that signaling was likely driven by a labile secondary messenger following IL-3 activation (22). Interestingly, the relatively rapid RPS6 dephosphorylation in GM-CSF-activated EOS was

phosphatase 1 (PP1)-dependent, although total PP1 activity in cell lysates was the same in GM-CSF- and IL-3-activated EOS (22). This suggests that PP1 activity toward RPS6 may be negatively regulated only in IL-3-activated but not in GM-CSF-activated EOS. Of note, a 23 KDa ribosomal-associated inhibitor of PP1, termed ribosomal-associated inhibitor of phosphatase 1 (RIPP1) has been identified but remains incompletely described (84, 85).

As mentioned above, RPS6 can be phosphorylated downstream of the PI3K/Akt/mTOR/p70S6K pathway (77). However, in EOS neither rapamycin, PI3K nor p70S6K inhibitors prevented IL-3-induced RPS6 phosphorylation (22). On the contrary, p90S6K (RSK) inhibitors significantly reduced IL-3-induced, RPS6 phosphorylation on both Ser235 and Ser236 (22). GM-CSF activation of p90S6K peaked after 10 min, and p90S6K was already largely dephosphorylated by 1 h (22). Conversely, progressive phosphorylation of p90S6K occurred after IL-3, peaking, at 16–20 h and still detectable until IL-3 was removed or neutralized in the culture medium (22). P90S6K was the first RPS6-phosphorylating kinase described in *Xenopus* oocytes (86), but has since been implicated in cell proliferation and survival (87). P90S6K includes three isoforms (RSK1, 2, and 3), all with inducible phosphorylation-dependent activity and similar functions. P90S6K phosphorylation is downstream of ERK and phosphorylated p90S6K has been found associated with polyribosomes (88). Phosphorylation on Thr573 is sequentially followed by Thr359, Ser363, and finally Ser380. All four sites are strongly phosphorylated following IL-3-activated EOS (22). Ultimately 3′–phosphoinositol-dependent kinase-1 (PDK1) phosphorylates Ser221 leading to maximal p90S6K activation (89). In addition to RPS6, p90S6K also phosphorylates eIF4B and GSK3 (12, 90). Phosphorylated eIF4B interacts with eIF3A, enhancing translation initiation (91). P90S6K inactivates GSK3, which would in turn dephosphorylate and activate eIF2B, thus promoting eIF2 recycling and increasing translation initiation [(90); **Figure 2**]. While the dephosphorylation of eIF2B possibly occurs *via* changes in PP1 activity (90), differential activation of p90S6K by the different β-chain cytokines was not accompanied by changes in PP1 activity (22), suggesting that IL-3-induced and prolonged p90S6K activation does not affect translation *via* the GSK3/PP1/eIF2B pathway. As proposed above, the β-chain cytokines could differentially regulate a ribosomal specific PP1 regulatory protein (85).

Upstream, p90S6K phosphorylation is known to be regulated by the MAPK and particularly by ERK1/2 (92). Consistent with these data, a selective inhibitor of both MEK1 and MEK2 (U0126) added 3 h after IL-3, blocked the phosphorylation of p90S6K on Ser380 and RPS6 in EOS in culture. Another MAPK, p38, has also been implicated as a potential activator of p90S6K in dendritic cells (93). However, a p38 inhibitor (SB203580) had no effect on p90S6K phosphorylation in IL-3-activated EOS.

In addition to semaphorin-7A, we have more recently shown that the low-affinity IgG receptors, FCGR2B and FCGR2C (CD32B and CD32C) were upregulated at the translational level by IL-3, in a p90S6K-dependent manner (94). Therefore, we have so far identified two transcripts whose translation is exclusively enhanced by the prolonged effect of IL-3 through ERK/p90S6K signaling. MS proteomic analysis of EOS treated with IL-3 with and without ERK inhibitors will yield insight into the identity of other similarly regulated mRNA.

mRNA-BINDING PROTEINS AND CONTROL OF PROTEIN TRANSLATION

Overview

RNA-binding proteins (RBP) regulate all aspects of RNA metabolism, including biogenesis, cellular localization and transport, stability, and translation. With the emergence of high throughput screening and quantitative proteomics, several hundred (approximately 500) potential RBP have been identified (95). Given their obvious importance, enormous effort has been directed to expand our knowledge on how RNA-protein interactions determine RNA function and cell fate. It bears reiterating that mRNA is not a rod but a complex 3-dimensional shape. As such, RBP can interact with mRNA *via* structure, sequence or structure, and sequence elements. A simple example is 5′-cap binding protein eIF4E. A more complex example is PABP, which interacts with poly A tails, a combination of sequence (Poly A) and structure. The iron-response binding protein (IRE-BP) interacts with a sequence presented on a stem-loop and bulge (96). Alterations in the size of the loop, the distance between the loop and bulge or the loop sequence ablates binding. Given these levels of target specificity, some RBPs will no doubt be successfully targeted with therapeutics to treat human disease.

Once transcribed from genomic DNA, newly produced pre-mRNAs are immediately covered by a number of nuclear RBP to protect from degradation by nucleases, guide splicing and prepare for cytoplasmic transport. As mature mRNA are translocated, the inventory of bound proteins are often replaced with a new set of RBP that determine intracellular location, define degradation rates as well as translatability (see above) in the cytoplasm. In response to extrinsic and intrinsic stimuli, free and bound RBP are subject to post-translational modifications (PTM) (e.g., phosphorylation, ubiquitination, acetylation, and methylation) that may induce conformational changes and alter the association between RBP and target mRNA (97, 98). Depending on stimulus and cell type, the modified RBP may associate with or dissociate from mRNA, affecting the transcript stability as well as its translation, clearly affecting protein production. RBP bind to RNA *via* a variety of domains, including the so-called RNA-recognition motif (RRM), zinc finger motives, K-homology domains (KH), RGG boxes, and DEAD/DEAH boxes (97). Often more than one binding domain are present allowing simultaneous interactions with multiple mRNAs, multiple sites within one mRNA target or between specific mRNA sequences and organelles such as ribosomes or stress granules. RBP can also form higher order structures through protein–protein interactions either as homo- or heterodimers/trimers, etc. As a rule, RBP that interact with 5′ or 3′ ends of mRNA often regulate translation initiation (e.g., translation initiation factors and their partners; PABP) while those that bind to coding regions can affect translation, localization, or mRNA decay (e.g., IRE-BP). 3′ UTR RBP (e.g., AUF1, HuR, TTP, TIA-1, TIAR, FMRP, PTB, KSRP, hnRNPs, nucleolin,

and CUGBP) are most often involved in mRNA localization and decay (99).

Regulation of mRNA Binding Proteins in EOS and Their Potential Effect on Protein Translation

It is well known that many rapidly inducible mRNA coding for pro-inflammatory cytokines and oncoproteins are very short-lived. Inevitably, these mRNA contain *cis*-acting sequences into their 3′-UTR (100). The best-characterized instability determinant is composed of adenosine-uridine (AU)-rich element (ARE) repeats that are found in 3′-UTR of GM-CSF, IL-3, IL-5, IL-2, IFN-γ, and TNF-α and other cytokine mRNA. The life-span of ARE mRNA are regulated by a subset of binding proteins (AUBPs) that preferentially target the ARE and stabilize or further destabilize the transcripts. To date, approximately 20 AUBP have been identified. EOS express 7 AUBP (AUF1, hnRNP C, YB-1, nucleolin, TIA-1, HuR, and BRF1) (3) and their role in the regulation of mRNA stability has been demonstrated by many studies (101–105). In response to an exogenous pro-survival signal, Y-box binding protein 1 (YB1) and heterogeneous nuclear ribonucleoprotein C (hnRNP C) became associated with, while heterogeneous nuclear ribonucleoprotein D (hnRNP D or AUF1) dissociated from the ARE of GM-CSF mRNA (101, 106). These interactions were accompanied by the multiple phosphorylation of AUF1 (Ser83, Ser87, and Thr91) likely by ERK, CK1, GSK3β, or PKA (103, 107–109). Presumably, phosphorylation reduced the affinity of AUF1 for the ARE. AUF1 also undergoes post-transcriptional, alternative splicing events (110), yielding four AUF1 mRNAs and isoform variants (p37, p40, p42, and p45), all of which are detectable in human EOS (106). Thus, the regulatory control by AUF1 isoforms appears to be highly complex and includes their potential to form heterodimers (111) with a different affinity for ARE containing mRNAs (p37 > p42 > p45 > p40) (112, 113). While AUF1 has additional functions, its best-characterized function is to accelerate the decay of associated ARE-rich mRNAs. The p37 isoform has been shown to interact with the exosome in EOS (103) and exhibit the greatest destabilizing activity toward ARE-containing mRNAs compared to other isoforms (114).

mRNA turnover is often linked to translation (115) and the role of AUBP in RNA translation has been extensively studied in many systems. Similar mechanisms may occur in EOS although no direct evidence has yet been published. As mentioned above, PI3K/Akt/mTOR and MAPK cascades are the major signaling pathways that control global RNA translation upstream of the ribosomal machinery. These pathways have also been linked to AUBP-mediated mRNA decay in many cell types. EOS possess all translational machinery (**Table 1**) and can activate those kinase pathways when stimulated with various agonists (fMLP, RANTES, eotaxin, IL-5, IL-3, and PGE2) (30, 57). For example, ERK is activated by hyaluronic acid, IL-3 or IL-5, and likely drives AUF1 phosphorylation (102, 103), impacting the translation and decay of multiple ARE mRNA, including GM-CSF. These data suggest that ARE mRNA will be subject to translation control in EOS. This has been investigated by analysis of transfected mRNA, which has revealed striking differences in protein production despite similar cytosolic steady state levels of coding mRNA (116). Below, we discuss well-defined AUBP and their potential roles in target mRNA translation in EOS.

Heterogeneous Nuclear Ribonucleoprotein D

In eukaryotic cells, AUF1 (hnRNP D) is one of the best-characterized AUBPs and has a multiplicity of functions. It is a positive regulator of mRNA translation (117) but can also accelerate transcript decay. These two events may or may not be coupled. For example, AUF1 weakly targets Myc mRNAs for an accelerated decay but strongly promotes its translation by successfully competing with the cytotoxic granule-associated RNA binding protein TIA-1 and TIA-1-like 1 (TIAR) for a common binding site (118). Consistent with this observation, cells lacking AUF1 exhibited an increase binding of the translation-inhibitory TIA-1/TIAR to ARE mRNA, resulting in translation repression of the mRNA encoding TGF-β-activated kinase 1 (TAK1) and IL-10 (119, 120). Depending on the cell and its activation state, AUF1 can also assemble factors necessary for mRNA translation, including eIF4G, chaperones (hsp27 and hsp70), and PABP, thereby affecting translation (121–123). In EOS, eIF4G is phosphorylated by a brief (5 min) exposure to IL-5 (3), a condition that favors AUF1 phosphorylation and global protein translation (22). As EOS express high levels of PABP-C1 (major cytoplasmic PABP isoform) (3, 124), activated AUF1 may facilitate the displacement of TIA-1/TIAR by PABP-C1 and promote phospho-eIF4G-mediated translation initiation. Taken together, these results strongly indicate that modulation of translation efficiency by AUF1 is a common cellular event, which may not necessarily couple with ARE-mediated decay. Interestingly, AUF1 can also function as an inhibitor as was reported in EV71 virus translation. In this model, AUF1 binding to a stem–loop structure within IRES displaced HuR and Ago2, whose association promotes IRES-dependent translation and subsequent viral replication.

Y-Box Binding Protein-1

In EOS, an increase in YB-1 content led to the stabilization of GM-CSF mRNA. Binding was mediated through 3′ UTR ARE and resulted in increased GM-CSF translation and release with subsequent pro-survival signaling (101, 102). YB-1 can also stabilize non-ARE containing mRNA (125, 126), suggesting that it associates with other cis-elements or acts through another protein effector(s). Consistent with this notion, as the YB-1/mRNA ratio increases, so does the translation efficiency of the affected mRNA (125, 126). At high YB-1/mRNA ratios associated with maximal mRNA stabilization, YB-1 displaces eIF4F from the messenger ribonucleoprotein (mRNP) complex, possibly inhibiting the translation of the stabilized mRNA (127). This mechanism was not observed in EOS for GM-CSF expression, however (101, 102), which may reflect the ordinarily high basal levels of YB-1 in these cells. Thus, it is not entirely clear how endogenous AUBP such as YB-1 influence eIF4F-mRNA interactions and regulate mRNA stability and translation in these cells. In cells, at low YB-1/mRNA ratios, eIF4F is known to bind effectively to mRNA near the 5′ cap-structure and drive translation (125, 126). YB-1 can

be phosphorylated at a single site (Ser316) within the C-terminal domain (CTD) by multiple kinases (Akt, ERK2, GSK3-β, and JNK) (3), which leads to increased IL-2 mRNA stability and cytokine production (128). YB-1 binds to mRNA as a monomer through the cold-shock domain (CSD) and the CTD (125), which can unfold mRNA secondary structures, likely facilitating interactions with the translation initiation machinery. Inhibition of translation is mainly attributed to the CTD. Similarly to the full length YB-1, CTD displaces eIF4G from mRNP while the CSD displaces eIF4E, eIF4A, and eIF4B by interacting with the 5′-Cap-structure or with its adjacent region (125, 126). After EOS exposure to IL-5, eIF4G (Ser1238, Thr1218, and Ser1194) and eIF4B (Tyr233, Ser406, Ser359, and Ser459) are rapidly phosphorylated (3). YB-1 can also be phosphorylated by Akt (98, 129, 130), which lowers its affinity for the 5′-cap-structure (or/and adjacent mRNA region) (130). This may also facilitate the assembly of the translation initiation complex. Of note, circadian changes of YB-1 binding to GM-CSF mRNA have been observed in circulating EOS from subjects with nocturnal asthma, with lower YB-1/GM-CSF mRNA interaction at 04.00 a.m., suggesting possible increased GM-CSF protein production and EOS activation at night (131).

Heterogeneous Nuclear Ribonucleoprotein C

Heterogeneous nuclear ribonucleoprotein C has been predominantly associated with the regulation of mRNA stability although several reports describe translational regulation through 5′ UTR interactions (132–135). This function was first identified in rabbit reticulocyte lysate supplemented with exogenous hnRNP C. Those studies revealed hnRNP C bound to a non-ARE domain, stabilizing APP mRNA and increasing its translation (132). In neurons, hnRNP C and FMRP were shown to compete for binding to a coding region element of APP mRNA that modulated APP mRNA translation in opposite directions (136). Further study clarified that increased APP translation by hnRNP C was accompanied by enhanced mRNA polyadenylation, which was mediated by a functional IRES found in the 5′ UTR of the transcript (137). Thus, the mRNA-specific translational activation by hnRNP C is generally independent of ARE and is through interactions with distinct 3′ or coding region (132, 136) target sequences, IRES (133), 5′ UTR, or heptameric U sequence in IRES (138). Whether similar mechanisms occur in EOS is unknown although hnRNP C was reported to bind GM-CSF mRNA and associated with transcript stability (103). To date, neither cytoplasmic kinases nor phosphosites on hnRNP C have been identified although several RNA-dependent protein kinases (PKA, PKC, CDK-II, and PKR) have been associated with hyperphosphorylation of hnRNP C1 (small isoform of hnRNP C) in nuclear extracts (139).

Other AUBPs

HuR (stabilizer of ARE mRNA) and TIA-1 (U-rich binding protein) bind to GM-CSF and TGF-β mRNA and are associated with transcript stability in EOS. While the role of HuR in mRNA translation has not been reported, TIA-1 is believed to repress the translation of TNF-α (140), COX-2 (141), cytochrome c (142), and 5′ TOP mRNAs (143). TIA-1 binds to the ARE of TNF-α mRNA, but has no effect on the mRNA decay. Instead, TIA-1 represses TNF-α translation by promoting its sequestration in non-polysomal mRNP complexes or the so-called stress granules (144). TIA-1 can also recruit multifunctional RBP, including PTB, La, hnRNP K, and hnRNP A1, all of which are expressed by EOS (145). However, it remains unknown whether this recruitment is associated with TIA-1-mediated translational repression. TIA-1 can be phosphorylated by FASTK but the phosphorylation sites have not been mapped (146, 147). On a similar note, the mRNA stabilizing protein, Sjögren syndrome type B antigen (SSB or La) plays a unique role in translation initiation (148–151). La is largely nuclear but acts as an RNA chaperone in the cytoplasm when translation starts. La binds in close proximity to the translation start site and unwinds second structure of mRNA to expose embedded AUG start codons. Similar actions were also observed for the translation of virus-encoded mRNA (152–154). This unique feature of La is critically important in facilitating translation initiation because the translation start sites of certain mRNA are buried in strong stem–loop or secondary structures and are not efficiently recognized by the scanning 43S ribosomal subunit. La is phosphorylated on Thr301, Ser366, and Thr389 by AKT and CK2 (151, 155, 156), which contributes to its nuclear or cytoplasmic distribution (157).

REGULATION OF TRANSLATION AND POTENTIAL THERAPEUTIC TARGETS

Endogenous GM-CSF Effects on EOS Biology and the Use of Pin-1 As a Potential Therapeutic Target

We have described above how RBP regulate mRNA stability and translatability, particularly of GM-CSF mRNA in EOS. GM-CSF plays a pivotal role in the modulation of EOS differentiation, function, and survival. The cytokine is upregulated in eosinophilic diseases and a major contributor to enhancing EOS survival in the lungs of patients during active asthma (158). Recombinant GM-CSF promotes EOS survival about five times as potently as equal concentrations of IL-5 (159). In asthmatics, GM-CSF is produced by a wide spectrum of cell types, including lung epithelial cells, lymphocytes, alveolar macrophages, EOS, endothelial cells, and fibroblasts. As EOS typically increase by 20-fold in the lung within a few days of an allergen challenge (160), autocrine GM-CSF is an important source in order to support survival. The level of endogenous GM-CSF in BAL fluid is low (161–163) compared to IL-5 in both mice and humans (164). However, intranasal delivery of Adeno-GM-CSF to the airways of OVA-sensitized mice resulted in sustained accumulation of various inflammatory cell types, most noticeably EOS, in the lung for more than 2 weeks post OVA aerosol challenge (165, 166). Conversely, neutralization of endogenous GM-CSF during aeroallergen exposure significantly inhibited eosinophilic inflammation and airway hyper-responsiveness. This suggests that small amount of endogenous GM-CSF can significantly contribute to the development and persistence of eosinophilic airway inflammation. *In vitro*, purified peripheral blood EOS synthesize small amounts (~1 pg/ml) of anti-apoptotic GM-CSF

(167, 168), after stimulation with a variety of factors (fibronectin, hyaluronic acid, TNF-α, IL-3, IL-5, IL-15, integrins, IFN-γ, calcium ionophore, cross-linking of cell surface molecules) (67, 102, 167, 169–174). Activation-induced survival was blocked by the addition of neutralizing anti-GM-CSF even 2 days after the initiation of culture, indicating that the cells continuously release low levels of GM-CSF on which survival depends (103, 172–174). Similarly, the majority of BAL EOS obtained 48 h after segmental allergen challenge died *in vitro* at 6 days in the presence of neutralizing anti-GM-CSF. Both *in situ* hybridization (tissue EOS) (175) and qPCR (purified EOS) (67, 172, 173) analyses have demonstrated that increased GM-CSF mRNA was associated with GM-CSF protein secretion and prolonged EOS survival.

Pin1

While all ARE mRNA have relatively short half-lives (20 min-2 h), GM-CSF mRNA is extremely labile ($t1/2 < 6$ min) in resting EOS but show significantly increased stability (increased by fourfold to sixfold) after cell activation. This conversion likely reflects alterations in the composition of interacting AUBP (67, 103, 172, 173). Multiple AUBP, including AUF1, HuR, YB-1, and hnRNP C associate with and regulate the decay of GM-CSF mRNA in EOS (101–103). Cell activation triggered occupancy of GM-CSF mRNA by YB1, hnRNP C, and HuR, which displaced AUF1. ERK-mediated phosphorylation likely caused a decrease in affinity for GM-CSF mRNA by AUF1 (103), which led to remodeling of the GM-CSF mRNP complex. Co-immunoprecipitation and gene knockout studies have found that Pin1, a *cis-trans* peptidyl prolyl isomerase, interacts with multiple AUBPs, including AUF1, HuR, KSRP, SLBP, and the translation regulators eIF4E and 4E-BP1/2 (103, 176–178). Pin1 is essential for cell-cycle progression through interactions with cyclinD (179). Pin1 is the only known eukaryotic isomerase with specificity for Ser-Pro or Thr-Pro peptide bonds. Isomerization is bidirectional with *cis* to *trans* or *trans* to *cis* conversions but occurs approximately 1000-fold faster when the N-terminal Ser or Thr has been phosphorylated (180). Structurally, Pin1 has two domains, including a ~40 amino acid *N*-terminal WW domain and a C-terminal isomerase domain. The WW domain binds pSer/pThr-Pro motifs while the catalytic domain is responsible for substrate isomerization. Pin1-mediated isomerization has profound effects on target-protein folding, altering subsequent protein–protein and protein–nucleic acid interactions, protein stability and subcellular localization thereby altering a variety of cellular processes, including cell cycle progression, apoptosis, innate and acquired immunity, and gene regulation. We showed that Pin1 was reproducibly pulled down with AUF1 in human EOS and T cells irrespective of cell activation (103, 105). Cell activation also increased Pin1 activity, which likely isomerized phosphorylated AUF1. These events occurred with a simultaneous reduction of AUF1 binding to GM-CSF mRNA. Conversely, inhibition of Pin1 reduced isomerase activity, reconstituted the AUF1–GM-CSF mRNP complex and accelerated transcript decay. Consistent with this *in vitro* data, EOS obtained from the blood or BALF from patients with active asthma showed significantly elevated Pin1 isomerase activity. *In vivo* Pin1 blockade significantly reduced pulmonary EOS counts, GM-CSF production, and cell viability in rat models of asthma (181). These observations indicate that Pin1 is a critical regulator of GM-CSF mRNA turnover and production, which in turn controls the survival of activated EOS in the lungs of asthmatics.

In addition to its role in mRNA stabilization, Pin1 signaling amplifies or suppresses the action of kinases, phosphatases, transcription factors, cell cycle regulators, and apoptotic effectors (124, 180). This broad targeting specificity of Pin1 arises from its short consensus target (pSer/pThr-Pro) as well as the phosphorylation frequency of S/T-P sites, which are found in numerous proteins. Pin1 activity can be modulated either positively or negatively without change in protein content, in response to injury or environmental cues. Chronic activation or suppression can be pathologic as seen in immune disorders, fibrosis, cancer, and neurodegeneration (105, 182, 183). Specifically, Pin1 overexpression or amplification is highly correlated with cancer progression and metastasis while Pin1 loss is seen in evolving Alzheimer Disease. Pathology may result from loss of regulation of RBP with alterations in cytokine mRNA stability and translation. Thus, pharmacologic modulation of Pin1 activity with small molecule inhibitors may provide a novel approach to eosinophilic diseases, such as asthma. Unfortunately, current Pin1 inhibitors lack specificity or are excessively toxic.

IL-3 Signaling in EOS

TPI ASM8 is a drug in development, targeting the common β-chain receptor for all IL-5, GM-CSF, and IL-3, in the form of RNA-targeted inhaled oligonucleotide antisense phosphorotioates (184). Although TPI ASM8 seems to be well tolerated and leads to some reduction of EOS and eosinophilic hematopoietic progenitor ($CD34^+IL5R^+$), other alternative therapeutic targets more specific to each of the 3 cytokine should be developed.

So far, we have identified semaphorin-7A and FCGR2B/C (CD32) as specific genes exclusively responding to IL-3 activation *via* prolonged ERK/p90S6K signaling. It is probable that additional genes are similarly regulated at a translation level by IL-3/ERK/p90S6K. Likely other candidate genes may share specific mRNA *cis*-elements whose identity may be inferred by homology searches among IL-3 upregulated mRNA. We started analyzing how semaphorin-7A or FCGR2 affect EOS function. We found that IL-3-activated EOS adhere to the only known semaphorin-7A ligand, plexin-C1 (27). Plexin-C1 is expressed by many cell types, including lymphocytes, monocytes, dendritic cells, and neutrophils (185), and has an important role in the migration of these inflammatory cells. Plexin-C1 is also expressed by stromal cells (186), which could facilitate migration or activate EOS in fibrotic tissue. Interestingly, IL-3-activated EOS migration on plexin-C1 was largely resistant to semaphorin-7A blockade while neutralizing anti-αMβ2 integrin were far more inhibitory (187). Migration in the absence of chemotaxis indicates that a haptotaxis process is operative for plexin-C1- or periostin-mediated migration (187, 188). Semphorin-7A signaling may also skew fibroblasts toward a pro-fibrotic, more mesenchymal phenotype (27, 189, 190), although we recently demonstrated anti-fibrotic functions for endogenous semaphorin-7A expressed by lung fibroblasts (191).

The upregulation of CD32 by IL-3 on EOS has a profound impact on EOS function. EOS-driven pathology in tissue requires both EOS migration from circulating blood to the site of inflammation and the release (degranulation) of preformed toxic proteins and mediators of the inflammation. IL-3-activated EOS strongly degranulate on heat-aggregated (HA)-IgG, with extrusion of ~25% of their total cellular eosinophil-derived neurotoxin (EDN) in 6 h (94) compared to less than 10% after IL-5 (94). Degranulation on HA-IgG was CD32-dependent (94). Thus, IL-3 and its downstream intracellular effectors may be potential therapeutic targets to limit EOS degranulation and EOS-driven pathologies. The use of anti-IL-5 therapies on patients with severe eosinophilic asthma has reduced asthma exacerbations and blood eosinophilia (192, 193), *see other article by Nair in this issue*. However, airway EOS are still present and active despite treatment (194, 195). This partially reflects loss of the surface IL-5 receptor expression by airway EOS (22, 196, 197). Conversely, IL-3 and the surface IL-3 receptor are upregulated and highly expressed on airway EOS (27, 198). Therefore, combined targeting of the IL-3 and IL-5 pathways may provide additive or synergistic benefits.

Ribosomal S6 Protein

Whether RPS6 phosphorylation in EOS induces a unique profile of proteins (e.g., semaphorin-7A, CD32, etc.), downstream of IL-3/ERK/P90S6K signaling, is unclear. If so, phospho-RPS6 would be a possible therapeutic target to reduce EOS-related pathology. On the positive side, knock-in mice lacking the ability to phosphorylate RPS6 have modest deficits (199) and show limited changes in global protein synthesis *in vivo* and in embryonic fibroblasts (78). However, β-cell development may be adversely affected by RPS6 knock-in (78). RPS6 phosphorylation can be blocked in EOS by small molecules inhibitors of p90S6K, such as BI-D1870 (200). However, the consequences of p90S6K inhibition probably include transcriptional silencing, blockade of cell proliferation, and cell death (87, 201). Thus, while potentially attractive, inhibition of this pathway remains hypothetical.

CONCLUSION

The three β-chain cytokines, IL-3, IL-5, and GM-CSF are all present in human eosinophilic diseases and have both highly redundant and yet critically unique roles in the EOS biology. Their signaling affects differentiation, maturation, survival, migration, piecemeal release of immune-mediators, and degranulation. IL-3 is unique among the β-chain cytokines in generating prolonged intracellular signaling leading to the translation of a subset of EOS mRNA. Signaling requires ERK and p90S6K activation and culminates in the phosphorylation of RPS6. The control of both translation and decay of cytokine mRNA ultimately involves an interplay between mRNA-BP, especially those that target ARE. The AUBP in turn are often regulated by the action of Pin1, leading to multi-level control over cytokine gene expression. Critical, unanswered questions include the identification of RPS6-dependent mRNA as well as additional Pin1 RBP interactors and whether drugs can be developed to target these important pathways.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Proteomics of Eosinophil Activation

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We recently identified and quantified >7,000 proteins in non-activated human peripheral blood eosinophils using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) and described phosphoproteomic changes that accompany acute activation of eosinophils by interleukin-5 (IL5) (1). These data comprise a treasure trove of information about eosinophils. We illustrate the power of label-free LC–MS/MS quantification by considering four examples: complexity of eosinophil STATs, contribution of immunoproteasome subunits to eosinophil proteasomes, complement of integrin subunits, and contribution of platelet proteins originating from platelet–eosinophil complexes to the overall proteome. We describe how isobaric labeling enables robust sample-to-sample comparisons and relate the 220 phosphosites that changed significantly upon treatment with IL5 to previous studies of eosinophil activation. Finally, we review previous attempts to leverage the power of mass spectrometry to discern differences between eosinophils of healthy subjects and those with eosinophil-associated conditions and point out features of label-free quantification and isobaric labeling that are important in planning future mass spectrometric studies.

Keywords: eosinophils, mass spectrometry-based proteomics, phosphorylation sites, interleukin-5, STAT3, integrins, immunoproteasome

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INTRODUCTION

Eosinophils derive from precursors set aside early in hematopoietic differentiation (2) and are easily identified in a Giemsa-stained blood smear by their abundant plump red granules and bilobed nucleus. Eosinophils have nuanced roles in normal physiology and responses to injury or pathogenic agents (3, 4), contributing to tissue homeostasis in the gut and adipose tissue and featuring prominently in inflammation associated with allergic diseases, malignancies, viral and helminthic infections, and orderly tissue repair (4–8). Eosinophils have the potential to participate in the pathogenesis of disease by diverse mechanisms, including release of a unique set of granule components, secretion of cytokines, and elaboration of mediators (3, 4). The need for better understanding of eosinophils in the context of eosinophil-associated diseases was highlighted in the report of a taskforce assembled by the National Institutes of Health (9).

Eosinophils were not among the >200 tissues, cell lines, and purified cell populations analyzed to assemble draft human proteomes published in 2014 (10, 11). We recently reported two high-resolution mass spectrometric investigations of human peripheral blood eosinophils: (1) identification and quantification of the proteins of non-activated eosinophils and (2) description of phosphoproteomic changes that accompany acute activation by interleukin-5 (IL5) (1). These data represent important information about eosinophils. An explicit goal of this review is to facilitate

access to and increase the usefulness of the data presented in supplementary spreadsheets of our paper.

QUANTITATIVE ANALYSIS OF THE EOSINOPHIL PROTEOME

The workhorse of modern global proteomics is reversed-phase liquid chromatographic (LC) separation of proteolytically generated peptides coupled to online nano-electrospray ionization of the effluent and identification of peptide mass/charge (m/z) and sequence by tandem mass spectrometry (MS/MS) (12). Peptide cations detected in the MS^1 scan are subjected to dissociation and subsequent MS^2 scan, yielding an ion series that can indicate amino acid sequence and the presence of post-translational modification. The resulting spectra are compared with peptides generated *in silico* to determine which of the $\sim 2 \times 10^4$ encoded human proteins and many more proteoforms (13) arising from differential mRNA splicing are present in the sample based on peptide spectral matches. To increase the probability of identification of any given peptide, peptides can be fractionated by a preliminary LC separation after which each fraction is analyzed in a separate liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) run. This paradigm routinely identifies thousands of proteins and proteoforms from biological samples (10–12). Estimates of relative abundance of identified proteins, called label-free quantification (LFQ), can be made by intensity-based absolute quantification (iBAQ), which sums signal intensities of all identified peptides for a given protein and divides by the number of theoretically observable peptides based on the *in silico* digest (14). The technology has been improving continuously to increase proteome coverage, speed of analysis, and quality of data with the goal of increasing applicability to biological experimentation and clinical samples. For instance, our group is able to quantify nearly 90% of the estimated 4,500 proteins in the yeast proteome in ~1 h of analysis (12, 15).

We assembled a map of the proteome of 75×10^6 non-activated peripheral blood eosinophils pooled from three different human volunteer donors with allergic rhinitis or asthma (1). Heparinized blood, 200 mL, was obtained from each, granulocytes were isolated by centrifugation in a Percoll gradient, and eosinophils were isolated from the granulocyte fraction by negative selection with magnetic beads bearing antibodies to CD3, CD14, CD16, and glycophorin-A (16, 17). Cells were lysed *via* probe sonication in a urea buffer, and proteins were digested with trypsin. Phosphopeptides were enriched by immobilized metal affinity chromatography (IMAC). Non-enriched and enriched samples were separated by high pH reversed-phase chromatography into 30 and 20 fractions, respectively, and fractions were analyzed by LC–MS/MS on an Orbitrap Fusion. The UniProt human proteins-plus-proteoforms database as of April 4, 2014, was queried using MaxQuant with the Andromeda search engine that included the iBAQ algorithm (14, 18, 19), yielding iBAQ intensities that can be translated into absolute molar abundances by assuming direct proportionality.

We identified 7,086 proteins based on 100,892 different tryptic peptides (1). Estimates of cellular abundance correlated well with the intensities of the protein spots seen in the two-dimensional gels of an earlier published proteomic study (20), with actin being the most abundant protein in both. The 15 most abundant proteins accounted for 25% of protein molecules. These include the granule proteins RNASE2 (eosinophil-derived neurotoxin), RNASE3 (eosinophil cationic protein), C-terminal remnant of PRG2 (major basic protein 1), C-terminal remnant of PRG3 (major basic protein 2), and CLC (Charcot-Leyden crystal protein, galectin 10); proteins associated with actin cytoskeleton (ACTB, PFN1, and CFL1); and histones. The abundances (molecules per eosinophil) of RNASE2 (1.8×10^8), RNASE3 (2.5×10^8), PRG2 (6.4×10^8), and eosinophil peroxidase (1.7×10^8) previously had been quantified by radioimmunoassay (21), thus allowing calculations of the absolute abundances of other proteins. The iBAQ intensities in Sheet 1 of the paper's supporting XLSL file entitled "Summary of proteins identified in global analysis… ordered from most to least abundant" ranged from 1.3×10^{11} for ACTB (cytoplasmic actin) to 3.1×10^3 for KIAA1211 (1). The ratios of the iBAQ intensities to cellular abundances of the four granule proteins average 360, and division by this number can be used to convert iBAQ score to molecules per cell. We also localized 4,802 sites of phosphorylation as described in the paper's supporting XLSL file entitled "Summary of phosphosites identified in global analysis…" (1).

Selected entries from the "global analysis" file have been pasted into Sheet 1 of the XLSX in the supplement of this review. A single entry may describe a single protein or a group that may consist of proteins of the same or nearly identical sequence encoded by separate genes, as for several of the histones; different proteoforms encoded by a single gene; or a single proteoform. In addition, frequently peptides will be matched to several entries in a protein database rather than to a single group (22). Each entry, therefore, contains information about rank in abundance; UniProt ID(s) of all proteins and majority proteins in the group; protein names(s); gene name(s); number of proteins in the group; number of peptides matching the group; number of peptides defined as "razor," i.e., specific for the protein group, and "unique," i.e., specific for a given proteoform within the group; % sequence covered by the identified peptides; molecular weight and sequence length of the longest proteoform within the group; posterior error probability of misidentification of the protein group; sum of peptide ion intensities; and iBAQ intensity score.

To drill down and exploit this information, one needs to consult UniProt[1] and, because UniProt is uneven in its annotation of possible proteoforms, one may need also to consult the literature and transcriptomic and genomic databases and perhaps to perform directed experiments. We illustrate such issues with the entries on eosinophil STATs in Sheet 1. STAT2, STAT5A, STAT5B, and STAT6 are encoded by separate genes, and each has a single entry. The entries for STAT2, STAT5A, and STAT6 describe groups of two or three proteoforms differentially spliced at or near the N-terminus that cannot be distinguished by the available proteomic data. STAT5A and STAT5B share sequence similarity such that of the identified peptides, 23 are assigned to both, 13

[1] http://www.uniprot.org/.

are unique to STAT5B, and 11 are unique to STAT5A. STAT1 and STAT3 each have two entries that describe differentially spliced proteoforms originating from single genes. For STAT1, the dominant proteoform was canonical 750-residue STAT1α, and the minor proteoform was 712-residue STAT1β with a truncated C-terminus due to a frameshift introduced by splicing. The analysis identified four peptides unique for STAT1α and one unique for STAT1β. The dominant proteoform of STAT3 was 770 residues in length, and the minor proteoform was 769 residues; these each have a single unique peptide in which Ser701 is present (S) or absent (ΔS). STAT3, such as STAT1, is subjected to splicing that generates α and β proteoforms, as was first observed at the transcript level in eosinophils (23). However, our analysis did not identify a peptide spectral match unique for STAT3β. The splicing events responsible for inclusion (S) or exclusion (ΔS) of the codon for Ser701 and the α or β variants are close to one another, such that we were able to use quantitative PCR to demonstrate the presence and proportions of the four possible STAT3 transcripts, S-α, ΔS-α, S-β, and ΔS-β, in eosinophils (24). In accord with the iBAQ data, ΔS-encoding transcripts were in the minority. We note that even if a tryptic peptide defining the β variants had been detected, we would not have known whether the peptide was derived from the S or ΔS variant or a mixture. Examination of the amino acid sequences of the four splice variants, however, indicates that such information likely could be obtained by substituting AspN protease for trypsin. AspN should generate four different peptides that span the sequences determined by the two splicing events.

The iBAQ intensities in Sheet 1 inform thinking about the complexity inherent in signaling by different eosinophil STATs. The intensities and hence abundances of STAT1, STAT3, and STAT5B are similar, with approximately 600,000 copies of each protein per eosinophil based on comparisons to the iBAQ intensities of the four granular proteins. STAT6 and STAT2 were present at approximately 70 and 12% the abundances of the three major STATs but at greater abundance than STAT5A. The complete "global analysis" file (1) allows comparisons of the abundances of numerous other classes of eosinophil proteins that have similar and perhaps overlapping functions, such as the tyrosine kinases that activate STATs.

The other entries in Sheet 1 concern proteasome subunit beta-type (PSMB) subunits of the 20S proteasome and illustrate the power of quantitative proteomics in dealing with complexes with known structure and stoichiometry. Such complexes account for a considerable fraction of the proteome (25) and are described on the CORUM website.[2] The PSMB5, PSMB6, and PSMB7 subunits of the constitutive 20S proteasome are replaced by the PSMB8, PSMB9, and PSMB10 subunits of immunoproteasomes in T-cells and monocytes (11). The switch involves the three catalytic proteasome subunits and results in preferential generation of peptides with a hydrophobic C-terminus that can be processed to fit in the groove of MHC class I molecules (26). The ratios of iBAQ intensities of PSMB8/PSMB5, PSMB9/PSMB6, and PSMB10/PSMB7 for eosinophils are 18, 3.7, and 2.1, respectively, comparable to the values of 31, 2.9, and 2.1 reported for monocytes in ProteomicsDB[3] [an easily navigated repository of human proteomics data (10)]. The comparable enrichment in immunoproteasome subunits in monocytes and eosinophils bears on the issue of whether eosinophils are important antigen-presenting cells (27).

Figure 1 illustrates a second example of insights to be gained from quantitative proteomics. Shown are our data and recently published transcriptomic RNA-Seq data (28) for the eight α-integrin (ITGA) and four β-integrin (ITGB) subunits detected in eosinophils. Lines connect the nine αβ dimers (29) that are possible between these subunits, and iBAQ intensities and mRNA abundance as RPKM (reads per kilobase per million mapped reads) are given (**Figure 1**). Several features are noteworthy. First, the iBAQ intensities are compatible with the proposed pairing of dimers. Second, the iBAQ intensities in general correlate with mRNA abundance. Third, protein and mRNA are missing for ITGAD, which is inconsistent with the prevailing view that there is a pool of αDβ2 that can be mobilized acutely to the eosinophil surface (29, 30). Fourth, the ITGA2B and ITGB3 subunits of αIIbβ3, the major integrin of platelets, are abundant as proteins but not as mRNA.

Issue of Contaminating Platelets

Platelets, which adhere to a fraction of circulating eosinophils (31, 32), carry an idiosyncratic mix of RNAs (33). To investigate whether the higher-than-expected abundance of ITGA2B and ITGB3 as proteins but not as transcripts was due to contamination by platelets, we purified eosinophils by negative selection with antibody to ITGB3 in addition to the standard antibody "cocktail" described above. We then compared the proteomes of purified platelets, eosinophils purified by the standard method, and

[2]http://mips.helmholtz-muenchen.de/corum/.

[3]https://www.proteomicsdb.org/.

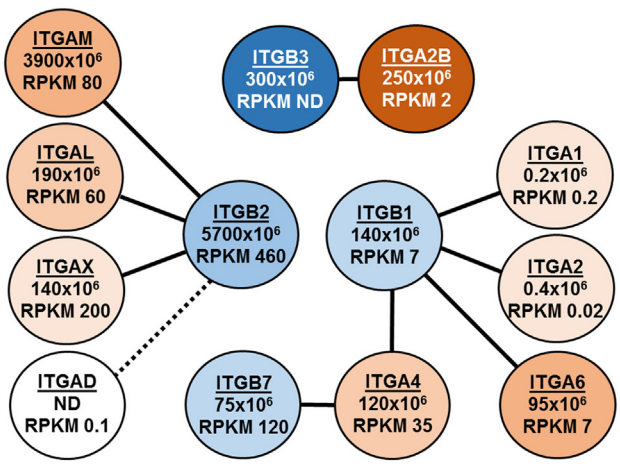

FIGURE 1 | Depiction of integrin expression in eosinophils as assessed by intensity-based absolute quantification (iBAQ) intensities and RNA-Seq. Lines connect the nine αβ dimers that are possible between these subunits, and iBAQ intensities and mRNA abundance as RPKM (reads per kilobase per million mapped reads) are given.

eosinophils purified with the extra depletion with anti-ITGB3 (1). ITGA2B and ITGB3, along with several platelet granule specific proteins, were outliers in a plot of ratios of standard eosinophils versus depleted eosinophils on one axis and platelets versus depleted eosinophils on the other. These data, which are compiled in our paper's supporting XLSL file entitled "Summary of LFQ of eosinophil, platelets, and platelet-depleted eosinophils…" (1) can serve as a guide to assess platelet contamination in future proteomic studies of eosinophils.

PHOSPHOPROTEOME OF UNSTIMULATED AND IL5-STIMULATED EOSINOPHILS

We used 10-plexed isobaric labeling to identify phosphorylation sites that change in eosinophils acutely activated with IL5. The advantage of isobaric labeling over LFQ is that if a phosphopeptide is identified, its relative abundance in relation to the same peptide in other samples can be estimated (34). Half of 20×10^6 cells collected from each of five donors remained non-stimulated, and half were incubated for 5 min with IL5, 20 ng/mL. Incubations were stopped by plunging the tubes into liquid nitrogen. The 5-min stimulation induces maximal polarization and activation of MAPK1/3, STAT1, and STAT5 (17). When all 10 samples had been collected, cell pellets were thawed, cells were lysed, trypsin was added, and peptides from each sample were labeled separately with one of a 10-chemical set of tandem mass tags. Each of the 10 tags had the same mass, allowing the same peptides from multiple samples to be observed during the MS^1 scan as a single m/z peak and isolated together for fragmentation. Each tag, however, had a unique distribution of heavy isotopes such that each tag yields a unique reporter ion upon fragmentation, and thus the relative amounts of the peptide in different samples can be determined based on relative intensities of the reporter ions in the MS^2 scan. Peptides were enriched for phosphorylated peptides using IMAC before LC–MS/MS, and both unenriched and enriched peptides were analyzed. The Open Mass Spectrometry Search Algorithm (OMSSA) search algorithm was used along with our in-house software suite Coon OMSSA Proteomic Analysis Software Suite (COMPASS) (35, 36). Phosphorylation localization was performed with the Phospho RS 3.0 algorithm implemented into COMPASS. Statistical significance was determined using a two-tailed and equal variance t-test, $n = 5$.

Results from the isobaric labeling study were tabulated in Data Sheet in Supplementary Material entitled "Summary of proteins and phosphosites quantified in comparative analysis of unstimulated and acutely activated eosinophils" (1). The numbers of identified proteins (4,446) and phosphosites (1,819) were less than in the label-free analysis described above. The comparison of five individuals afforded the opportunity to assess individual-to-individual variation in protein abundances. The major differences were in HLA proteins. As would be expected given the short time of stimulation, only 16 proteins (0.3%) changed significantly ($p < 0.001$) between the resting and activated states. In contrast, 220 phosphorylation sites (12.1%) in 171 proteins changed significantly ($p < 0.001$) upon activation, 173 increasing and 47 decreasing (**Table 1**). Motif-X (37, 38) identified recurrent

TABLE 1 | Summary of phosphorylation changes described in Sheet 2 of the Supplementary XLSX.

Process	Sites	Proteins	Up	Down	Notable examples
Chromatin	7	7	7	0	Known CDK2 site in BAP18
Replication	2	2	2	0	–
Transcription, templated	3	3	3	0	pS300 of LRRFIP1 up 22-fold
Transcription, general	11	10	9	2	pS43 of PBXIP up 12-fold
mRNA, splicing	17	14	10	7	4 decreased sites in SRRM2
mRNA, nuclear export	3	2	3	0	2 increased sites in ZC3H11A
mRNA, translation	9	8	5	4	pY233 of EIF4B up 13.6-fold
miRNA, processing	3	1	3	0	Single region of DDX17 helicase
Signaling, kinase	18	14	14	4	pS226 of MAP2K2 up 3.1-fold
Signaling, phosphatase	5	5	2	3	pY546 of PTPN11 up 20-fold
Signaling, scaffold	13	12	12	1	pS1134 of SOS1 up 6.4-fold
Signaling, small GTPase	21	19	17	4	S1834 of DOCK5 up 9.1-fold
Signaling, PI	7	6	5	2	pS1259 of PLCG2 up 9.1-fold
Signaling, ubiquitin	7	6	7	0	2 increased sites in HECTD1
Cytoskeleton, IF	11	5	10	1	6 sites in VIM including pY11
Cytoskeleton, microfilament	34	17	31	4	Multiple sites in EVL and RCSD1
Cytoskeleton, microtubule	7	7	4	3	pT154 of MAPRE1 up 3.1-fold
Vesicle-related	12	12	7	5	pT154 of PACS up 15-fold
Podosome-related	2	1	2	0	2 sites in BIN2
Membrane protein	5	5	2	3	pS405 of SELPLG tail up 5.9-fold
Metabolic	7	3	5	2	4 sites in NCF1
Unknown	16	12	14	2	5 sites in NHSL2

The entries have been parsed for numbers of changed sites and proteins harboring the sites associated with each process and numbers of sites for which the phosphorylation increased or decreased. In addition, noteworthy examples of changes are given for all except one of the processes.

PI, phosphotidyl inositol; IF, intermediate filament.

In Sheet 2, the phosphorylated residues in FAM21B, LMNB2, PI4KA, and ARL6IP4 are renumbered compared with the entries for these proteins in original paper. The renumbering is in response to changes in the annotations of these proteins in UniProt.

increased phosphorylation of Ser or Thr in motifs that are targets for MAPK or CAMIIK kinases.

The top 18 most upregulated sites (8- to 25-fold increase) included phosphorylation of Tyr546 of tyrosine-protein phosphatase non-receptor type 11 (PTPN11, also known as SHP2) and Ser5 of plastin-2 (LCP1) both known to be critical early activation events in eosinophils (39, 40); Ser1259 of phospholipase C-γ2 (PLCG2); and Ser320 of $p47^{PHOX}$ (NCF1), which controls activation of the respiratory burst oxidase (41). Sheet 2 lists all 220 sites with information about gene name, residue modified, fold change, *p*-value for the change, iBAQ intensity of the protein in the "global analysis" XLSX of our paper (1), name of protein, assignment of the protein to a single pathway or function, and implications of the phosphorylation. The last two determinations were made after inspection of information on the protein organized in UniProt and PhosphoSite.[4] Significant changes were found in proteins that varied in abundance by as much as 22,000-fold. Only 13 of the significantly changed sites are unknown, i.e., not presently described in PhosphoSite. The proteins were assigned to 22 different processes (**Table 1**), and a notable example of a changed site is given for all except one category. The significance of the changed phosphorylation site varies from obvious to obscure. For instance, the increased phosphorylation of S226 of MAP2K2, the dual specificity kinase that activates MAPK1/3 (ERK2/1), involves one of the serines in the activation loop targeted for *O*-acetylation by *Yersinia* YopJ, a modification that inactivates MAP2K2 (42). In contrast, the four sites of decreased phosphorylation in SRRM2, a highly repetitive nuclear matrix protein involved in mRNA splicing, constitute a miniscule subset of the >280 sites listed in PhosphoSite as being phosphorylated in SRRM2. Changes in proteins associated with cytoskeleton were the most common but accounted for <25% of changed sites. Overall, the data are perhaps best interpreted as a snapshot at 5 min of a cell that is activated by IL5 to undergo simultaneous shape change, oxidative burst, new gene transcription, new mRNA processing and translation, and extensive shuttling of components among membrane compartments.

PROSPECTS FOR THE FUTURE

We have identified and quantified 7,086 proteins associated with non-activated peripheral blood eosinophils and demonstrated significant changes in 220 phosphosites in response to IL5 (1). For comparison, 10,225 proteins have been identified in HeLa cells (43), 7,952 in human embryonic stem cells (36), and ~4,200 in human platelets (44). Can the analyses of eosinophils be improved, what are the relative advantages of LFQ versus multiplexed isobaric labeling, and can such analyses lead to a better understanding of eosinophils in the context of eosinophil-associated diseases?

Our global data constitute a "version 1.0" of the eosinophil proteome that surely merits a "version 2.0." More comprehensive coverage is important for finding peptides that define presently poorly characterized proteins and proteoforms such as ΔS and β variants of STAT3 described above and enabling one to exploit ongoing refinement of the human proteins-plus-proteoforms databases against which peptide sequence matches are made. Analyses of peptides generated by proteases other than trypsin such as chymotrypsin, LysN, LysC, AspN, and GluC can greatly increase % coverage of the sequences of identified proteins as well as increasing the number of identified proteins (12, 45). Such deep coverage requires preliminary fractionation of peptides and multiple LC–MS/MS analyses of the fractions. About 10^8 purified eosinophils are needed, necessitating pooling of eosinophils from multiple donors inasmuch as only 2.5×10^7 eosinophils will be purified from 200 mL of blood from a donor with a high normal eosinophil count of 250/μL if the yield is 50%. Our experience indicates that the analysis should be done on eosinophils purified by negative selection with antibody to ITGB3 in addition to antibodies to CD3, CD14, CD16, and glycophorin-A. We emphasize that no analysis will be ideal. The negative selections cannot remove other cell types completely, and the deeper one goes into the proteome the greater the chance of finding proteins from contaminating components of blood. In addition, workflows that avoid membrane-disrupting detergents, as ours does, may miss multipass membrane proteins with short loops and tails.

Prior proteomic studies have described alterations in amounts of eosinophil proteins in subjects with atopic dermatitis and eosinophilia (46), mildly elevated eosinophil counts associated with seasonal birch pollen allergy (47), and eosinophilia associated with *Fasciola hepatica* infection (48). In these studies, eosinophil proteins from affected individuals or healthy controls were separated by two-dimensional electrophoresis to produce high-resolution maps of protein-stained spots. The maps were compared by image analysis programs plus manual input, spots that stained differentially were identified, proteins in these spots were subjected to in-gel trypsinization, and tryptic peptides were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The numbers of proteins identified were considerably less than the number of spots subjected to trypsinization and MALDI-TOF because the same protein often was identified in multiple spots, presumably because of multiple proteoforms as described above or posttranslational modifications. One only has to examine the 2-dimensional maps upon which the quantification is based to realize the enormous amount of careful work that went into the studies. Nevertheless, the protein changes reported were not consistent, and the papers fail to identify a set of eosinophil proteins associated with increased eosinophil counts. The most complete study employing 2-D electrophoresis and MALDI-TOF identified 426 unique eosinophil proteins (20) as compared with the >7,000 and >4,400 that we identified in our LFQ and multiplexed isobaric labeling studies, respectively (1). With its vastly deeper coverage, ability to distinguish proteoforms and pinpoint post-translational modifications, standardized workflows, and intensity-based readouts that are amenable to facile statistical analyses, LC–MS/MS combined with LFQ or multiplexed isobaric labeling offers powerful and complementary approaches to the question of whether certain proteins in blood eosinophils are altered or predict therapeutic outcomes in patients with eosinophil-associated diseases.

[4] http://www.phosphosite.org.

The MaxLFQ algorithm, which is part of MaxQuant software suite, allows comparisons of protein abundance in different samples even though peptides from each sample are analyzed separately and the mix of quantifiable peptides from a given protein may vary from sample to sample (49). A recent study of individual variations in the 1,000 most abundant blood plasma proteins is an excellent example of the utility of LFQ (50). With 20×10^6 eosinophils that can be purified routinely from individual subjects, it should be possible to perform LFQ of the ~5,000 eosinophil proteins that account for >99% of cellular molar abundance (1). One advantage of LFQ is the ability to analyze samples upon collection and, should data have clinical significance, communicate results within a clinically useful turnaround time. Multiplexed isobaric labeling would work well for comparisons of well-defined sets of subjects, such as those with mild versus severe asthma, for which samples could be archived over time and analyzed in batch. As above, the outstanding advantage of isobaric labeling is that the same peptide from all individuals will be detected and allow determination of the relative abundances of the peptide in the different individuals based on ion intensities in the reporter region (34). Being able to compare abundance of a given peptide in all individuals would be especially important in analyses of changes in specific phosphosites. The method suffers from contamination of the reporter region by reporter ions derived from co-isolated contaminating ions with resultant compression of ratios of reporter ion intensities (34). This problem, however, would lead to underestimation rather than overestimation of differences (51, 52). Once biomarkers are identified by either LFQ or isobaric labeling, it should be possible to devise a focused proteomic screen that employs multiple reactions monitoring for selected peptides with a set of these peptides labeled with heavy atoms serving as internal standards in an absolute quantification strategy (53, 54).

Planners of disease-oriented studies face the decision of whether to analyze eosinophils purified by the standard method, purified eosinophils also depleted of eosinophils to which platelets are adherent, or both types of purified eosinophils. Because platelets may modify eosinophil activity (31, 32), we favor not depleting eosinophils of eosinophil–platelet complexes, thereby not removing what may be the most interesting population of blood eosinophils. Abundances of proteins known to be specific for platelets can be used to estimate the contribution of platelets to observed proteomic differences.

A recent time course study using two-dimensional electrophoresis combined with MALDI-TOF to identify spots that stained differentially with a phosphoprotein-specific dye, ProQ Diamond, demonstrated that IL5-family cytokines increased phosphorylation of >20 eosinophil proteins in a pattern that was different from the effects of eotaxin or other agonists (55). Sites of phosphorylation were not determined. Multiplexed isobaric labeling should be a powerful method for pinpointing residues attacked by kinases or phosphatases upon eosinophil activation with different agonists and learning the effects of inhibitors and therapeutic agents on these phosphorylation events. We studied IL5 effects at only a single time point (1). Recently, 4,797 phosphosites were profiled temporally in an isobaric labeling study of platelets responding to ADP (56). The 1,819 phosphosites detected and quantified in our IL5 study are several-fold lower than in the platelet study. We believe that the latter number is achievable with eosinophils such that isobaric labeling studies can lead to a full "systems biology" understanding of the molecular events that underlie eosinophil activation in response to multiple agonists and how these events can be perturbed therapeutically.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fmed.2017.00159/full#supplementary-material.

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REVIEW
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The Biology of Eosinophils and Their Role in Asthma

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This review will describe the structure and function of the eosinophil. The roles of several relevant cell surface molecules and receptors will be discussed. We will also explore the systemic and local processes triggering eosinophil differentiation, maturation, and migration to the lungs in asthma, as well as the cytokine-mediated pathways that result in eosinophil activation and degranulation, i.e., the release of multiple pro-inflammatory substances from eosinophil-specific granules, including cationic proteins, cytokines, chemokines growth factors, and enzymes. We will discuss the current understanding of the roles that eosinophils play in key asthma processes such as airway hyperresponsiveness, mucus hypersecretion, and airway remodeling, in addition to the evidence relating to eosinophil–pathogen interactions within the lungs.

Keywords: eosinophils, asthma, IL-5, eosinophil receptors, respiratory tract infections, asthma exacerbation

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INTRODUCTION

The three main processes responsible for the clinical features of asthma are well recognized: bronchoconstriction, mucus hypersecretion, and airway inflammation. However, the underlying pathophysiology responsible for these processes is complex and nuanced, involving multiple cell types and cytokines (1). Furthermore, the activity and clinical impact of each cellular and subcellular component varies considerably between individuals and can change over time, as well as in response to drug therapy and environmental/lifestyle influences.

Among these myriad cellular interactions and this extremely heterogeneous patient group, it is possible to identify certain key cells that are commonly involved—of which, arguably, the eosinophil is the most important.

Eosinophil precursors originate in the bone marrow and following differentiation traffic to the lungs (among other sites) *via* the bloodstream (2). While high concentrations of circulating eosinophils are often measured in asthmatic patients, of more clinical relevance is the lung tissue eosinophilia that is also frequently present.

The phenotype of "severe eosinophilic asthma" refers to a subgroup of asthmatic patients with evidence of eosinophilia that often require high maintenance doses of oral corticosteroids to maintain reasonable disease control. The notoriously non-specific mechanisms of action of corticosteroid therapy give rise to numerous well-documented adverse effects (3), which have driven decades of research focused on the development of targeted anti-eosinophil drug therapies. In order to understand how to better assist this group of patients, who currently have an unmet clinical need, it is helpful to understand the eosinophil itself, and the role that it plays in asthma. Targeted anti-eosinophil therapies will be touched upon but will be covered in greater detail by other reviews in this Research Topic.

EOSINOPHIL CELL STRUCTURE

Eosinophils are granulocytes, typically measuring 10–16 μm in diameter. They possess segmented (usually bi-lobed) nuclei and their nucleus: cytoplasm ratio is approximately 30%. Eosinophils stain with acidophilic dyes—a feature noted in 1879 by Paul Ehrlich, who first described eosinophils and appreciated their increased presence in patients with asthma and helminth infections, among other conditions (4). See **Figure 1** for an overview of the eosinophil ultrastructure.

The presence of large specific granules, also known as secondary granules, is a characteristic feature that distinguishes eosinophils from other granulocytes (neutrophils and basophils). Specific granules consist of a dense crystalline core and a matrix, surrounded by a membrane (5). They contain a large number of mediators capable of inducing inflammation and/or tissue damage, including basic proteins, cytokines, chemokines, growth factors, and enzymes. The predominant substances are the proteins: major basic protein (MBP) is located in the core, while the matrix contains eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN) (6).

Primary granules tend to be smaller than specific granules. They are the principal location of Charcot–Leyden crystal protein (galactin-10): hexagonal bipyramidal crystals, which exhibit lysophospholipase activity and have been identified in tissues subject to eosinophilic inflammation (7).

Lipid bodies are particularly important when considering the role of eosinophils in asthma, due to their involvement in the production of eicosanoids, including cysteinal leukotrienes, prostaglandins, and thromboxane (2). Lipid bodies are a key site of arachidonic acid esterification and eicosanoid production due to their high concentrations of relevant enzymes such as cyclooxygenases, 5-lipoxygenase, and leukotriene C4-synthase (5).

Golgi bodies, endoplasmic reticulum, and mitochondria are also present and fulfill the fundamental duties of protein and adenosine triphosphate production within the eosinophil.

The histological appearance of eosinophils varies depending on the level of activation. For example, higher numbers of vesicles such as eosinophil sombrero vesicles may be seen when the cell is undergoing piecemeal degranulation (PMD), a process described in detail further on.

EOSINOPHIL SURFACE MOLECULES AND RECEPTORS

The varied roles of the eosinophil are reflected in its wide repertoire of surface molecules and receptors, which integrate eosinophils with both the innate and adaptive immune systems.

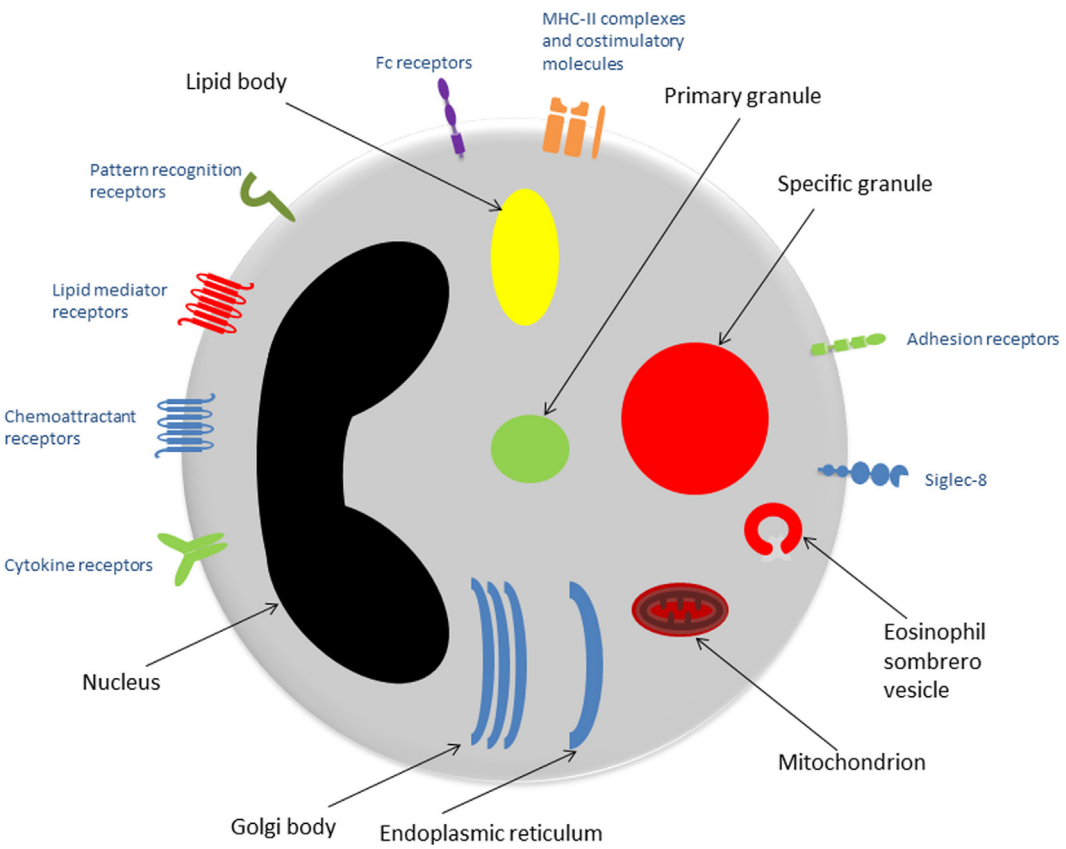

FIGURE 1 | Eosinophil ultrastructure. Schematic representation of an eosinophil showing the major organelles (black labels) and cell surface receptors (blue labels). Abbreviation: MHC-II, major histocompatibility complex-II.

Cytokine and Growth Factor Receptors

The heterodimeric receptor for IL-5 is thought to be the most important cytokine receptor expressed by eosinophils. The beta-subunit is identical to the beta-subunit of the receptors for granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-3 (both of which are also present on eosinophil cell membranes). The alpha-subunit, IL-5Rα, is specific to IL-5 and has been identified as a therapeutic target for severe eosinophilic asthma and other eosinophil-mediated conditions. The IL-5 receptor is also expressed by basophils.

Eosinophils also express receptors for multiple other cytokines and growth factors, including for IL-4, IL-13, IL-33, thymic stromal lymphopoietin, and transforming growth factor-β (TGF-β).

Chemoattractant Receptors

Chemokines are small cytokines, which stimulate the migration of specific subsets of leukocytes. Chemokines are divided into four groups, depending on the presence or absence of one or more interposing amino acid(s) between two cysteine residues (known as CXC-, CX3C-, and CC-chemokines), or the presence of only one cysteine residue (XC-chemokines) (8).

CC-chemokine receptor-3 (CCR3) is an important G protein-coupled receptor expressed on eosinophil cell membranes. CCR3 binds to all three subtypes of eotaxin (a variety of CC-chemokine that functions as a selective eosinophil chemoattractant). CCR3 also binds to several other chemokines including monocyte chemoattractant protein-3 (MCP-3) and MCP-4. The airways of patients with asthma have been shown to contain higher numbers of cells expressing mRNA for CCR3 and its ligands, compared to non-asthmatic control subjects (9). In mouse models of allergic airway inflammation, antigen-induced airway eosinophilia may be inhibited by the administration of either a monoclonal antibody against CCR3 (10) or a low molecular weight CCR3-antagonist (11).

CCR1 is another key chemokine receptor on eosinophils, which is activated by chemoattractant cytokine ligand-3 (CCL-3) and CCL-5 (also known as RANTES—regulated on activation, normal T cell expressed and secreted).

Lipid Mediator Receptors

Eosinophils possess cell surface receptors for lipid mediators such as leukotrienes, prostaglandins, and platelet-activating factor, all of which have been shown to have a role in asthma pathophysiology (12–14).

Pattern Recognition Receptors (PRRs)

Pattern recognition receptors react to microbial pathogen-associated molecular patterns (PAMPs) or host-derived damage-associated molecular patterns (DAMPs) and regulate the immune response to these indicators of infection and/or tissue damage (15).

Toll-like receptors (TLRs) are a family of PRRs, which are expressed by eosinophils, as well as multiple other cell lines. In humans there are 10 types of TLRs. TLRs are transmembrane glycoproteins, some of which are located at the cell surface and some in endosomes. The cytoplasmic domain resembles that of the IL-1 receptor, and the intracellular signals generated are therefore similar (16).

Eosinophils also express several other families of PRR, including retinoic acid-inducible gene-I-like receptors, nucleotide-binding oligomerisation domain-like (NOD-like) receptors, and the receptor for advanced glycation endproducts (RAGE) (15).

Fc Receptors

Fc receptors to IgA, IgD, IgE, IgG, and IgM are expressed on the surface of eosinophils, facilitating interaction with the adaptive immune system.

The high-affinity FcεR1 receptor binds IgE and signals *via* intracellular tyrosine kinases. On mast cells and basophils, where FcεR1 is expressed as a tetramer ($\alpha\beta\gamma 2$), stimulation by IgE results in degranulation. However, on eosinophils, FcεR1 is usually expressed in very small quantities as a trimer (without a β chain) and has no role in eosinophil activation (17). In contrast, cross-linking of FcαRI and FcγRII, with IgA and IgG, respectively, has been shown to trigger eosinophil activation (18).

Major Histocompatibility Complex-II (MHC-II)

Eosinophils have an additional role as antigen-presenting cells, facilitated by the presence of MHC-II molecules and co-stimulatory molecules such as CD80 and CD86. In allergic patients, evaluated after segmental antigen challenge, expression of HLA-DR (a subtype of MHC-II molecule) was found to be approximately four times greater in lung eosinophils compared to blood eosinophils (19).

Adhesion Receptors

Adhesion receptors, as their name suggests, allow cells such as the eosinophil to adhere to the extracellular matrix (ECM) and to other cells. They also allow the eosinophil to sense its surroundings and respond accordingly. Adhesion receptors are divided into four main groups: integrins, cadherins, selectins, and immunoglobulin-like cell adhesion molecules (Ig-CAM). Integrins and selectins are the main forms of adhesion receptors expressed on eosinophil cell membranes.

Eosinophils express seven types of integrins, which are transmembrane glycoproteins, consisting of an α and a β chain (20). Examples include very late antigen-4 (VLA-4, CD49d/CD29) and the complement receptor CR3 (CD11b/CD18), which is otherwise known as macrophage-1 antigen (Mac-1).

Selectins are single-chain transmembrane glycoproteins with multiple domains. There are three families: E-, L-, and P-selectin; the latter two are expressed by human eosinophils, whereas E-selectin is present on activated endothelium (21).

Siglec-8

Siglec-8 is a sialic acid immunoglobulin-like lectin (a carbohydrate-binding protein) expressed by eosinophils, mast cells, and basophils. Its physiological role has not yet been identified, although it is thought to represent a potential therapeutic target for eosinophil-mediated disease, due to the observation that administration of an antibody targeted against Siglec-8 can induce selective eosinophil apoptosis and inhibit mast cell degranulation (22).

EOSINOPHIL DIFFERENTIATION, MATURATION, MIGRATION, ACTIVATION, AND DEGRANULATION

Eosinophils develop from pluripotent CD34+ granulocyte progenitor cells.

Differentiation and maturation occurs as follows:

Myeloblast → Promyelocyte → Eosinophil myelocyte → Eosinophil metamyelocyte → Eosinophil.

Allergen challenge of mild asthmatics results in increased expression of IL-5Rα on CD34+ cells in the bone marrow, associated with blood and sputum eosinophilia (23). Eosinophil differentiation usually occurs in the bone marrow. However, eosinophil precursors have been isolated from the peripheral blood of atopic subjects at significantly higher concentrations compared to non-atopic controls (24). Increased numbers of CD34+/IL-5Rα+ eosinophil precursors have also been identified in bronchial biopsies of atopic asthmatics, compared to non-asthmatic control subjects (both atopic and non-atopic) (25). Eosinophil-lineage committed cells have also been identified in lung tissue in a mouse model of allergic airway inflammation (26). More recently, eosinophil progenitors isolated from the blood of patients with severe eosinophilic asthma have been shown to have an exaggerated clonogenic response to IL-5 *in vitro*, compared to eosinophil precursors from mild asthmatics, suggesting that *in situ* eosinophilopoiesis may have a clinically relevant role in severe eosinophilic asthma (27).

The differentiation of eosinophils is regulated by the transcription factors GATA-binding protein 1 (GATA-1), PU.1, and the CCAAT-enhancing binding protein (c/EBP) family. GATA-1 and PU.1 synergistically promote transcription of MBP (28). GATA-1 is thought to have the most important role, as disruption of the GATA-1 gene in mice results in a strain completely devoid of eosinophils (29).

The cytokines IL-3, IL-5, and GM-CSF also synergistically contribute to the development of mature eosinophils (30). IL-5 is the most eosinophil-specific and also promotes the release of eosinophils from the bone marrow to the bloodstream, acting synergistically with eotaxin (31, 32). Eosinophils are present in relatively low numbers in peripheral blood, usually accounting for no more than 5% of the total white blood cell count (33). They have a relatively short blood half-life of approximately 18 h (34). Migration to specific body sites, including the lungs and intestines, is mediated by eosinophil chemoattractants such as eotaxins. Eotaxins are a variety of CC-chemokines. There are three known subtypes: eotaxin-1 (CCL-11), eotaxin-2 (CCL-24), and eotaxin-3 (CCL-26). These bind to CCR3 receptors on the cell membranes of eosinophils and induce chemotaxis. 5-oxo 6, 8, 11, 14-eicosatetraenoic acid (5-oxo-ETE) is another eosinophil chemoattractant.

In vitro, the presence of prostaglandin-D2 (PGD2) has been shown to significantly enhance the chemoattractant effects of eotaxin-1 and 5-oxo-ETE on eosinophils and—unlike eotaxin-1 or 5-oxo-ETE—PGD2 retains its chemoattractant effect in the presence of blood or plasma (35). It is therefore proposed that PGD2 acts as the initial chemoattractant, triggering the migration of circulating eosinophils to specific tissues, where eotaxins and 5-oxo-ETE then predominate. PGD2 is released from activated mast cells (36) and acts *via* CRTh2 (chemoattractant receptor-homologous molecule expressed on T_H2 cells).

In allergic inflammation and asthma, circulating eosinophils adhere to the vascular endothelium and roll along it, before extravasating to the lung tissue. Initial tethering to the endothelium occurs as a result of the eosinophil cell membrane adhesion receptor P-selectin binding to P-selectin glycoprotein ligand-1 on the endothelium (37). Binding of the integrin VLA-4 to vascular cell adhesion molecule-1 promotes eosinophil activation and extravasation (37). IL-13 results in increased eosinophil expression of P-selectin and increased P-selectin mediated adhesion to endothelial cells (38).

The eosinophil's ability to store several preformed cytotoxic mediators ready for rapid release upon appropriate stimulation facilitates a much quicker reaction to pro-inflammatory stimuli, compared to other cells, whose responses depend on upregulating the transcription of genes coding for such proteins.

The bronchial epithelium produces the cytokines IL-25, IL-33, and thymic stromal lymphopoietin, collectively known as the alarmins, in response to irritants such as allergens, pollutants, and pathogens. These cytokines trigger an inflammatory cascade involving, among others, T helper-2 (T_H2) cells and type-2 innate lymphoid cells (ILC2s), resulting in increased production of numerous cytokines including IL-4, IL-5, and IL-13, therefore prompting eosinophil activation (1, 39).

High mobility group box 1 protein, acting *via* receptors TLR-2, TLR-4, and RAGE, also promotes eosinophilia, although less is known regarding its mechanism of action (2).

Specific granule contents may be released *via* three different degranulation processes. Conventional exocytosis entails the specific granules fusing with the eosinophil cell membrane, resulting in the release of the entire contents of the specific granule. Alternatively, the eosinophil may be lysed (cytolysis), releasing all the cell contents, including the intact specific granules. These extracellular granules can be found in tissues affected by eosinophil-mediated disease and may subsequently release their contents in response to pro-inflammatory stimuli (40). However, the most common mechanism of eosinophil granulation is termed piecemeal degranulation (PMD). In this process, vesicles (both round and tubular) are released from specific granules and travel to the cell membrane to discharge their contents to the extracellular domain (41). The tubular vesicles tend to curl into a hoop-like morphology, giving rise to the term "eosinophil sombrero vesicles" (42). Vesicles with particular contents may be selectively released in response to particular cytokines, allowing eosinophils to supply a specific combination of cytotoxic mediators on demand (42, 43).

The activation of TLRs on eosinophils has been shown to promote adhesion and the release of certain cytokines and superoxides (44). Activation of TLR-2 and TLR-9 triggers eosinophil degranulation (44, 45). *In vitro*, eosinophils from atopic subjects have been shown to produce more IL-8 and EDN in response to stimulation of TLR-7 and TLR-9, compared to healthy controls (45).

Eosinophil survival is promoted by IL-3, IL-5, GM-CSF, and eotaxin (37). Activation of TLR-7 (the most abundant TLR subtype expressed by eosinophils) also enhances eosinophil survival (45).

IL-3, IL-5, AND GM-CSF

Among the type-2 cytokines, IL-3, IL-5, and GM-CSF are particularly important for the initiation and perpetuation of eosinophilic airway inflammation. These three cytokines are closely linked, in that the genes for all three are all located on chromosome 5, and their receptors also share a common β-subunit (βc).

Monoclonal antibodies against IL-5 have been developed in order to treat eosinophil-mediated diseases such as eosinophilic asthma. Although inhibition of IL-5 activity in this manner (using mepolizumab) results in significant depletion of circulating eosinophils, the effect on bronchial tissue eosinophilia is less marked, with a median reduction of 55% (46). The residual tissue eosinophilia may reflect ongoing effects mediated by IL-3 and GM-CSF.

In a mouse model of allergic airways inflammation, allergen-induced lung tissue eosinophilia was abolished in mice bred to lack the common β-subunit, therefore incapable of responding to IL-3, IL-5, and GM-CSF (47). In the same study, lung tissue from βc-deficient mice was found to contain fewer myeloid dendritic cells, and the local T_H2 cells showed a reduced ability to proliferate and produce type-2 cytokines (47). These findings suggest a multifactorial role for the common β-subunit in the regulation of allergic airway inflammation.

THE ROLE OF THE EOSINOPHIL IN HEALTH

In comparison to the roles that eosinophils play in diseases and infections, relatively little is known about their purpose in health. However, an increasing number of homeostatic mechanisms have been attributed to—or at least associated with—eosinophils in recent years. This has prompted a call for a fundamental change of the perception of eosinophils purely as cytotoxic effector cells (48, 49).

In health, eosinophils are found in the thymus, spleen, lymph nodes, and gastrointestinal (GI) tract (50). The number of eosinophils in the thymus declines with age (51). Eosinophils may have a role in T cell selection. In a mouse model of MHC I-restricted acute negative selection, eosinophil recruitment to the corticomedullary region of the thymus and association with apoptotic bodies has been demonstrated (52). Eosinophils also enhance the ability of macrophages to phagocytose apoptotic thymic cells (53).

Eosinophils migrate to the GI tract during embryonic development, i.e., prior to the development of any viable gut flora (54). In health, they are present throughout the GI tract—with the notable exception of the esophagus. Eosinophils contribute to the immune defense against gut microorganisms, due to multiple antimicrobial properties. (The antimicrobial properties of eosinophils are discussed in detail further on, with specific relation to respiratory pathogens.) Other potential homeostatic roles for eosinophils within the gut are not currently well defined but may relate to their ability to interact with the enteric neuronal system and increase smooth muscle reactivity (*via* release of MBP) (55).

In murine white adipose tissue, a positive correlation was identified between eosinophil counts and the numbers of arginase-1-expressing macrophages (56). Macrophages expressing arginase-1 are thought to contribute to glucose homeostasis, although macrophage classification is contentious (57). In a more recent study involving more than 9,000 human participants, the peripheral blood eosinophil percentage was found to be inversely associated with the risk of type-2 diabetes mellitus and insulin resistance (58).

Eosinophils have also been implicated in the regeneration of liver tissue (59) and skeletal muscle (60). The increased presence of eosinophils in preovulatory ovarian follicles (61) and in endometrium (62) has prompted speculation that they may have a role in tissue remodeling related to ovulation and menstruation.

Eosinophils also perform several important immunomodulatory functions, discussed in the following section.

THE EOSINOPHIL'S ROLE IN ASTHMA PATHOPHYSIOLOGY

Asthma pathophysiology is complex, and the relative contributions of the various cytokine networks involved vary between patients. Core features include airway hyperresponsiveness (AHR), mucus hypersecretion, tissue damage, and airway remodeling. See **Figure 2** for an overview of the eosinophil's role in asthma pathophysiology.

It has long been observed that eosinophil counts in peripheral blood and bronchoalveolar lavage (BAL) fluid are higher in asthmatics compared to healthy controls (63). Analysis of BAL fluid obtained from patients with atopic asthma reveals increased expression of T_H2 cytokines (64), including IL-5, which are strongly associated with eosinophilic inflammation (65). In general, the degree of eosinophilia correlates with disease severity and exacerbation frequency (63, 66). However, non-eosinophilic asthma phenotypes are also recognized (67). Peripheral blood eosinophilia may also occur in numerous other conditions (see **Box 1**).

AHR and Mucus Hypersecretion

Eosinophils may be prompted to release a number of different mediators with the capacity to cause AHR. Human MBP is known to result in AHR when administered to primates (70) and rats (71). In the former study, administration of EPO also caused AHR, although ECP and EDN did not (70). Data from the latter study suggested the mechanism of action involved the stimulation of bradykinin production (71). MBP can also trigger mast cells and basophils to release histamine, a potent mediator of bronchial hyperreactivity (72, 73).

Eosinophils are a source of several cytokines including IL-13, which causes AHR, and also promotes mucus hypersecretion *via* enhanced differentiation of goblet cells (74). IL-13 is also

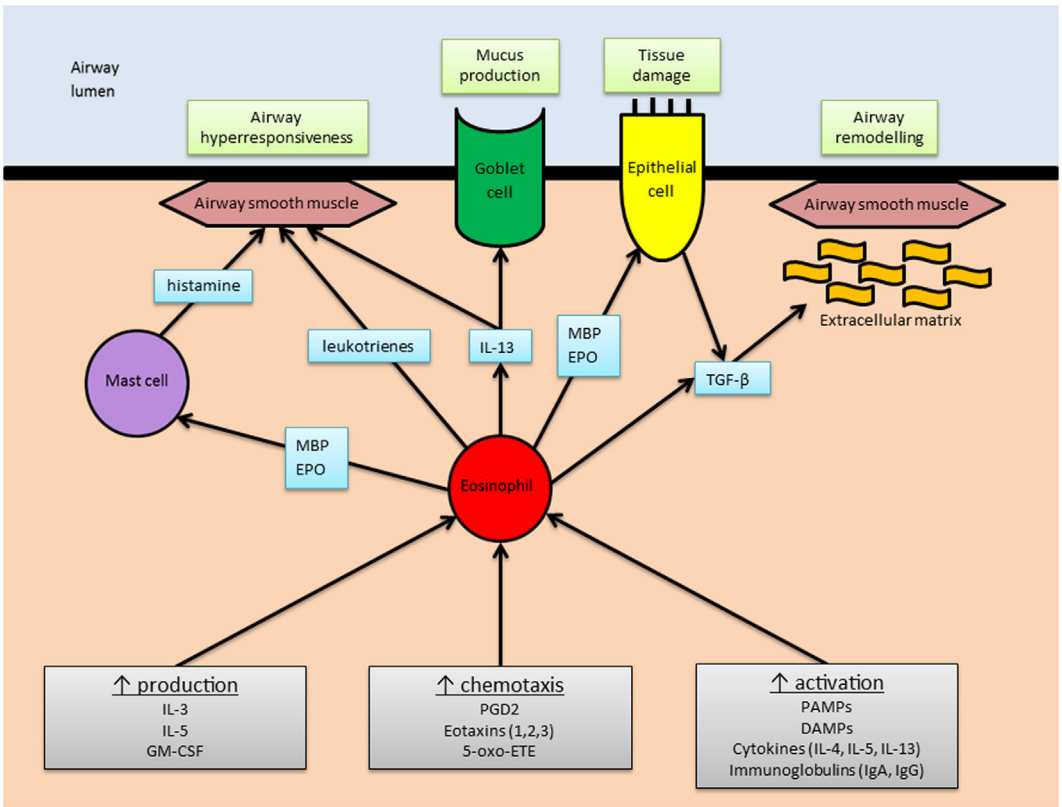

FIGURE 2 | The role of eosinophils in asthma. An overview of the main stimuli for eosinophilic airway inflammation (gray boxes) and the means by which eosinophils elicit the main pathophysiological changes associated with asthma (green boxes). Abbreviations: MBP, major basic protein; EPO, eosinophil peroxidase; IL, interleukin; TGF-β, transforming growth factor-β; GM-CSF, granulocyte–macrophage colony-stimulating factor; PGD2, prostaglandin-D2; 5-oxo-ETE, 5-oxo 6, 8, 11, 14-eicosatetraenoic acid; PAMPs, pathogen associated molecular patterns; DAMPs, damage associated molecular patterns; Ig, immunoglobulin.

BOX 1 | Alternative (i.e., non-asthma) causes of peripheral eosinophilia (68, 69).

Respiratory
- Eosinophilic granulomatosis with polyangiitis (EGPA)
- Allergic bronchopulmonary aspergillosis
- Sarcoidosis

Hematological and neoplastic
- Myeloproliferative hypereosinophilic syndrome
- Lymphocytic-variant hypereosinophilic syndrome
- Certain leukemias and lymphomas
- Systemic mastocytosis
- Solid tumors—adenocarcinomas, squamous cell carcinomas, large cell lung carcinomas, transitional cell carcinoma of the bladder

Infective
- Parasitic infection, in particular helminths
- Human immunodeficiency virus

Dermatological
- Eczema
- Scabies infestation

Iatrogenic
- Certain drug hypersensitivity reactions
- Graft vs host disease

produced by T_H2 cells and ILC2s. Lipid mediators such as leukotrienes, which are produced in eosinophil lipid bodies (and mast cells), also cause AHR and mucus hypersecretion (75).

Studies involving two different strains of eosinophil-deficient mice have attempted to clarify the role of eosinophils in an ovalbumin model of asthma but yielded seemingly contradictory results. In one study, performed by Lee et al., eosinophil-deficient mice were protected from AHR and mucus hypersecretion (76). However, another study, led by Humbles, found that eosinophil deficiency was not protective in this regard (77). Several theories have been put forward to explain the conflicting results, including evidence of residual lung eosinophils in the Humbles study and differences between the underlying mouse strains (78).

In terms of practical application, the existence in humans of eosinophilic bronchitis, a condition characterized by marked airway eosinophilia in the absence of AHR, calls into question the concept that eosinophils—acting alone—have a clinically significant impact on AHR. In patients with mild asthma, administration of a monoclonal antibody to IL-5 has been shown to reduce blood and sputum eosinophilia but had no effect on AHR (79). This may reflect the cellular redundancy of AHR pathophysiology, which involves several cell types including T_H2 cells, ILC2s,

and mast cells. In addition, the current evidence relating to AHR does not specifically study the pathophysiology of asthma exacerbations, during which it is possible that eosinophil degranulation may contribute to worsening AHR.

Tissue Damage and Airway Remodeling

Postulation that eosinophils are major effectors of lung tissue damage in asthma is well founded, given their propensity to release highly charged basic proteins, which exert multiple cytotoxic effects. MBP is toxic to respiratory epithelial cells *in vitro* and has been identified in postmortem lung tissue specimens of patients who have died of asthma, in association with epithelial damage (80). ECP and EDN share 67% amino acid sequence homology and tend to be grouped together as eosinophil-associated RNases, although ECP's RNase activity is much less potent (81). ECP binds to cell membranes and alters their permeability (82). EDN, as its name suggests, is neurotoxic. It was first identified following the observation that, in rabbits, the intracerebral administration of eosinophils resulted in the destruction of cerebellar Purkinje cells and neurological features named "the Gordon phenomenon" (83, 84). EPO catalyzes the oxidation of halides and thiocyanate, resulting in cytotoxic reactive oxidant species (85).

Cell damage triggers the activation of repair pathways which, if excessive, may contribute to structural changes referred to as airway remodeling. The underlying pathological processes include hyperplasia of fibroblasts, airway smooth muscle (ASM) and goblet cells, deposition of ECM proteins, and angiogenesis (86). Airway remodeling is associated with the severity of asthma (87). It has been hypothesized that airway remodeling is responsible for the accelerated decline in lung function and development of fixed airway obstruction observed in some asthmatic patients. However, bronchial biopsies of children with difficult asthma have been shown to display reticular basement membrane thickening to a similar degree compared with adult asthmatics (88). Furthermore, there is evidence that some pathological features of airway remodeling can become evident within 24 h of allergen exposure (89).

Eosinophils release multiple growth factors and fibrogenic mediators that promote airway remodeling (see **Table 1**). For example, eosinophils are known to produce TGF-β in disease states involving the skin (atopy) (90), nose (nasal polyposis) (91), and blood (idiopathic hypereosinophilic syndrome) (92). Eosinophils are the main source of TGF-β in bronchial biopsies taken from asthmatic patients (93) and can also stimulate epithelial cells to produce a number of mediators, including TGF-β (94). TGF-β is implicated in tissue remodeling *via* fibroblast proliferation and increased production of collagen and glycosaminoglycans (95–97).

Eosinophils isolated from asthmatics, when cocultured with ASM cells, promote enhanced ASM proliferation, which is inhibited by the addition of the leukotriene antagonist montelukast (117). It appears that eosinophils and ASM enjoy a reciprocal relationship, as ASM cells are also known to produce pro-eosinophil cytokines (118). Mouse studies lend further support to the assertion that eosinophils have an important role in airway remodeling, as eosinophil-deficient mice are protected against airway deposition of collagen and smooth muscle (77).

Treatment with the anti-IL-5 monoclonal antibody mepolizumab has been shown to reduce bronchial tissue eosinophilia, in association with decreased TGF-β1 in BAL specimens, and reduced reticular basement membrane procollagen III, tenascin, and lumican (119).

Asthma Exacerbations

Airway eosinophilia is an early feature of asthma exacerbations. In a study of steroid-dependent asthmatic patients, whose prednisolone dose was gradually reduced to below their maintenance requirement, the sputum eosinophil count started to rise 4 weeks before the blood eosinophil count and 6 weeks prior to spirometric and symptomatic deterioration (120). In fact, the adoption of an asthma treatment strategy based on sputum eosinophilia rather than traditional markers of disease activity (such as symptoms and spirometry) was found to reduce the frequency of exacerbations, with no overall increase in the average daily corticosteroid dose (121).

The primary action of anti-IL-5 therapies appears to be a reduction in exacerbation frequency. Administration of mepolizumab to selected patient groups reduced exacerbation rates by approximately 50% (122–124). A similar reduction in exacerbation rates was seen with reslizumab, another anti-IL-5 monoclonal antibody (125). Mepolizumab has also been found to have a moderate glucocorticoid-sparing effect in a phase III clinical trial (126).

Benralizumab is a monoclonal antibody targeted against the alpha subunit of the IL-5 receptor (IL-5Rα). As well as blocking the interaction between IL-5 and its receptor, benralizumab causes eosinophil cell death *via* antibody-dependent cell-mediated cytotoxicity (127), resulting in striking (95%) airway eosinophil depletion (128). Phase III clinical trials have demonstrated reductions in exacerbation rates (129, 130).

Immunomodulation

In addition to the direct effects of eosinophils on asthma pathophysiology, they have an important role in immunomodulation (2). MBP, released from eosinophil-specific granules, stimulates inflammatory responses from neutrophils (increased production of superoxide and IL-8) (131) and mast cells (increased histamine release) (72). Nerve growth factor (also released from specific granules) has also been shown to prolong the survival of neutrophils (132) and mast cells (133). EDN promotes the activation of dendritic cells (134), which in turn trigger the proliferation of T cells (both helper and cytotoxic) and B cells *via* antigen presentation. Eosinophils themselves can also present antigens to T cells (135, 136).

The cytokines released from eosinophil-specific granules have various immunomodulatory effects. For example, IL-4 and IL-13 simulate the proliferation of B cells and IgE production (137, 138), and IL-6 enhances survival of plasma cells (139, 140) Eosinophil-specific granules are also capable of releasing several chemokines. Depending on the stimulation they receive, these include CCL-17 and CCL-22, which attract T_H2 cells, and CXCL-9 and CXCL-10, which are T_H1 chemokines (141). In addition, eosinophils express indoleamine 2,3-dioxygenase (IDO), an enzyme that catalyzes the production of kynurenine,

TABLE 1 | Factors produced by eosinophils that are associated with airway remodeling.

Factor	Mechanism(s) and evidence
TGF-β	• Epithelial/submucosal expression of TGF-β correlates with basement membrane thickness and fibroblast numbers (98). • In allergen-challenged human atopic skin, eosinophils expressing TGF-β1 are associated with myofibroblast formation and deposition of tenascin and procollagen-1 (90). • TGF-β induces hypertrophy and increased contractility of ASM *in vitro* (99). • Administration of anti-TGF-β antibody to mice with established eosinophilic airway inflammation significantly reduces airway remodelling (100).
MMP-9 and TIMP-1	• MMP-9 breaks down ECM proteins; TIMP-1 inhibits MMP-9. • Sputum MMP-9 and TIMP-1 concentrations are higher in asthmatics compared to controls; The MMP-9/TIMP-1 ratio is lower in patients with asthma and chronic bronchitis, and positively correlates with FEV1 (101). • MMP-9 is required for angiogenesis in mice (102).
VEGF, bFGF, and angiogenin	• VEGF, bFGF, and angiogenin promote angiogenesis. • Bronchial biopsies of asthmatics exhibit greater immunoreactivity to VEGF, bFGF, and angiogenin; Immunoreactivity to these factors positively correlates with vascular area (103).
Specific granule proteins	• MBP and ECP are toxic to airway epithelial cells. • Damaged airway epithelium produces TGF-β (104). • ECP induces fibroblast migration (105) and inhibits fibroblast-mediated proteoglycan degradation (106).
IL-17	• Fibroblasts isolated from bronchial biopsies produce more IL-6 and IL-11 (profibrotic cytokines) when stimulated by IL-17 (107). • In a mouse model of asthma, administration of IL-17A results in increased vascular remodelling; *in vitro*, IL-17A accelerates EPC migration (108).
IL-13	• Mice bred to overexpress IL-13 exhibit eosinophilic airway inflammation, epithelial cell hypertrophy, mucus metaplasia, and subepithelial fibrosis (109). • *In vitro*, IL-13 induces human bronchial epithelial cells to release TGF-β (110).
HB-EGF	• Recombinant HB-EGF promotes migration of ASM cells *in vitro* and accelerates the thickening of the ASM layer in a mouse model of asthma (111).
NGF	• NGF causes migration of vascular smooth muscle cells and fibroblasts, and proliferation of epithelial cells and ASM cells (112). • In mice with chronic allergen-induced airway inflammation, anti-NGF antibodies reduce airway collagen deposition (113).
Cysteinyl leukotrienes	• In a mouse model of allergen-induced airway remodelling, administration of montelukast (a CysLT1 receptor blocker) reverses established ASM layer thickening and subepithelial fibrosis (114).
SCF	• SCF promotes mast cell proliferation and activation. • Mast cells produce TNF-α, which can damage bronchial epithelial cells (115) and stimulate fibroblasts to produce TGF-β (116).

TGF, transforming growth factor; MMP, matrix metalloproteinase; ECM, extracellular matrix; TIMP, tissue inhibitor of metalloproteinase; FEV1, forced expiratory volume in 1 s; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; MBP, major basic protein; ECP, eosinophil cationic protein; IL, interleukin; EPC, endothelial progenitor cell; HB-EGF, heparin-binding epidermal growth factor-like growth factor; ASM, airway smooth muscle; NGF, nerve growth factor; CysLT1, cysteinyl leukotriene 1; SCF, stem cell factor; TNF, tumor necrosis factor.

suppressing T_H1 activity and promoting a type-2 inflammatory milieu (142, 143).

See **Figure 3** for an overview of the eosinophil's immunomodulatory roles in asthma.

INTERACTIONS BETWEEN EOSINOPHILS AND RESPIRATORY PATHOGENS

Eosinophils have traditionally been regarded as end-stage effector cells, responding to infections directly, i.e., by releasing substances that are toxic to pathogens (in particular, helminths) and resulting in the unwanted secondary effect of human tissue damage. However, research performed over the last 30 years has revealed additional roles fulfilled by the eosinophil, involving links with both the innate and adaptive immune systems. These roles include antigen presentation and interaction with other parts of the immune system, such as the complement pathway (144).

Pattern recognition receptors on the cell membranes of eosinophils allow them to detect the presence of PAMPs such as lipopolysaccharide (LPS) and beta-glucans, cell wall components of bacteria and fungi, respectively (144). The cysteine and serine proteases produced by mites and fungi activate eosinophils *via* protease-activated receptors (5). TLR-7, the most common TLR expressed by eosinophils, is activated by viral single-stranded RNA (2).

The contents of eosinophil-specific granules are directly cytotoxic to pathogens. MBP causes disruption of cell membranes due to its highly basic nature (145). ECP has antiviral activity (146) and can also agglutinate Gram-negative bacteria by binding to LPS and peptidoglycans (147). EDN is only mildly toxic to helminths, compared to MBP and ECP (148). However, EDN significantly reduces the infectivity of respiratory syncytial virus group B, indicating a role in the immune response to viruses (149). EPO facilitates the generation of toxic reactive oxygen species (150).

In addition to releasing cytotoxic proteins, eosinophils have been shown to phagocytose bacteria (albeit less efficiently than neutrophils) (151). More recently, the "catapult-like" extrusion of "traps" consisting of mitochondrial DNA and eosinophil granule contents, in response to Gram-negative bacteria, has been observed (152).

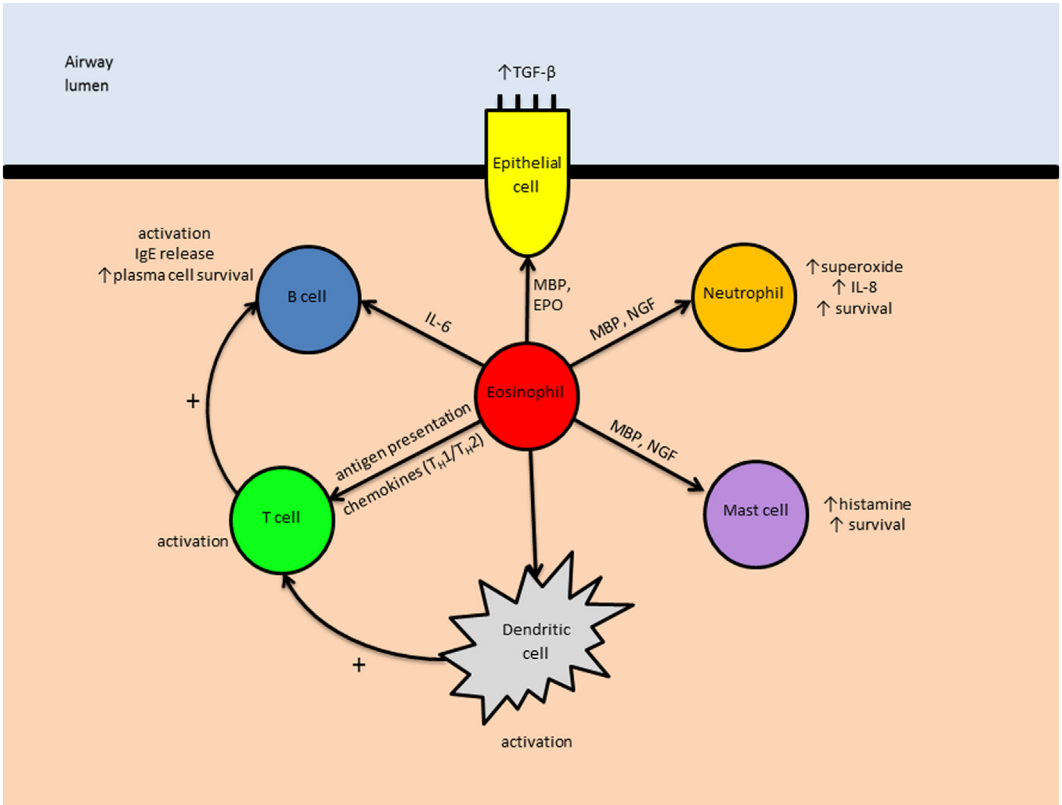

FIGURE 3 | The immunomodulatory role of eosinophils in asthma. Eosinophils may influence other leukocytes both directly (e.g., IL-6-induced B cell activation) and indirectly (e.g., by enhancing antigen presentation by dendritic cells). Abbreviations: TGF-β, transforming growth factor-β; IgE, immunoglobulin E; IL, interleukin; MBP, major basic protein; EPO, eosinophil peroxidase; NGF, nerve growth factor; T_H1, type 1 T helper cell; T_H2, type 2 T helper cell.

Eosinophils express receptors for various complement proteins, including C3a and C5a, which are known to promote eosinophil recruitment, extravasation, and activation (153, 154). Complement is thought to facilitate eosinophil adherence to, and damage of, nematode larvae (155), although the development of secondary immunity is unaffected in complement-deficient mice (156).

As fungi are known to trigger the production of T_H2-associated cytokines (i.e., type-2 cytokines) and eosinophilia, it has been hypothesized that subclinical fungal infection/colonization of the airways may play a role in the genesis of diseases characterized by eosinophilia. Such diseases include severe eosinophilic asthma, as well as related conditions (e.g., chronic rhinosinusitis). One study of patients undergoing sinus surgery found that 74% of those with T_H2-associated conditions had evidence of airway surface mycosis, compared to just 16% of controls (157). However, potential confounding factors such as inhaled and/or systemic corticosteroid usage must be considered.

The increased susceptibility to respiratory viral infections observed in patients with asthma has been linked to reduced production of type I and type III interferons (158, 159). Eosinophils may contribute to this impairment by producing TGF-β, which has been shown to diminish the ability of bronchial epithelial cells to produce interferons in response to human rhinovirus *in vitro* (160).

The lung is known to harbor communities of bacteria, known as the lung microbiome, during health, which are deranged in disease states including asthma (161, 162). Data have recently been published suggesting a possible link between the level of eosinophilia and microbiome community structure in asthma (163). Further dedicated studies, examining subject groups matched for baseline characteristics, are required.

CONCLUSION

Although eosinophils have been associated with asthma since their initial discovery, our understanding of their roles in health and disease has evolved significantly over time. The eosinophil's status as a cytotoxic effector cell appears to be justified, due its capacity to release potent destructive basic proteins, capable of antimicrobial effects as well as host tissue damage. However, its ability to modulate the innate and adaptive immune systems may be just as important.

An appreciation of the numerous receptors expressed by eosinophils offers some insight into the many different interactions this versatile cell is capable of. Not only is the eosinophil recruited to the lungs in the context of pro-inflammatory type-2 cytokines but it is also a promoter of the type-2 inflammatory milieu, taking on roles such as antigen presentation and cytokine-mediated modulation of local lymphocytes.

There is strong evidence that eosinophils contribute to airway remodeling in asthma. Mechanisms also exist by which eosinophils could promote AHR and mucus hypersecretion.

The development of new anti-eosinophilic drugs, capable of selective depletion of eosinophils, offers great potential to explore further questions relating to the role of eosinophils in asthma and the consequences of their eradication. Research into variation in eosinophil-related gene expression between individuals may provide further insights regarding the relative contributions of eosinophils in different asthma phenotypes and the potential application of personalized medicine to this field.

AUTHOR CONTRIBUTIONS

CM performed the initial literature review and drafted the article. AM-G provided critical review and additional content.

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MINI REVIEW
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Homeostatic Eosinophils: Characteristics and Functions

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Eosinophils are typically considered to be specialized effector cells that are recruited to the tissues as a result of T helper type 2 (Th2) cell responses associated with helminth infections or allergic diseases such as asthma. Once at the site of injury, eosinophils release their cytotoxic granule proteins as well as preformed cytokines and lipid mediators, contributing to parasite destruction but also to exacerbation of inflammation and tissue damage. Accumulating evidence indicates that, besides their roles in Th2 responses, eosinophils also regulate homeostatic processes at steady state, thereby challenging the exclusive paradigm of the eosinophil as a destructive and inflammatory cell. Indeed, under baseline conditions, eosinophils rapidly leave the bloodstream to enter tissues, mainly the gastrointestinal tract, lungs, adipose tissue, thymus, uterus, and mammary glands, where they regulate a variety of important biological functions, such as immunoregulation, control of glucose homeostasis, protection against obesity, regulation of mammary gland development, and preparation of the uterus for pregnancy. This article provides an overview of the characteristics and functions of these homeostatic eosinophils.

Keywords: eosinophils, homeostasis, immunomodulation, mucosae, innate immunity

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INTRODUCTION

Eosinophils have long been perceived as terminally differentiated cytotoxic and destructive cells that play an effector role mainly in helminthic infections and allergic reactions, such as asthma (1). However, several recent studies have challenged the simplistic view of eosinophils as being exclusively involved in parasite destruction and allergic inflammation. Indeed, at steady state, blood eosinophils rapidly migrate into the gastrointestinal tract, lungs, adipose tissue, thymus, uterus, and mammary glands, where they are now known to exert a variety of essential homeostatic functions (2, 3). In this Mini Review, we summarize the advances in our understanding of the biology (distribution, phenotypic and morphological features, and ontogeny) and functions of these homeostatic eosinophils (hEos).

DISTRIBUTION OF hEos

In both humans and mice, most hEos are found in the non-esophageal portions of the gastrointestinal tract, where they principally reside in the *lamina propria* of the small intestine (4–7). Depending on the bibliographic source, the numbers of hEos in the gastrointestinal tract of mice are estimated to be 1.5- to 10-fold higher than in the blood (i.e., ranging from 3×10^5 to 2×10^6 cells) (8, 9). Pulmonary hEos are located in the lung parenchyma of both humans and mice (10). In

C57BL/6 mice, the numbers of lung hEos exceed 4×10^5, which corresponds to two times the numbers of eosinophils present in the entire circulation (10). In the adipose tissue of mice, hEos account for 4–5% of the stromal/vascular fraction cells (11). In the other organs, hEos reside only transiently (8, 12–16). In mice, the numbers of thymic hEos increase drastically after birth to reach a peak at 2 weeks of age (15). Their numbers then diminish significantly but rise again at 16 weeks when thymic involution starts (15). During the first recruitment phase, hEos concentrate in the cortico-medullary region of the thymus, whereas they are more prominent in the medulla at latter time points (15). It is noteworthy that, in humans, hEos seem to be already present in the thymus of fetuses (14). In rodents, infiltration of the uterus by hEos coincides with the estrus cycle (12, 13). Numerous hEos are indeed observed in the uterus just prior to estrus, during estrus and 1 day postestrus, whereas only few hEos are present during diestrus (12, 13). The vast majority of these cells are located in the endometrium adjacent to the muscular layer (16). In mice, hEos also home to the mammary gland during postnatal development (17). Mammary hEos are principally found around the growing terminal end buds from 3 weeks until 8 weeks of age (17).

In vivo studies in humans and mice have shown that eosinophils spend only a short time (i.e., half-life between 3 and 24 h) in the circulation (8, 18, 19). By contrast, hEos remain for a longer time in the tissues. Indeed, their half-life is about 36 h in the lung and up to 6 days in the intestines, thymus, and uterus (8) (**Figure 1**). The longevity of tissue hEos seems to correlate with CD11c expression. Indeed, while intestine, uterus, and thymus hEos express CD11c, lung, and blood hEos do not express this marker (8, 10) (**Figure 1**).

Time-course studies in mice have revealed that hEos are not present in the lung at birth but gradually increase in numbers to reach a maximal density by day 7 (10). This observation suggests a link between the colonization of the lung by hEos and the development of the microbiota. Paradoxically, however, hEos recruitment to the gastrointestinal tract seems to be independent of the bacterial flora. Indeed, prenatal mice have detectable hEos in their intestines, and germ-free mice display hEos levels similar to those of control colonized mice (5).

The basal recruitment of hEos to tissues is mainly driven by eotaxin-1 (CCL11), a chemokine produced by local cells such as epithelial cells, endothelial cells, fibroblasts, and monocytes (20–23). Correspondingly, hEos numbers are drastically reduced in the gastrointestinal tract, thymus, and uterus of eotaxin-1-deficient mice (5, 16, 24). Loss of CCR3, the major eotaxin-1 receptor (25, 26), results in defective tissue homing of hEos to the intestines but has no effect on the numbers of lung and thymus hEos (27), which likely relates to the fact that eotaxin-1 may act through alternative receptors such as CCR5 (28). Interleukin (IL)-5 and IL-13, two T helper type 2 (Th2) cytokines, may also promote, although to a lesser extent than eotaxin-1, trafficking of hEos under normal conditions (3, 5). IL-13 enhances eotaxin-1 production (29), while IL-5 supports eosinophil generation from bone marrow progenitors, enhances their sensitivity to eotaxin-1, and sustains their survival (30–32). It has been recently shown that the major source of basal IL-5 and IL-13 in the gastrointestinal tract and the adipose tissue are type 2 innate lymphoid cells (ILC2s) (29, 33). Moreover, after food intake, the vasoactive intestinal peptide stimulates intestinal ILC2 to enhance their secretion of IL-5 and IL-13, linking eosinophil levels with metabolic cycling (29).

MORPHOLOGICAL AND PHENOTYPIC FEATURES OF hEos

hEos have been mainly characterized in mice. They display most of the typical features of eosinophils, including red staining granules containing toxic cationic proteins (e.g., major basic proteins) and combined expression of CCR3, Siglec-F, and CD125 (i.e., the subunit α of the IL-5 receptor) (8, 9, 34) (**Figure 1**). They may also express CD11b (intestines, thymus, and adipose tissue), F4/80 (mammary glands, lung, and adipose tissue), CD69 (intestines and thymus), and CD44 (intestines and thymus) (6, 8, 10, 11, 15, 17, 35). In addition, most tissue hEos have a segmented nucleus and express CD11c (8, 13, 15, 16, 24, 35). Lung hEos represent an exception and rather resemble resting blood eosinophils. Indeed, both blood and lung eosinophils have a ring-shaped nucleus (as is the case for mammary hEos as well), express CD62L, display only intermediate levels of Siglec-F, and are negative for CD11c (8, 10, 17, 36, 37) (**Figure 1**). In mouse eosinophils, such characteristics, especially the presence of a ring-shaped nucleus, are considered a sign of cell immaturity (38, 39), suggesting that pulmonary hEos retain an immature phenotype when spreading into the lungs. However, they undergo piecemeal degranulation and are capable of phagocytosis, demonstrating their functionality (10). Interestingly, the number, localization, and morphological, phenotypic, and transcriptomic features of lung hEos remain unchanged, and differ from those of inflammatory eosinophils (iEos), during allergic airway inflammation (10). iEos, which are abundantly recruited to the lung during airway allergy, are indeed defined as SiglecF^{hi}CD62L^{-}CD101^{hi} cells with a segmented nucleus (CD101 being an iEos marker that is not expressed by lung hEos) (10). These observations suggest that hEos and iEos represent distinct eosinophil subsets. In line with this hypothesis, hEos- and iEos-like eosinophils are present in the blood of asthmatic mice (10), indicating that the differentiation of both subsets occurs even before their recruitment to the tissues. Furthermore, the parenchymal hEos found in non-asthmatic human lungs (Siglec-8^{+}CD62L^{+}IL-3R^{lo} cells) are phenotypically distinct from the iEos isolated from the sputa of eosinophilic asthmatic patients (Siglec-8^{+}CD62L^{lo}IL-3R^{hi} cells), confirming the mouse findings (10).

ORIGIN OF hEos

Eosinophil development depends on a complex interplay of several transcription factors, including GATA-binding protein-1 (GATA-1), CCAAT/enhancer-binding protein-α and -ε (C/EBP-α and -ε), E26 family transcription factor PU.1 (PU.1), and X-box-binding protein-1 (1, 3, 40–42). Among these transcription factors, GATA-1 is the most selective, as attested by the fact that ΔdblGATA mice, in which the double palindromic GATA-1-binding site in the *Gata1* promoter has been genetically

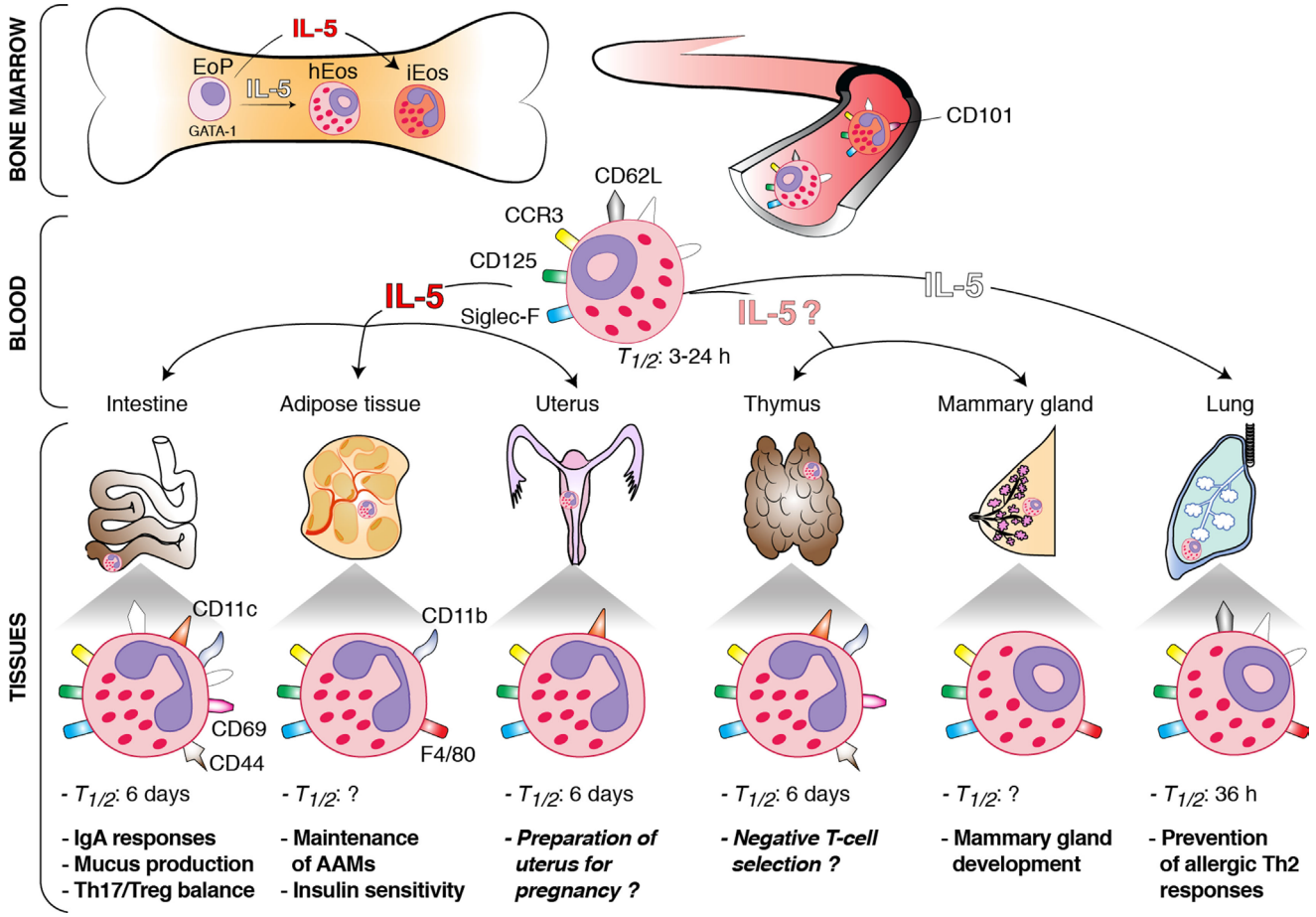

FIGURE 1 | Schematic overview of the origin, interleukin (IL)-5 dependence, phenotype, and functions of homeostatic eosinophils (hEos) in mice. hEos are produced in the bone marrow from the EoP precursor independently of IL-5. Conversely, inflammatory eosinophils (iEos) require IL-5 for their production. hEos are uniformly characterized by expression of Siglec-F, F4/80, CD125, and CCR3. hEos transit through the blood circulation to home into tissues at baseline. Blood hEos have a ring-shaped nucleus and express CD62L, while iEos have a segmented nucleus and do not express CD62L but express CD101. hEos homing to the tissues is either dependent (dark red) or independent (white) on IL-5. The IL-5-(in)dependence of thymic and mammary gland hEos remains unknown. Tissue hEos display distinct phenotype, half-life ($T_{1/2}$), and homeostatic functions. The surface phenotype depicted shows whether hEos express (colored symbols) or do not express (white symbols) the indicated surface markers. When marker expression is undefined, the symbol is not present. The function described in *italic* has been suggested, but a clear demonstration is still lacking. h, hours.

deleted, specifically lack eosinophils, including blood and tissue hEos (7, 10, 11, 36, 43). IL-5, which is the most specific cytokine for the eosinophil lineage, is dispensable for the steady-state production of eosinophils. Indeed, the basal numbers of blood eosinophils are only moderately reduced in IL-5-deficient mice, which are, however, unable to develop eosinophilia in the context of a Th2 response (44). Interestingly, recruitment of hEos to the tissues is independent (lungs), partly dependent (gastrointestinal tract and uterus), or entirely dependent (adipose tissue) on local IL-5 production (5, 10, 33, 44, 45) (**Figure 1**). Given that IL-5 enhances eosinophil survival following migration into the tissues, and that hEos that partly depend on IL-5 (gastrointestinal tract and uterus) have a higher half-life (see Distribution of hEos) than the IL-5-independent ones (lungs), one may speculate that the longevity of tissue hEos is directly linked to their dependence on IL-5. All these observations, if applicable to humans, could also explain why residual eosinophils are found in the blood and lungs of patients treated with anti-α-IL-5 antibodies (46–48).

FUNCTIONS OF hEos

Depending on the type of tissue they infiltrate, hEos are fulfilling completely different tasks, suggesting the local environment is able to drive hEos functions according to its specific needs. Here, we will review the tissue-specific homeostatic functions of hEos, also summarized in **Figure 1**.

Gastrointestinal Tract

Small intestinal hEos are now considered as actively contributing to intestinal homeostasis, allowing the host to cope with the constant and intense exposition to potentially pathogenic microorganisms and foreign and food antigens. In two

independent studies, hEos have been shown to be required for the development and maintenance of immunoglobulin (Ig) A-producing plasma cells (7, 35), concordant with the function of bone marrow eosinophils in supporting plasma cell survival (49). They also promote class switching toward secretory IgA, components involved in the neutralization and regulation of intestinal microorganisms (7, 35). In addition, eosinophil deficiency has been associated with altered gut microbiota composition (7, 35), altered development of Peyer's patches, and decreased mucus production in the small intestine (35), as well as increased numbers of Th17 cells (50) and decreased numbers of regulatory T cells and dendritic cells in gut-associated tissues (7). *In vitro*, Chen and colleagues have shown that small intestinal hEos are able to induce differentiation of naive T cells into $Foxp3^+$ regulatory T cells through IL-1β- and retinoic acid-dependent mechanisms (51). More recently, small intestinal hEos have also been shown to suppress the *in vitro* differentiation of Th17 cells and intestinal T cell-derived IL-17 production by secreting large amounts of the IL-1 receptor antagonist IL-1Rα (50). Altogether, these findings are concordant with the idea that small intestinal hEos contribute to intestinal homeostasis by regulating adaptive humoral IgA responses and cellular T cell responses.

Adipose Tissue

Eosinophils have been emerging as central regulators of adipose tissue metabolism and metabolic health. In adipose tissues, hEos are present together with alternatively activated macrophages (AAMs), and such hEos produce IL-4, thereby favoring the polarization of adipose macrophages toward the alternatively activated phenotype (11). AAMs play a crucial role in glucose homeostasis and development of beige fat, which improves glucose tolerance, insulin reactivity, and, hence, protects against obesity (11, 52, 53). In the absence of adipose hEos, AAMs are greatly reduced and biogenesis of beige fat is impaired (11, 54). Moreover, eosinophil-deficient mice on high-fat diet develop obesity, insulin resistance, and impaired glucose tolerance (11). Conversely, wild-type mice on a high-fat diet but infected with the gastrointestinal nematode *Nippostrongylus brasiliensis*, which triggered a greater eosinophil recruitment in the adipose tissues, exhibit a long-lasting improved sensitivity to insulin and glucose tolerance (11).

This important hEos/macrophage axis is regulated by ILC2s, which sustain adipose hEos and AAMs (33), and is promoted by microbiota depletion (55) and caloric restriction (56).

Uterus

It is known for decades that hEos infiltrate the non-pregnant uterus of rodents and humans in a cyclic manner, with a peak during estrus (13, 57, 58), but few studies have assessed their potential contribution to the physiology of uterus and to reproductive functions. Gouon-Evans and Pollard examined eotaxin-deficient animals, in which recruitment of hEos to the uterus was impaired, and found a delay in the establishment of the first estrus cycle along with the first age of parturition in those animals compared to wild-type controls (16). While these observations point toward a potential role for hEos in preparing the uterus for pregnancy (16), they must be balanced by the fact that the timing of establishment of subsequent estrus cycles in mature mice is not affected by the absence of eosinophils (16). Most importantly, no fertility issues have been reported in constitutively eosinophil-deficient mice (36, 59), demonstrating that hEos are not essential for normal reproduction.

Thymus

The presence of thymic hEos in the close vicinity of immature double-negative thymocytes and their abundance in neonates suggest that they may contribute to the process of central tolerance and negative T-cell selection (15). Supporting this, thymic hEos numbers rapidly increase and hEos cluster with apoptotic bodies in an acute model of MHC-I-dependent negative selection (15). Another report proposes that hEos may contribute to the clearance of apoptotic cells, as eosinophil-deficient mice subjected to irradiation-induced thymocyte death are impaired in their ability to phagocyte apoptotic cells (43). However, the definitive proof of a homeostatic role for thymic eosinophils in the process of negative T-cell selection is currently lacking.

Mammary Gland

A role for eosinophils in regulating postnatal mammary gland development has been proposed in mice (17). Indeed, ablation of hEos recruitment to the mammary glands in eotaxin-deficient animals resulted in a reduced number of branches of the mammary ductal tree and of terminal end buds (i.e., the precursors of alveolar buds) (17). A similar phenotype was observed in the mammary tissue of IL-5-deficient mice as compared to the one from wild-type mice, although the specific contribution of IL-5 itself vs. IL-5-dependent eosinophils has not been assessed in this model (60). Nevertheless, such IL-5-mediated developmental events appear to have functional consequences, as IL-5-deficient nursing dams gave rise to decreased litter size and weanling survival, a phenomenon rescued when IL-5-deficient pups were nursed by IL-5-sufficient dams (60).

Lungs

Microarray analyses revealed that lung hEos, unlike lung iEos, express several genes, such as *Anxa1*, *Nedd4*, *Runx3*, *Serpinb1a*, and *Ldlr*, that are implicated in the maintenance of lung immune homeostasis, and especially in the negative regulation of Th2 cell responses (10). In line with this observation, eosinophil-deficient ΔdblGATA mice exhibit increased sensitivity to house dust mites (10), confirming that lung hEos are endowed with the capacity to prevent Th2-driven airway allergy. This immunosuppressive function of lung hEos is linked to their unique ability to inhibit the maturation, and therefore the pro-Th2 function, of allergen-loaded dendritic cells (10).

CONCLUSION

Although hEos are far from being fully characterized, it is fascinating to see how fast our understanding of the complexity of their phenotype and functions is growing. The fact that these cells exert crucial homeostatic roles at multiple levels merits further investigations and is of medical importance. Indeed, anti-eotaxin-1 and eosinophil-depleting agents, such as humanized

anti-IL-5 receptor antibodies and anti-Siglec-8 molecules, are currently being developed to treat eosinophilic disorders such as allergic asthma (9, 61–66), and one has to keep in mind the possibility that such drugs may disrupt tissue homeostasis by preventing organ-specific homing of hEos or by affecting their survival or functions.

AUTHOR CONTRIBUTIONS

TM, CM, and FB conducted a review of the literature. TM and FB wrote the manuscript.

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Regulation of Eosinophil and Group 2 Innate Lymphoid Cell Trafficking in Asthma

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Asthma is an inflammatory disease usually characterized by increased Type 2 cytokines and by an infiltration of eosinophils to the airways. While the production of Type 2 cytokines has been associated with T_H2 lymphocytes, increasing evidence indicates that group 2 innate lymphoid cells (ILC2) play an important role in the production of the Type 2 cytokines interleukin (IL)-5 and IL-13, which likely amplifies the recruitment of eosinophils from the blood to the airways. In that regard, recent asthma treatments have been focusing on blocking Type 2 cytokines, notably IL-4, IL-5, and IL-13. These treatments mainly result in decreased blood or sputum eosinophil counts as well as decreased asthma symptoms. This supports that therapies blocking eosinophil recruitment and activation are valuable tools in the management of asthma and its severity. Herein, we review the mechanisms involved in eosinophil and ILC2 recruitment to the airways, with an emphasis on eotaxins, other chemokines as well as their receptors. We also discuss the involvement of other chemoattractants, notably the bioactive lipids 5-oxo-eicosatetraenoic acid, prostaglandin D_2, and 2-arachidonoyl-glycerol. Given that eosinophil biology differs between human and mice, we also highlight and discuss their responsiveness toward the different eosinophil chemoattractants.

Keywords: eosinophil, group 2 innate lymphoid cells, 2-arachidonoyl-glycerol, chemokine, eotaxin, asthma

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INTRODUCTION

Asthma is a respiratory disease characterized by inflammation and hyperresponsiveness of the airways and roughly affects 300 million people worldwide (1). Eosinophils play a pivotal role in asthma by generating many mediators inducing bronchoconstriction and/or contributing to inflammation and remodeling (2). Airway eosinophilia is observed in many subjects with asthma and increases with disease severity and exacerbations (3). The anti-inflammatory treatment of asthma is primarily based on inhaled corticosteroids (4). The dose is adjusted to decrease eosinophil counts in the blood and/or in induced sputum, which results in a reduction of asthma exacerbations. However, the chronic use of corticosteroids is linked with significant systemic side effects even at low doses, and some severe asthmatics remain symptomatic and have high sputum eosinophil counts despite the use of high doses of corticosteroids (5). This stresses the need of developing new therapeutics that could limit both bronchoconstriction and inflammation.

Increased eosinophil numbers are observed in many asthmatics, notably those characterized by a Type 2-like inflammation, characterized by an increased production of the cytokines interleukin (IL)-4, IL-5, and IL-13 (6). As such, it is well accepted that the Type 2 cytokines IL-4, IL-5, and IL-13 are linked to increased eosinophil numbers, either by promoting eosinophil survival (IL-5) or by inducing the production of eosinophil chemoattractants (IL-4 and IL-13) (7, 8). While T_H2 lymphocytes participate in the release of Type 2 cytokines, group 2 innate lymphoid cells (ILC2) are being increasingly recognized as a significant source of Type 2 cytokines as well (9, 10). Asthma treatments that focused on blocking Type 2 cytokines (IL-4, IL-5, and IL-13) decrease blood or sputum eosinophil counts and asthma symptoms in subjects with severe asthma presenting a high eosinophil count in their induced sputum (11–25). This article reviews the current evidence regarding eosinophil and ILC2 chemoattractants and their involvement in asthma and its severity.

DISCOVERY TIMELINE OF THE MAIN EOSINOPHIL CHEMOATTRACTANTS

The extensive investigation of how eosinophils were recruited really began in the 1970s. Complement component 5a (C5a) has been known to induce guinea pig eosinophil migration since 1970 (26–29), and its impact on human eosinophils was documented in 1973 (26). Histamine was next documented as an eosinophil chemoattractant in 1975 (30) although its effect is limited (31–34).

In 1980s, other eosinophil chemoattractants were characterized, notably platelet-activating factor (PAF), leukotriene (LT) B_4, and *N*-formylmethionyl-leucyl-phenylalanine (fMLP). Numerous reports indicate that PAF induces the migration of eosinophils (29, 35–41). Even if LTB_4 is mainly characterized as a neutrophil chemoattractant, it also induces human eosinophil migration (29, 37, 42, 43). fMLP is a weak chemoattractant for eosinophil migration: some studies unraveled a weak migration of eosinophils (29, 37, 44, 45) while others did not find any effect (38, 46).

The expansion of the chemokine field in the 1990s allowed the characterization of additional eosinophil chemoattractants. CCL5 [regulated on activation, normal T cell expressed and secreted (RANTES)] was the first chemokine documented as a human eosinophil chemoattractant in 1992 (47) and was shown to induce both the migration and transmigration of human eosinophils (48–57). The effect of CCL3 (MIP-1α) on human eosinophil migration was also evaluated in 1992 (47). However, the ability of CCL3 as an eosinophil chemoattractant is low, as later reports indicated that at optimal concentration, the CCL3-induced migration of eosinophil corresponded to about 33% of that induced by CCL5 (48, 52, 57). Of note, one study showed that ~20% of individuals responded to CCL3 to the same extent than CCL11, while the others poorly responded to CCL3 and this was linked to CCR1 (58). In mid-1990s, other chemokines were tested for their ability to elicit human eosinophil migration, notably CCL7 (MCP-3), CCL8 (MCP-2), and CCL13 (MCP-4) (34, 48, 50–53, 55–57, 59, 60). However, their impact on human eosinophil migration was limited.

The discovery of eotaxins was a substantial leap forward in understanding how eosinophils were selectively recruited into the tissues. CCL11 (eotaxin-1) was first discovered by Jose et al. in guinea pigs (61, 62). Two years later it was confirmed as a selective chemoattractant of human eosinophils in 1996 (63) and several studies confirmed its potency in several migration models (55, 64–66). A year later, CCL24 (eotaxin-2) was discovered (67) and was confirmed as being as efficient as CCL11 (34, 55–57, 65). Last but not the least, CCL26 (eotaxin-3) was discovered in 1999 (68, 69), and it is the most efficient eotaxin to induce the migration or transmigration of asthmatic eosinophils (65). Of note, CCL26 appears also critical for eosinophil migration/tissue eosinophilia in other human disorders characterized by eosinophil recruitment, notably eosinophilic esophagitis and Churg–Strauss syndrome (70, 71).

It was also in the mid-1990s that additional bioactive lipids from the 5-lipoxygenase pathway were documented as human eosinophil chemoattractants. 5-Oxo-eicosatetraenoic acid (5-KETE) was identified as a potent chemoattractant of eosinophils in 1996 (72, 73). To this date, 5-KETE is the most efficient human eosinophil chemotactic factor *in cellulo* (41, 43, 65, 66). LTD_4 was the first cysteinyl leukotriene (CysLTs) to be defined as a direct chemoattractant of human eosinophils (74) but induces a weak migration (75–78). It was also reported that LTC_4 and LTE_4 induce an eosinophil migration comparable to LTD_4 (79).

The new millennia also expanded our knowledge on how human eosinophils could be recruited into the tissue. In that regard, CXCL12 (SDF-1) was shown to induce the recruitment of eosinophils (65, 80, 81). Furthermore, a 2001 study demonstrated that prostaglandin (PG) D_2 selectively induced the migration of eosinophils, Th2 lymphocytes cells, and basophils (82), and increasing evidence support the development of DP_2/CRTH2 antagonists for the management of asthma (83). However, PGD_2 seems to induce a limited recruitment of eosinophils (66, 84–88). Of note, PGD_2 increases CCL11- and 5-KETE-induced-eosinophil migration (87). Finally, in 2004, the endocannabinoid 2-arachidonoyl-glycerol (2-AG) was identified as an eosinophil chemoattractant (89); this effect of 2-AG involves the CB_2 receptor and is largely potentiated by IL-3, IL-5, and GM-CSF (66, 90, 91).

HUMAN EOSINOPHIL RECRUITMENT AND ASTHMA

As underscored in the previous section, many soluble mediators and chemokines can induce human eosinophil recruitment and thus participate in asthma pathogenesis. In this section, we review how these chemoattractants contribute to eosinophil recruitment in a context of asthma. A differential eosinophil recruitment could be observed in asthma severity and/or during asthma exacerbations if there is a dysregulation in the release of the different chemoattractants or their receptors, notably by desensitization or internalization. To this end, our data (**Figure 1**) indicate that with the exception of the CXCR4 and the CB_2 receptors, the expression of chemoattractant receptors do not change, at the mRNA level, in human eosinophils isolated from the blood

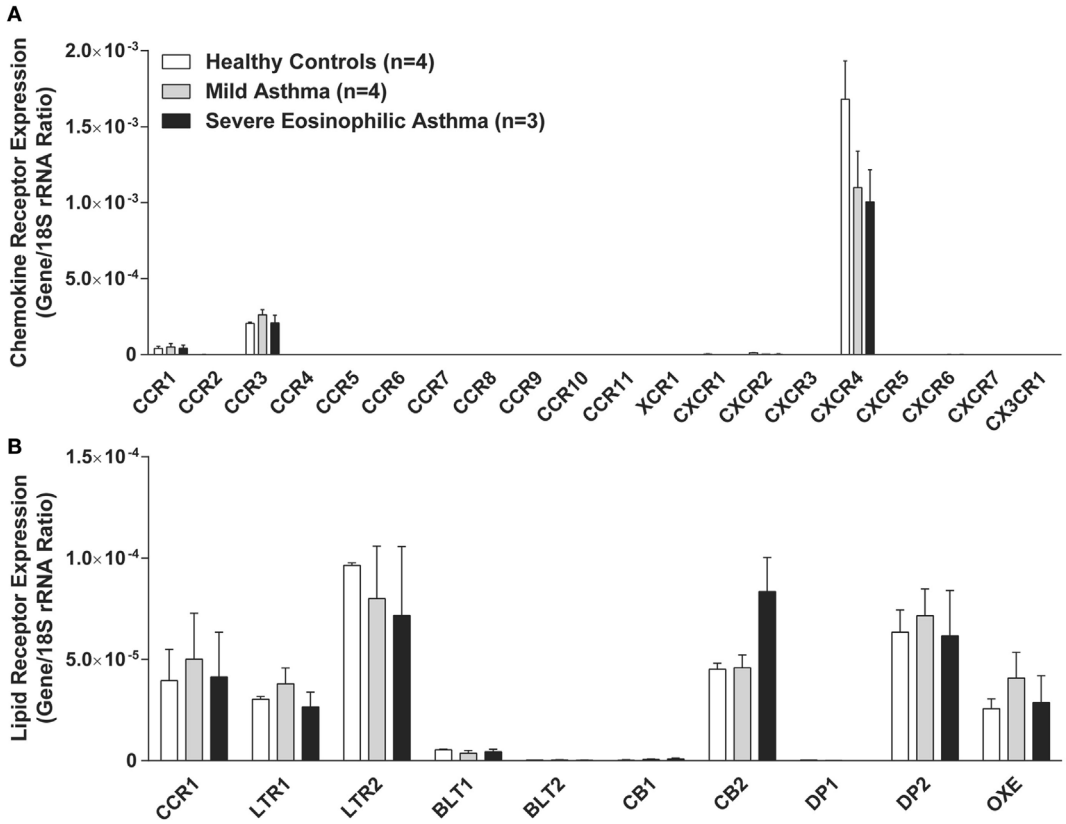

FIGURE 1 | Expression of chemokines and lipid mediator receptors by human eosinophils. Human eosinophils were isolated from the blood of healthy controls, mild asthmatics, and severe eosinophilic asthmatics as defined and described in Ref. (92). mRNAs were quantitated by qPCR array using a custom qPCR array (RT^2 Profiler qPCR Multiplex Array Kit, Qiagen, ON, Canada). Chemokine receptor expression **(A)** and bioactive lipid receptor expression **(B)** are represented by the ratio between mRNAs and 18S rRNA control. Results are the mean (±SEM) of 3–4 donors for each group. Approval from the local ethics committee was obtained, and all volunteers signed an informed consent form.

of healthy subjects, mild and severe eosinophilic asthmatics, as defined in Ref. (92). This supports the notion that perhaps the increased recruitment of eosinophils is rather the consequence of increased chemoattractants in the bronchial tissue.

CHEMOKINES

The most studied chemokines in asthma are CCL5 and eotaxins, probably because their levels are usually increased in asthmatics compared to healthy controls in all body fluids tested, namely bronchoalveolar lavages (BAL), induced sputum, blood, and bronchial biopsies (92–115). Moreover, these chemokines are linked to poor asthma control and increased eosinophil recruitment to the airways. Indeed, CCL5 levels are greater in induced sputum from poorly controlled asthmatics than from controlled asthmatics (116, 117); subjects undergoing acute exacerbations have higher CCL11 levels in induced sputum and plasma samples than subjects with stable asthma or healthy controls (111, 118–120); and CCL24 and CCL26 expression in airway epithelial cells are associated with lower forced expiratory volume in 1 s (FEV_1), more asthma exacerbations, and increased sputum eosinophil counts (92, 121). It is not clear whether one chemokine is more important than the others and if we could target these chemotactic proteins to limit eosinophil recruitment and asthma exacerbation. In that regard, different studies evaluated the expression of these chemokines during allergen challenges, and the obtained data rather indicate that eosinophil-recruiting chemokines are not necessarily present at the same time and might have different as well as overlapping roles. CCL5 levels correlate with eosinophil counts in BAL 4 h after the challenge (122), but not 24 h after the challenge (123). CCL11 levels are increased in BAL, induced sputum and bronchial biopsies of asthmatics, and are associated with eosinophil numbers 4 and 24 h after the challenge (104, 124, 125). That being said, one study reported that CCL11 levels are similar in bronchial biopsies from asthmatics before and 24 h after allergen challenge (103). CCL24 expression is significantly increased in bronchial mucosa from asthmatics 48 h after allergen challenge (126), but is similar before and 24 h after allergen challenge (103). As for CCL26, its expression in bronchial biopsies increases 24 and 48 h after allergen challenge (103, 126), but its expression in bronchial submucosa did not correlate with eosinophil counts 48 h after allergen challenge (126). Additionally, some research groups documented the impact of these chemokines on eosinophil migration in asthma *in cellulo*. CCL11 and CCL26 induce a greater migration of eosinophils from asthmatics than from healthy subjects (65, 127). Finally, while most evidence reflects an

important role of CCL5 and the eotaxins in asthma, some studies reported that there was no increase in CCL5 or eotaxin expression in BAL, airway epithelium brushings, or bronchial biopsies between asthmatics and healthy controls (92, 103, 121, 128, 129).

Studies on CCL3, CCL7, CCL8, CCL13, and CXCL12 in relation with asthma are limited. Among the latter, CCL13 is better associated with eosinophils and asthma. Its expression is higher in BAL, bronchial biopsies, induced sputum, and plasma samples from asthmatics than from healthy controls (99, 100, 105, 130, 131). One study reported increased CCL3 levels in BAL from asthmatics compared to healthy controls (93). Increased CCL7 levels and CCL7-expressing cells are found in bronchial biopsies and BAL from asthmatics compared to healthy controls (94, 95, 100), and serum CCL8 levels are higher in asthmatics compared to healthy controls (132). CXCL12 levels in bronchial mucosa and BAL are greater in asthmatics than in healthy controls (133, 134), and CXCL12 levels in BAL correlate with eosinophil numbers (134).

LIPID MEDIATORS AND OTHERS

Other soluble mediators might also participate in the recruitment of eosinophils in asthma. In that regard, $CysLT_1$ receptor blockade usually decreases eosinophil counts, although it is not clear whether this is a direct or indirect effect (135–144). LTB_4, histamine, C5a, and PGD_2 are all associated with asthma, but their involvement in eosinophil recruitment in asthma is not well defined. Even if LTB_4 levels in blood and exhaled breath condensate are increased in asthma (145–147), the LTB_4 receptor antagonist, LY293111, decreases neutrophil but not eosinophil counts in BAL from asthmatics (148). As for PGD_2, some studies demonstrated similar PGD_2 levels in BAL or induced sputum of asthmatics, atopics, and healthy subjects (149–152), but its levels can increase in the BAL after an allergen challenge (149, 153, 154). Of note, the antagonism of the PGD_2 receptor 2 (DP_2/CRTH2) improves lung function and the quality of life of asthmatics compared to placebo (155, 156). Finally, C5a levels are increased in BAL and in induced sputum from asthmatics compared to healthy controls after an allergen challenge (157, 158), and a haplotype of the C5a gene was identified to be protective against asthma (159).

As for PAF, 5-KETE, fMLP, and 2-AG, their association with asthma is not well documented and this requires further investigations. For example, we have no idea to which extent 2-AG and 5-KETE levels are modulated in asthma and its severity.

ASTHMA SEVERITY

As underscored with the data from the allergen challenges presented in the previous section, it is not possible to pinpoint one chemoattractant explaining the recruitment of human eosinophils. They rather indicate that they collaborate together and that they might be involved at different times during the asthmatic response. In addition, it is possible that the mediators responsible for eosinophil recruitment might also change as the disease worsens. For example, CCL11 and/or CCL26 levels are greater in induced sputum from severe or moderate asthmatics than from mild asthmatics or healthy controls (92, 160). In plasma samples, CCL11 levels are associated with asthma severity and are not significantly affected by corticosteroid treatment (161). Coleman et al. demonstrated that CCL24 and CCL26, but not CCL11, mRNA expression in bronchial epithelium increases with asthma severity and is associated with sputum eosinophil counts, lower FEV_1, and more asthma exacerbations (121). In contrast, subjects with severe eosinophilic asthma have lower CCL24 levels in bronchoalveolar lavage fluids and similar CCL24 levels in bronchial epithelial cells compared to healthy controls (92, 121). For CCL5, Saad-El-Din demonstrated that serum CCL5 levels are greater in subjects with severe or moderate asthma as compared to subjects with mild asthma and are associated with blood eosinophil number (114). As for CXCL12, it induces a greater migration of corticosteroid-treated eosinophils than untreated eosinophils and that the expression of the CXCL12 receptor, CXCR4, increases in corticosteroid-treated eosinophils (80), raising the possibility that CXCL12 plays a more important role in unstable severe eosinophilic asthmatics which are taking large doses of corticosteroids.

In asthma, CysLTs levels in induced sputum are increased in moderate asthmatics compared to severe asthmatics and healthy controls (162). Also, similar sputum CysLTs levels were found in severe eosinophilic and non-eosinophilic asthmatics (162). In contrast, exhaled breath condensate levels of CysLTs correlate with asthma severity (163). In mild-to-moderate asthmatics or eosinophilic asthmatics, the CysLT1 antagonist montelukast, alone or in combination with corticosteroids, decreases sputum or blood eosinophil counts (136, 138, 141, 164). On the other hand, severe eosinophilic asthmatics, severe non-eosinophilic asthmatics, and moderate uncontrolled asthmatics have similar sputum or blood eosinophil counts between montelukast-treated and placebo-treated individuals or between montelukast/corticosteroid-treated and corticosteroid-treated asthmatics (165–167). Of note, PGD_2 and DP_2/CRTH2 levels are increased in asthma severity in BAL (151, 152), and the DP_2/CRTH2 antagonist OC000459 improves FEV_1 and the quality of life of subjects with eosinophilic uncontrolled asthma and steroid-free subjects with moderate persistent asthma (155, 156). Finally, C5a receptor expression on bronchial epithelium is greater in subjects with fatal asthma than mild asthmatics and healthy controls (168).

OF MICE AND MEN

The potential and/or documented roles of multiple chemoattractant involved in eosinophil recruitment in asthma underscore the need to revisit this concept and to establish when and how those actors are involved. The development of experimental asthma models with mice, rats, or guinea pigs has been very helpful to broaden our knowledge about asthma pathogenesis and to identify some eosinophil and ILC2 chemoattractants in allergic asthma. However, eosinophils and their functional responses are very different between species (169). In that regard, some chemoattractants and their receptors in humans are not expressed in mice. For instance, the 5-KETE receptor OXE is not expressed in mice (170, 171), resulting in an absence of 5-KETE-induced eosinophil migration (170). Additionally, CCL26 is not expressed

in mice (170) and human CCL26 does not induce the migration of mouse eosinophils (172, 173). Furthermore, CCL5 does not induce the migration of mouse eosinophils (172, 174–176). Globally, three of the most efficient human eosinophil chemoattractants described so far (CCL5, CCL26, and 5-KETE) do not induce the migration of eosinophils from mice, illustrating major differences in eosinophil recruitment between mice and humans and underscoring that transposing eosinophil recruitment data from mice to humans might be hazardous. The impact of the different chemoattractants on the migration of eosinophils from humans and mice is summarized in **Table 1** in which the number of migrated eosinophils in different migration assays is compared. It should be kept in mind that the presented data involve different eosinophil migration assays and that a true comparison between the presented chemoattractant is somewhat subjective. This is why we defined the different efficiencies using %migration intervals.

MEDIATORS PROMOTING ILC2 RECRUITMENT

First identified in 2010, ILC2 are defined as lymphoid cells lacking specific lymphocytes lineage markers and the expression of the DP_2/CRTH2 and ST2, the IL-33 receptor (214–218). They produce, in response to IL-25, IL-33 or thymic stromal lymphopoietin (TSLP), large amounts of the T_H2 cytokines IL-5, IL-13 and, to a lesser extent, IL-4. Of note, the number of ILC2 correlate with sputum eosinophils in allergic asthma (219). This suggests that ILC2 might play an important role in asthma (220, 221), especially by directly or indirectly modulating eosinophil survival/recruitment. However, the cellular mechanisms by which ILC2 are recruited to the lungs remain poorly defined and few studies addressed the impact of chemokines or bioactive lipids on the migration of ILC2.

Since IL-25, IL-33, and TSLP are potent activators of ILC2, their ability to induce the migration of ILC2 was first evaluated. IL-33 and TSLP induce a weak migration of human ILC2 (218, 222, 223). However, the impact of IL-25 remains a matter of debate, as one study reported a weak IL-25-induced ILC2 migration (223), while another found no effect of IL-25 (218). PGD_2 and CysLTs are defined as potent chemoattractants of ILC2. Indeed, PGD_2 is almost five times more potent than IL-33 (218, 224), and the PGD_2-induced migration is greater in ILC2 from allergic subjects compared to healthy subjects (224). Furthermore, mice lacking DP_2/CRTH2 or treated with a DP_2/CRTH2 antagonist have lower ILC2 levels in the lungs after intranasal administration of PGD_2 (225). As for CysLTs, ILC2 express the receptor $CysLTR_1$ and its expression is increased in atopic subjects (223, 226, 227). Interestingly, a research group recently demonstrated that all CysLTs induce the migration of human ILC2 *in vitro*, $LTE_4 \gg LTD_4 > LTC_4 \approx$ IL-33, indicating that perhaps another CysLT receptor might be involved in this process (223).

Although only IL-33, TSLP, PGD_2, and the CysLTs have been identified as chemoattractants of ILC2, some studies reported that human ILC2 express the chemokine receptor CCR4 and mouse ILC2 express the LTB_4 receptor BLT_1 (222, 227). Furthermore, TGF-β increases the basal migration of murine ILC2, which suggests that it could enhance their response to other chemoattractants (228). Other studies are thus needed to delineate how ILC2 migrate to the bronchial tissue.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

This review highlights that many chemokines and soluble mediators are very good to excellent at inducing the migration of eosinophils *ex vivo* and their recruitment *in vivo*. This

TABLE 1 | Eosinophil chemoattractants and their receptors of human and mice.

Eosinophil chemoattractants	Human		Mice	
	Receptors	Efficiency	Receptors	Efficiency
CCL11/eotaxin-1	CCR3 (177–179)	++ (55, 56, 64, 66)	CCR3 (172, 180)	++ (181)
CCL24/eotaxin-2	CCR3 (179, 182)	++ (55, 56)	CCR3 (172, 180)	+ (172, 173)
CCL26/eotaxin-3	CCR3 (68, 69)	+++ (65, 68, 69)	CCR3 (172, 180)	– (172, 173)
CCL5/RANTES	CCR1, CCR3 (58, 177, 178, 183, 184)	++ (47, 52, 55, 56)	CCR1, CCR3, CCR5 (172, 180)	– (172, 174, 175)
PAF	PAFR (185, 186)	++ (29, 37, 39, 41)	PAFR (187)	+ (181)
C5a	C5aR (188–190)	++ (29, 47, 52)	C5aR (191, 192)	++ (174, 193)
2-AG	CB_2 (89, 194)	+ (66, 90)	n/a	n/a
5-KETE	OXE (171, 195, 196)	+++ (41, 43, 66)	n/e	– (170)
LTB_4	BLT_1 (197, 198)	+ (29, 37, 64)	BLT_1 (197)	+ (199)
PGD_2	DP_2/CRTH2 (82, 87)	+ (87)	DP_2/CRTH2 (200, 201)	+ (202, 203)
fMLP	FPR (204–206)	+ (29, 37, 52)	n/a	+ (193, 207)
CCL3/MIP-1α	CCR1, CCR3 (58, 177, 178, 183, 184)	± (47, 48, 52, 57)	CCR1, CCR3 (172, 180)	± (172, 173, 181, 208)
CCL7/MCP-3	CCR1-CCR3 (178, 183, 209)	+ (52, 55)	CCR1–CCR3 (172, 180)	n/a
CCL8/MCP-2	CCR1–CCR3 (183, 184, 209)	+ (52)	CCR1–CCR3 (172, 180)	n/a
CCL13/MCP-4	CCR1–CCR3 (177, 183, 209)	+ (56)	CCR1–CCR3 (172, 180)	n/a
CXCL12/SDF-1	CXCR4 (80, 210)	++ (65, 80)	CXCR4 (172)	n/a
LTD_4	$CysLT_1$, $CysLT_E$? (211, 212)	+ (74–77)	$CysLT_1$, $CysLT_E$? (213)	– (199)

–: no migration, ±: weak or no migration, +: migration usually between 10 and 30%.

++: migration usually between 30 and 50%, +++: migration over 50%.

2-AG, 2-arachidonoyl-glycerol; fMLP, N-formylmethionyl-leucyl-phenylalanine; n/a, not available; n/e, not expressed; PFA, platelet-activating factor; PG, prostaglandin.

underscores that targeting eosinophil recruitment as a therapeutic approach in asthma might not be readily successful, as suggested with the attempt at blocking the eotaxin receptor CCR3 (229). Additionally, many questions remain unanswered. For instance, it remains unclear when all those chemoattractants actually play a role during the asthmatic response and this needs to be addressed, notably by defining the presence of all eosinophil and ILC2 chemoattractants in the same samples and at different stages of the disease/exacerbation. Experimental restrictions such as specie (mouse vs. humans) or the number of chemoattractants being investigated in a given study make the obtained data a little blurry, sometimes raising more questions than answering them. In addition, the involvement of the different chemoattractants as the disease worsens remains anecdotal. Given that severe asthmatics are frequently older than mild and moderate asthmatics, it is possible that the set of chemoattractant changes with age and perhaps, with gender as well [keeping in mind that aging modulates sex hormones, which could affect the synthesis of the different chemoattractants as it is the case for 5-lipoxygenase derivatives (230)]. Another important aspect of this review is the illustration that some of the best chemoattractants for human eosinophils are not present or are effectless in murine models (**Table 1**), raising the question that perhaps data obtained from animal models should be taken cautiously until they are validated in humans. Finally, if ILC2 play a prominent role in asthma as it is proposed from mouse data, it will be of crucial importance to rapidly understand the regulation of their recruitment into the airways, by defining which chemokines, lipids, and other chemoattractants are promoting their recruitment both in mice and humans, as well as all the receptors involved in that process.

AUTHOR CONTRIBUTIONS

All the authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Glycobiology of Eosinophilic Inflammation: Contributions of Siglecs, Glycans, and Other Glycan-Binding Proteins

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The historical focus on protein–protein interactions in biological systems, at the expense of attention given to interactions between other classes of molecules, has overlooked important and clinically relevant processes and points of potential clinical intervention. For example, the significance of protein–carbohydrate interactions, especially in the regulation of immune responses, has recently received greater recognition and appreciation. This review discusses several ways by which cell-surface lectin–glycan interactions can modulate eosinophil function, particularly at the levels of eosinophil recruitment and survival, and how such interactions can be exploited therapeutically. A primary focus is on discoveries concerning Siglec-8, a glycan-binding protein selectively expressed on human eosinophils, and its closest functional paralog in the mouse, Siglec-F. Recent advances in the synthesis of polymeric ligands, the identification of physiological ligands for Siglec-8 and Siglec-F in the airway, and the determination of the basis of glycan ligand discrimination of Siglec-8 are discussed. Important similarities and differences between these siglecs are outlined. Eosinophil expression of additional glycan-binding proteins or their glycan ligands, including interactions involving members of the selectin, galectin, and siglec families, is summarized. The roles of these molecules in eosinophil recruitment, survival, and inflammation are described. Finally, the modulation of these interactions and potential therapeutic exploitation of glycan-binding proteins and their ligands to ameliorate eosinophil-associated diseases are considered.

Keywords: eosinophils, Siglec-8, Siglec-F, selectins, galectins, glycans

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Eosinophils are innate immune cells that contribute to host defense responses against parasitic infections and appear to have been retained in evolution throughout vertebrate species (1–3). Yet, there is a sizable body of evidence that eosinophils, under other circumstances, can be pro-inflammatory and are thus thought to be major effector cells in allergic and other type 2 immune responses. These include common conditions such as asthma, often manifesting with comorbid upper airways diseases of chronic rhinosinusitis with or without nasal polyposis, disorders that similarly manifest prominent type 2 inflammatory signatures and features including elevated Th2 and ILC2 cells with IL-4, IL-5, IL-13, eotaxins, and other downstream mediators (4–7). Another common eosinophil-associated disease is atopic dermatitis, where eosinophils contribute to some but perhaps not all stages of the disease (8). Less common disorders where eosinophils are felt to play a major role include eosinophilic granulomatosis with polyangiitis (formerly known as Churg–Strauss

syndrome); eosinophilic gastrointestinal disorders (EGID), namely eosinophilic esophagitis, gastritis, and colitis, existing alone or in combination (9–13); and other systemic and organ-specific hypereosinophilic syndromes and disorders (14).

Current treatments for these eosinophil-associated conditions include glucocorticosteroids, mediator receptor antagonists, and other anti-inflammatory drugs that reduce eosinophil numbers and activity, but they are neither fully effective nor curative or disease modifying, hence the need for additional therapies (15, 16). Advanced efforts to indirectly target eosinophils [e.g., with agents that antagonize TSLP (17) and IL-4 and IL-13 biology with FDA-approved dupilumab (18–21)] or more specifically target eosinophils (e.g., with the FDA-approved anti-IL-5 biologics mepolizumab and reslizumab, and perhaps someday with the anti-IL-5 receptor antibody benralizumab and the oral agent dexpramipexole) offer hope for improved management of these disorders (22–24). Despite these promising agents, and advancements in our understanding of the pathophysiology of each of these disorders, many patients remain refractory to treatment, or in the case of EGID, there are as yet no FDA-approved drugs. These and other unmet needs led to collaborative efforts to find additional eosinophil-selective targets, and in recent years, these have included the only known pure eosinophil-specific surface target EMR1 (25, 26), and Siglec-8, expressed not only on eosinophils but also on mast cells and weakly on basophils (27–29). The focus of this review is not only on the latter molecule but also includes discussions of other lectin–glycan interactions known to influence eosinophil responses.

SIGLEC-8

Receptor Discovery, Characteristics, and Expression Patterns

Siglec-8 [also originally named sialoadhesin family 2 (SAF-2)] is an I-type single pass transmembrane protein that was discovered from a human eosinophil cDNA library generated from a patient with hypereosinophilic syndrome and first described in the year 2000. Eosinophil mRNA was examined by random sequencing of expressed sequence tags, which led to the identification of a protein 431 amino acid residues (aa) in length that was highly homologous to others in the sialoadhesin/siglec family. The highest levels of homology were found with Siglec-7 (68%), Siglec-3 (49%), and Siglec-5 (42%). The extracellular region of Siglec-8 contains 358 aa with a hydrophobic signal peptide and three Ig-like domains, with the N-terminal Ig domain possessing an arginine at position 125 that is putatively necessary for sialic acid binding (27, 28). When originally described, the cytoplasmic domain was found to be unusually short, and no known signaling motifs were observed. Subsequent investigations by Foussias et al. led to the observation that Siglec-8 exists in two isoforms (the 431-aa originally identified Siglec-8 "short form" and a 499-aa Siglec-8 "long form"), both containing identical extracellular and transmembrane regions. However, like most other CD33-related siglecs, the long form of Siglec-8 contained two characteristic tyrosine-based motifs: a membrane-proximal immunoreceptor tyrosine-based inhibitory motif (ITIM) resembling a classical ITIM (ILVxYxxLV) and a membrane-distal immunoreceptor tyrosine-based switch motif (ITSM) resembling a motif (TxYxxIV) found in the intracellular region of signaling lymphocyte activation molecule (SLAM) (30). The Siglec-8 long form is now just called Siglec-8 because it was found to be the primary form of the receptor, with a molecular weight of ~54 kDa (30), although eosinophils usually but not always express the short form, the function of which remains unknown (31).

While quantitative PCR analysis for the Siglec-8 mRNA not surprisingly detected signals in hematopoietic organs, expression in lung was unexpected. Using monoclonal antibodies recognizing the extracellular region, it was soon discovered that Siglec-8 was not just an eosinophil marker. It was selectively expressed on the surface of eosinophils, mast cells, and at low levels on basophils, but not on any other cells, making it the first receptor to be exclusively expressed on these three allergic effector cell types (28). Using human CD34+ cell-derived culture systems, it was determined that Siglec-8 is a terminal differentiation marker in both eosinophils and mast cells, with maximum protein expression in each cell type occurring at 21 and 30 days of culture, respectively. In contrast, none of the eosinophilic cell lines express Siglec-8 and only modest expression was observed on the mast cell line HMC-1.2, furthering the concept that Siglec-8 is a terminal differentiation marker on these cell types (32, 33).

The *SIGLEC8* gene, like other CD33-related siglecs, is located in the centromeric region of chromosome 19q13 (27, 30). However, little is known about regulation of *SIGLEC8* expression at the transcriptional level. In a recent report, Hwang et al. identified Olig2, a basic helix-loop-helix transcription factor, as a potential regulator of *SIGLEC8* gene expression. They showed that *OLIG2* and *SIGLEC8* are coexpressed late in eosinophil differentiation and that both proteins are expressed in terminally differentiated eosinophils. Furthermore, they showed that Olig2 siRNA reduced *SIGLEC8* mRNA and Siglec-8 surface protein levels, suggesting that Olig2 is a transcriptional regulator of the *SIGLEC8* gene (34). However, all of the currently available human eosinophilic cell lines express Olig2 protein but fail to express Siglec-8, as noted earlier. In addition, Olig2 is not expressed in cord blood-derived mast cells that express Siglec-8. Thus, it appears that *SIGLEC8* gene expression is only partially regulated by Olig2 and further work is needed to determine the exact combination of transcription factors responsible for Siglec-8 expression (33, 34).

Ligands for Siglec-8

All siglecs contain an amino-terminal V-set Ig lectin domain that binds sialic acid, but each siglec has a characteristic specificity profile for binding only certain conformations of sialic acid. Most siglecs recognize α2-3- and α2-6-linked sialic acids, although some can also recognize α2-8-linked sialic acids (35, 36). Initial experiments to characterize Siglec-8 ligand-binding preferences demonstrated that Siglec-8 preferentially binds α2-3-sialic acids linked to Galβ1-4GlcNAc (27). Using a glycan array generated by the Consortium for Functional Glycomics, 172 glycan structures were screened, and it was discovered that

Siglec-8 specifically bound 6′ sulfated sialyl Lewis^X (6′-sulfo-sLe^x or NeuAcα2-3Galβ1-4(Fucα1-3)(6-*O*-Sulfo)GlcNAcβ1). Siglec-8 did not bind sialyl Lewis^X, a common ligand for L-, P-, and E-selectins, demonstrating that the 6′-position sulfate on the galactose was absolutely necessary for Siglec-8 lectin-glycan binding (37). A subsequent re-screen of an expanded array containing over 600 structures revealed that the fucose was dispensible (38) (**Figure 1**). Experiments using heparinized whole blood showed that a polyacrylamide polymer decorated with 6′-sulfo-sLe^x bound only eosinophils in a Siglec-8-dependent manner, further demonstrating that this glycan is a specific ligand for Siglec-8 (39).

The structural basis of how Siglec-8 interacts with its glycan ligand had been unexplored until a recent report by Propster et al., where they provide a detailed description of how Siglec-8 selectively recognizes its ligand, 6′-sulfo-sLe^x. First, using NMR spectroscopy, they determined the 3D structure of the lectin domain of Siglec-8. The structure is a V-set Ig-like β-sandwich of two antiparallel β-sheets formed by β-strands ABED and C′CFG, with the conserved arginine, responsible for providing a salt bridge interaction with sialic acid, located on β-strand F. Ligand specificity is mediated by two motifs, where the primary motif recognizes the terminal Neu5Ac, similar to other siglecs, and the secondary motif recognizes the subterminal Gal6S, which was found to be unique among siglecs. Although amino acid mutations failed to affect the overall structure of Siglec-8, a mutation in the conserved arginine eliminated Neu5Ac binding and completely abrogated Siglec-8–ligand interactions. In accordance with previous work done in our lab, this group also demonstrates that the sulfate modification was absolutely necessary for enhanced ligand affinity and revealed the key determinants for glycan specificity (40) (**Figure 2**).

Siglec-8 Function on Human Eosinophils

Initial clues regarding how Siglec-8 might function came from an examination of its structural motifs. The cytoplasmic domain of Siglec-8 contains one ITIM and one ITSM, thought to be responsible for initiating downstream receptor function. Using peripheral blood human eosinophils, it was shown that multimeric engagement of Siglec-8 with a mouse monoclonal antibody (mAb) causes Siglec-8-dependent eosinophil apoptosis. However, this required the presence of a secondary anti-mouse cross-linking antibody; without secondary antibody, no cell death was seen (41). Further studies to delineate the mechanisms through which Siglec-8 induces eosinophil apoptosis revealed that Siglec-8 cross-linking promoted reactive oxygen species (ROS) production, loss of mitochondrial membrane potential and caspase cleavage (41, 42). Additionally, Siglec-8-dependent eosinophil apoptosis was paradoxically amplified under conditions of eosinophil priming with IL-5, GM-CSF, or IL-33, eliminating the need for secondary cross-linking antibody and changing the apoptotic mechanism to one dependent on ROS rather than caspase activity (41–44). Furthermore, incubation of IL-5-primed eosinophils with a synthetic polyacrylamide polymer decorated with 6′-sulfo-sLe^x induced eosinophil apoptosis (39), suggesting that Siglec-8 functions through different mechanisms in the presence or absence of cytokine priming.

In addition to studies using a mAb and a synthetic ligand, von Gunten et al. discovered that exposure of IL-5-primed eosinophils to intravenous immunoglobulin (IVIG), often used at high doses for the treatment of autoimmune and allergic diseases, resulted in eosinophil cytotoxicity. Further experiments revealed that IVIG contained autoantibodies against Siglec-8 that were responsible for this cytokine-dependent apoptotic effect of IVIG and that this effect was ROS-dependent (45), similar to what was observed when using mAbs to Siglec-8.

Although the intracellular signaling pathways for most siglecs are not well characterized, several studies have shown that engagement of CD33-related siglecs leads to downstream activation of Src family kinases (SFKs) that provide docking sites

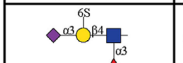
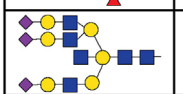
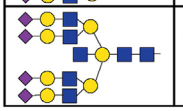
Structure	Name	Binds Siglec-8?	Binds Siglec-F?
	6'-S-Sialyl-LacNAc	Yes	Yes
	6'-S-Sialyl-Le^x	Yes	Yes
	Tri-antennary bisected	No	Yes
	Tetra-antennary bisected	No	Yes

FIGURE 1 | Glycans recognized by Siglec-8-Fc and Siglec-F-Fc, as determined by using glycan microarray analysis, are shown. Reproduced and modified from Ref. (38) with permission.

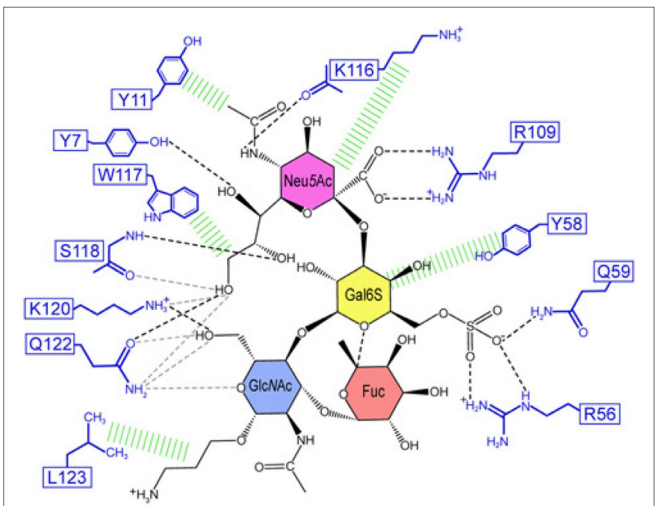


FIGURE 2 | Structural basis for 6′ sulfated sLex recognition by human Siglec-8 illustrated by a representative structure (lowest energy) of the NMR ensemble. Schematic illustration of the Siglec-8–6′ sulfated sLex interaction network. Black dashed lines indicate hydrogen bonds in the depicted structure; gray dashed lines indicate hydrogen bonds abundantly observed in other structures of the ensemble. Hydrophobic contacts are shown in green. Reproduced from Ref. (40) with permission.

for Src-homology region 2 domain-containing phosphatases such as SHP-1 and SHP-2 that then propagate downstream functions (46–49). Indeed, ongoing work to further explore Siglec-8 signaling on eosinophils revealed that Siglec-8 engagement on IL-5-primed eosinophils leads to phosphorylation of SFKs, and use of SFK pharmacological antagonists inhibited Siglec-8-mediated eosinophil ROS production and apoptosis, although the SFKs involved in Siglec-8 function have yet to be determined (50). Furthermore, preliminary data show that Siglec-8 associates with SHP-2 and that pharmacological inhibition of protein tyrosine phosphatases inhibits Siglec-8-mediated eosinophil apoptosis (51). Together, these studies support the notion that Siglec-8 functions similar to other CD33-related siglecs.

The presence of an ITIM suggests that Siglec-8 should be involved in negative cell signaling; however, some of the latest observations suggest that Siglec-8 can, under certain circumstances, function as an activating receptor, such as after IL-5 priming. Initial evidence supporting this hypothesis showed that Siglec-8 cross-linking leads to enhanced phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 and activation of ERK1/2 was necessary for Siglec-8-mediated eosinophil apoptosis (50).

SIGLEC-F

In view of the usefulness of mouse models for functional manipulations, it was imperative to identify a suitable mouse homolog of Siglec-8. In mice, there is no Siglec-8 ortholog, but Siglec-F has been found to be the closest functional paralog. Siglec-F is a 569-aa, CD33-related siglec that contains four Ig-like domains (Siglec-8 contains three) and, like Siglec-8, it contains both ITIM and ITIM-like motifs in its cytoplasmic tail. Using sequence homology comparisons, it was initially proposed that Siglec-F was the likely ortholog of human Siglec-5, but the homology was limited to the extracellular domains of both receptors (47). Furthermore, initial studies revealed that Siglec-F was predominantly expressed in bone marrow cells of the myelomonocytic lineage, and it was not expressed on mature neutrophils and monocytes (47), which have been shown to express Siglec-5 (52), further suggesting that Siglec-F may not be the true ortholog of Siglec-5.

Later efforts to fully characterize the expression pattern of Siglec-F and determine its functional counterpart in humans revealed that Siglec-F shared 38% similarity with human Siglec-8 (53). Using monoclonal antibodies to Siglec-F, it was found that Siglec-F, like Siglec-8, was predominantly expressed on the surface of mature eosinophils and on bone marrow eosinophils (54). However, Siglec-F is not expressed on mouse mast cells and surprisingly is instead expressed on mouse alveolar macrophages and subpopulations of intestinal epithelial cells (55–57) (**Figure 3**). Although they are structurally different and are expressed on different cell types, Siglec-F, like Siglec-8, has a binding preference for α2-3-linked sialic acids and recognizes 6′-sulfo-sLe^x (58). While subsequent studies have reproduced these findings, access to additional glycan structures for screening has allowed the identification of several multi-antennary structures that are recognized by Siglec-F but not Siglec-8 (38)

	Siglec-8	Siglec-F
Eosinophils	+++	+++
Mast cells	++	-
Basophils	+	-
Alv Mϕ	-	++
T cells	-	+/-
Neutrophils	-	+/-
Monocytes	-	-
Tuft/M cells	-	+

FIGURE 3 | Comparison of cellular surface expression patterns for Siglec-8 and Siglec-F. Alv Mϕ, alveolar macrophage.

(**Figure 1**). Indeed, this may explain why mouse lung ligands are recognized by Siglec-F but not by Siglec-8 (see below) (59). Based on these reports, it was concluded that Siglec-F and Siglec-8 are functionally convergent paralogs rather than orthologs.

Subsequent studies to examine the biological roles of Siglec-F *in vivo* revealed that its expression is upregulated following allergen challenge in a mouse lung allergy model and the congenital deficiency *via* genetic deletion of the Siglec-F gene led to enhanced eosinophil numbers in the bone marrow, peripheral blood, and lungs during allergic inflammation but not at baseline. Furthermore, Siglec-F-null mice had diminished eosinophil death, suggesting a role for Siglec-F in mediating eosinophil apoptosis (60). Indeed, administration of anti-Siglec-F antibody reduced peripheral blood and tissue eosinophil numbers in wild-type mice, IL-5 transgenic mice, and in mouse models of hypereosinophilic syndrome and eosinophilic esophagitis, which was attributed to induction of eosinophil apoptosis. Additionally, the effect of the anti-Siglec-F antibody was specific to eosinophils and had no effect on other cells, not even Siglec-F-expressing alveolar macrophages (61–64). Despite our advances in understanding the role of Siglec-F in eosinophil survival *in vivo* and *in vitro*, little is known about the signaling mechanism of this receptor. A report by Mao et al. showed that Siglec-F engagement on mouse eosinophils led to caspase cleavage; however, unlike Siglec-8, there was no detectable ROS production, and Siglec-F function did not involve the activation of SFKs or SHP-1 (65). Therefore, further studies are needed to fully characterize the signaling pathways for Siglec-F.

TISSUE LIGANDS FOR SIGLEC-F AND SIGLEC-8

Although both Siglec-F and Siglec-8 preferentially recognize the glycan 6′-sulfo-sLe^x, the identity of their natural ligands is still under investigation. Initial studies to identify endogenous tissue ligands in mice using Siglec-F-Fc chimeras and immunohistochemistry showed that Siglec-F ligands were constitutively expressed on airway epithelial cells and their expression was

dependent on ST3Gal-III, a sialyltransferase that can add α2,3 terminal sialic acids to glycans (66). Expression of these ligands was increased upon induction of allergic airway inflammation (67, 68). Glycoproteomic analysis of material derived from mouse tracheal epithelial cells revealed that Siglec-F-Fc bound to glycans displayed on Muc5b and Muc4, but not Muc5ac. Mouse lungs deficient in Muc5b had reduced Siglec-F-Fc binding, and mice conditionally deficient in Muc5b showed enhanced eosinophilic inflammation in response to airway instillation of IL-13, further validating that Muc5b carries glycan ligands for Siglec-F and suggesting that only subsets of airway mucins display the glycan structures necessary for Siglec-F binding (38). Although less is known about Siglec-8 tissue ligands, a recent study showed that Siglec-8 ligands were expressed selectively on serous cells, a subpopulation of submucosal gland cells in the inferior turbinate, and inflammation that occurs in chronic sinusitis led to increased expression of Siglec-8 tissue ligands in the upper airways (69). Additional studies show that mouse airways do not express Siglec-8 ligands and Siglec-8-Fc binding in human tracheal sections is restricted to serous cells in submucosal glands and cartilage (59) (**Figure 4**). The exact identity of these ligands is still under investigation, but given the fact that the galactose 6-*O*-sulfotransferase CHST-1 is dispensable for generating Siglec-F ligands in the mouse, it appears that the 6′ sulfation needed for Siglec-8 binding is not required for Siglec-F binding (70).

	Siglec-8-Fc Binding	Siglec-F-Fc Binding
Mouse airway submucosal glands	No	Yes
Mouse airway epithelium	No	Yes
Muc5b glycoform	No	Yes
Human airway submucosal glands	Yes	Yes
Human airway epithelium	No	Yes

FIGURE 4 | Comparison of binding of Siglec-F and Siglec-8 to mice and human tissue-based sialoside ligands. Based on data from Ref. (59).

ENDOCYTOSIS OF SIGLEC-F AND SIGLEC-8

Beyond the physiological role of Siglec-8 in inducing cell death of eosinophils, Siglec-8 represents a promising target through which to deliver therapeutic payloads into eosinophils and other Siglec-8-expressing cells. Several studies have shown that siglecs are endocytic receptors and, once engaged, can carry their ligand—and presumably any associated cargo—into the cell (71–74). This strategy has been employed in the development of cancer therapeutics by targeting preferentially upregulated receptors such as the receptors for transferrin or folate or through antibodies targeting slightly more selectively expressed antigens, such as CD33 (Siglec-3) in acute myelogenous leukemia (75–77). An antibody-targeting CD22 (Siglec-2) is also under investigation to treat diseases involving B cells, such as B-cell acute lymphoblastic leukemia or systemic lupus erythematosus (78, 79). Due to the restricted expression pattern of Siglec-8, targeting of eosinophils through Siglec-8 offers promise. However, the capability to exploit Siglec-8 in this manner depends on whether Siglec-8 is endocytosed and is present and accessible on the surface of eosinophils in various circumstances. Siglec-F endocytosis has been studied in mouse eosinophils. Siglec-F is internalized in response to antibody ligation *via* a clathrin- and lipid raft-independent pathway that relies on ARF6 but not dynamin-1 (72). New data indicate that Siglec-8 is indeed internalized in response to antibody or synthetic ligand engagement on peripheral blood eosinophils and that this pathway can be exploited to deliver a toxin (the ribosome-inhibiting protein saporin) to eosinophils to induce cell death under conditions in which Siglec-8 engagement alone would be insufficient (i.e., in the absence of IL-5 priming) (80). Despite some similarities, including the lysosomal localization of the internalized siglec, the pathway utilized by Siglec-F internalization appears to be distinct from that of Siglec-8. The pathway of internalization can have profound effects on receptor function, leading to distinct signaling mechanisms and downstream functions or alterations in receptor turnover. For example, endocytosis of SR-A *via* a lipid raft/caveolae-dependent pathway is required for macrophage apoptosis in a ligand-dependent manner, whereas clathrin-mediated SR-A endocytosis is expendable for this effect (81). While there is abundant evidence linking endocytosis to the organization of signaling events (82), it remains to be determined whether the endocytosis of Siglec-8 affects its function.

Siglec-8 may also achieve part of its function by internalizing other surface proteins. Upon antigen stimulation, the B cell receptor (BCR) engages clathrin in lipid raft domains and thus is internalized *via* a mixed pathway (83, 84). While the siglec CD22 is initially excluded from lipid rafts, it colocalizes with the BCR and promotes its internalization when unmasked (85, 86). This downregulation of the BCR is thought to be one mechanism underlying the inhibitory function of CD22. Of note, the IL-5 receptor, which is critically important to the activation and survival of eosinophils, has been found to be internalized *via* distinct clathrin- and lipid raft-dependent pathways and is targeted for proteolytic degradation through the lipid raft-mediated endocytic pathway (87). It is an intriguing possibility that the endocytosis and trafficking of Siglec-8 and the IL-5 receptor may be linked in a way that influences the function of each receptor.

OTHER SIGLECS FOUND ON MOUSE AND HUMAN EOSINOPHILS

While Siglec-8 has garnered much attention as a cell-surface marker of eosinophils, there are a number of other glycans and glycan-binding proteins present on eosinophils that regulate their survival, trafficking, and adhesion and that may be useful markers of eosinophilic inflammation. In both mice and humans,

eosinophils express siglec family members other than Siglec-F and Siglec-8, respectively.

CD22, Siglec-E, Siglec-G, and Mouse Eosinophils

Interestingly, mouse eosinophils in the gastrointestinal tract were found to express the siglec CD22 on their cell surface, a siglec previously thought to be restricted to B cells (88). CD22 expression was highest on eosinophils in the jejunum, although it was also found on eosinophils in the stomach, duodenum, or ileum. CD22 was not found on eosinophils in the blood or other tissues. The function of CD22 on these eosinophils is not yet clear, although CD22 ablation led to an increase in eosinophils in the jejunum, an effect that did not appear to be due to increased eosinophil differentiation from hematopoietic precursors or augmented IL-5 or eotaxin-2 signaling. In studies of mice overexpressing IL-5, which gives rise to eosinophilia, it was found that these eosinophils expressed mRNA for Siglec-E (orthologous to Siglec-9 in humans) and Siglec-G (orthologous to Siglec-10 in humans) (53). However, the surface expression and function of these siglecs on mouse eosinophils have not been studied.

CD33, Siglec-7, Siglec-10, and Human Eosinophils

Immature human eosinophils express low levels of CD33 (Siglec-3) and downregulate this receptor upon maturation (89). Human eosinophils also express modest levels of Siglec-7 both in the peripheral blood and in nasal polyps (90, 91). In addition, Siglec-10, which was identified by four different groups through genomic analysis and screens of cDNA libraries (including one from asthmatic eosinophils), is expressed by human eosinophils (92–95). Siglec-10 possesses three tyrosine-containing cytoplasmic motifs—a membrane-proximal Grb2 binding motif, a central ITIM, and a membrane-distal ITSM or ITIM-like motif—and has been found to interact with SHP-1 and SHP-2 but not with SLAM-associated protein (48, 94). While Siglec-10 was detected on B cells using polyclonal antibody (93), a mAb detected Siglec-10 expression only on eosinophils, neutrophils, and monocytes and failed to detect expression on B cells (94), suggesting either that the polyclonal antibody was not specific to Siglec-10 (perhaps binding to another siglec family member present on B cells) or that a unique variant of Siglec-10 is expressed on these cell populations and not on B cells. Interactions between Siglec-10 and CD24 (heat stable antigen) (96), vascular adhesion protein-1 (97), and CD52 (98) have been demonstrated *in vitro* or on other cell types; however, the function of Siglec-10 in eosinophils has not been described. Indeed, little is known about the functions of any of these three siglecs on human eosinophils. Antibody ligation of Siglec-7 on eosinophils failed to induce apoptosis or prevent chemotaxis under conditions in which Siglec-8 ligation produced these effects (91), and the role of CD33 on eosinophils has similarly not been determined. Given the lack of functional data and the broader cell expression patterns for these siglecs, there has been less interest in exploiting these receptors to address eosinophilic inflammation.

SELECTINS AND SELECTIN LIGANDS ON EOSINOPHILS

Protein–glycan interactions are exceptionally important in the processes of cell adhesion and trafficking. In the initial steps of leukocyte extravasation, the cell must tether to and roll along the endothelium, which requires the interaction between selectins and their glycan ligands. Eosinophils depend to differing degrees on P-, E-, and L-selectin interactions in this process. Relative to neutrophils, eosinophils bind less well to E-selectin and bind to a greater extent to P-selectin through cell-surface glycan ligands (99), presumably due to increased levels of P-selectin glycoprotein ligand (PSGL)-1 (100). This same study found that L-selectin on the surface of eosinophils was important in tethering of eosinophils to human umbilical vein endothelial cells (HUVECs) but only due to establishing inter-eosinophil interactions rather than binding to the endothelial cells (99). However, others have found that diminished L-selectin expression or the use of blocking antibodies to L-selectin reduce eosinophil rolling and adhesion on HUVECs or on rabbit mesenteric venule endothelial cells under conditions of flow (101, 102). As demonstrated by the study by Sriramarao et al., human selectins are capable of interacting with ligands expressed in other species. Indeed, the selectin-binding sites in the best characterized P-selectin ligand, PSGL-1, are evolutionarily well conserved (103). However, distinct patterns of expression render cross-species comparisons more difficult. For example, P-selectin expression in mice but not in humans is induced by TNF-α or LPS, and cytokine regulation of human and primate P- and E-selectins is more selective than in mice (104, 105). However, mouse strains in which selectins have been knocked out have permitted elegant studies of their importance in mouse eosinophil migration. Using these mouse strains, several studies have shown that P-selectin is critical in eosinophil recruitment to the lung and peritoneum (106–108).

P-selectin glycoprotein ligand-1 is the best characterized P-selectin ligand on eosinophils, and its expression has been shown to be increased in patients with allergic asthma relative to healthy controls. This increase results in enhanced binding to P-selectin and IL-4-treated HUVECs (109). PSGL-1 contributes to eosinophil, but not neutrophil, adhesion to IL-13-activated HUVECs under conditions of physiological flow (110). Paradoxically, PSGL-1 expression is reduced on activated eosinophils and is shed from leukocytes (111). Although there have been reports that PSGL-1 can act as a ligand for E-selectin as well (112, 113), no changes in E-selectin binding were observed with eosinophils from allergic asthma patients in this study. There appear to be other significant ligands for P-selectin on eosinophils, however. In patients with atopic dermatitis, eosinophils are capable of binding substantially more soluble P-selectin than eosinophils from healthy donors but do not display more PSGL-1 on their surface (114).

Although the selectins bind to related sialylated glycans, these glycans can be present on a variety of different proteins or lipids that may be cell type- or tissue-specific. Eosinophils, for example, display far less sialyl Lewis^x antigen, a selectin ligand carbohydrate structure, than neutrophils, but a greater proportion is in the form

of sialyl dimeric Lewis^X and is sensitive to endo-β-galactosidase treatment (115). Interestingly, neither the display of sialyl Lewis^X on eosinophils nor adhesion to immobilized E-selectin is protease-sensitive, indicating that these carbohydrate ligands may not be present on cell-surface proteins (115). Consistent with this, glycosphingolipids extracted from leukocytes were found to interact with E-selectin, and their biosynthesis was required for E-selectin-dependent, but not P-selectin-dependent, neutrophil adhesion (116). P-selectin ligands present on eosinophils clearly differ from those of E-selectin in that binding to immobilized P-selectin is protease-sensitive and endo-β-galactosidase-resistant (117). Indeed, although the expression of sialyl Lewis^X on eosinophils is not changed by cellular activation with platelet-activating factor (PAF), P-selectin binding is reduced following PAF activation and L-selectin is shed following transendothelial migration (117, 118).

Due to their critical role in eosinophil trafficking to peripheral tissues, selectins represent a potentially useful therapeutic target for diseases of eosinophilic inflammation. In fact, selectin antagonists interfere with eosinophil (and neutrophil) adhesion (119), and a pan-selectin antagonist glycomimetic agent is in clinical trials to modulate selectin-based adhesion in acute sickle cell crisis (120). Although lack of cell specificity is a concern, a more selective P-selectin antagonist or one that interacts with a eosinophil-selective P-selectin ligand would likely be effective in preventing further eosinophilic inflammation in the tissues with fewer potential complications.

GALECTIN FAMILY MEMBERS AND THEIR GLYCAN LIGANDS ON EOSINOPHILS

The galectin family of proteins, previously known as S-type lectins, has a binding preference, generally, for β-galactosides, although there appear to be exceptions to this. Most of the members of the family are secreted but can cross-link cell-surface receptors due to the presence of more than one carbohydrate recognition domain (CRD) or through multimerization of a monomer containing one CRD (galectin-3).

The galectin family member most commonly associated with eosinophils is galectin-10, also known as Charcot–Leyden crystal (CLC) protein. Galectin-10 makes up about 10% of the total protein content of human eosinophils (121), and CLC deposition in tissues has long been considered a marker of eosinophilic (or basophilic) inflammation (122). The protein localizes to both the cytosol and a subset of core-less granules (123). The CLC protein was initially believed to function as a lysophospholipase within the eosinophil (121); however, this enzymatic activity has since been ascribed not the CLC protein but to another enzyme that can associate to a degree with it (124). Due to sequence identity, structural homology, and genomic structure, CLC protein became known also as galectin-10 (125–127). Unlike other members of the galectin family, however, galectin-10 appears not to bind β-galactosides to any substantial degree but instead appears to bind to mannose-containing carbohydrate moieties (128). The natural ligand or ligands of galectin-10 and the functional significance of its ability to bind to carbohydrates remain undetermined. Despite the lack of functional data, galectin-10 mRNA and protein levels remain useful biomarkers for eosinophilic airway inflammation, active eosinophilic esophagitis, aspirin-exacerbated respiratory disease, CRTH2 activation, and celiac disease (129–133).

On the eosinophil cell surface, galectin–ligand interactions have been found to be important in eosinophil recruitment, activation, and survival. The granulocyte-specific and heavily glycosylated protein CD66b (also known as CEACAM8) is expressed on eosinophils and is upregulated in response to cellular activation (134, 135). Sialylated glycans on CD66b interact with E-selectin, and this interaction has been shown to be important for neutrophil adherence to activated endothelium (136). Glycans on CD66b also interact with galectin-3, and engagement of CD66b using either soluble galectin-3 or antibody induced ROS production and degranulation (137). Cross-linking of CD66b also caused the eosinophils to become more adherent, perhaps through the clustering of the integrin subunit CD11b.

Several other galectin interactions may be important in eosinophil adhesion and chemoattraction, including those with galectins-1, -3, and -9 (138–140). However, the role of galectins in eosinophil recruitment is covered in greater detail in a review in this volume by Sriramarao et al. and will not be discussed further here. It should be noted, however, that galectins play other important roles in eosinophil biology and are biomarkers of disease activity. High concentrations of galectin-1, for example, can induce eosinophil cell death (140), and levels of galectin-3 before treatment in patients with severe asthma predict treatment responses to omalizumab (141).

EOSINOPHIL GLYCOMICS

While many of the cell-surface and intracellular eosinophil proteins have been identified and extensively described, the glycans that coat the various cell-associated proteins and likely play important roles in numerous biological pathways remain largely shrouded in mystery. As part of an effort to characterize these glycans, the glycome of human eosinophils has been analyzed in cell lysates and compared to those of basophils and mast cells to elucidate the identities of these glycans, their relative abundances, and key differences between these cell types (142). Although mast cells possess substantial amounts of terminally sialylated epitopes on their various glycoproteins, eosinophils and basophils have far more part-processed terminal *N*-acetylglucosamine (GlcNAc)-containing structures. For example, the most abundant *N*-glycan by far in both eosinophils and basophils is a bi-antennary structure with two terminal non-extended GlcNAc sugars, which is far less abundant in mast cells. While the functional relevance of these patterns is unclear, it is unlikely that these modifications to the cell surface are random. In addition, it is uncertain how cytokine priming and other signals that may be present under inflammatory conditions may affect glycan synthesis and processing. However, such changes may well affect processes such as adhesion, activation, cell–cell interaction, and even survival.

CONCLUSION

There remains a clinical need to effectively and selectively treat diseases of eosinophilic inflammation. Due to their roles in recruitment, adhesion, activation, and survival, glycan–glycan-binding protein interactions are beginning to garner attention as therapeutic prospects. Siglec-8 and its ligands offer a cell-selective pathway to induce cell death in primed eosinophils and deliver therapeutic payloads into the cell. Antagonists of selectin interactions may help limit eosinophilic inflammation, but significant hurdles remain for achieving a safe, cell-selective effect. Monomeric β-galactosides or glycomimetics may also be clinically useful in antagonizing eosinophil lectin interactions that are involved in cell adhesion and activation. While targets and biomarkers have been identified, further studies are necessary to elucidate the functions of glycan-binding proteins on eosinophils, such as those of other members of the siglec family; to identify their natural glycan ligands and how they are modulated; and to determine the functional significance of the glycans displayed on the eosinophil cell surface.

AUTHOR CONTRIBUTIONS

JO completed the sections on Siglec-8 endocytosis, selectins, galectins, and other members of the siglec family. DC completed all other sections regarding Siglec-8 as well as Siglec-F. BB organized the effort and wrote the introduction. All authors contributed to revisions of the manuscript.

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Regulation of Eosinophil Recruitment and Activation by Galectins in Allergic Asthma

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Eosinophils are differentiated granulocytes that are recruited from the bone marrow to sites of inflammation *via* the vascular system. Allergic asthma is characterized by the presence of large numbers of eosinophils in the lungs and airways. Due to their capacity to rapidly release inflammatory mediators such as cytokines, chemokines, growth factors, and cytotoxic granule proteins upon stimulation, eosinophils play a critical role in pro-inflammatory processes in allergen-exposed lungs. Identifying key players and understanding the molecular mechanisms directing eosinophil trafficking and recruitment to inflamed airways is a key to developing therapeutic strategies to limit their influx. Recent studies have brought to light the important role of glycans and glycan binding proteins in regulating recruitment of eosinophils. In addition to the role of previously identified eosinophil- and endothelial-expressed adhesion molecules in mediating eosinophil trafficking and recruitment to the inflamed airways, studies have also indicated a role for galectins (galectin-3) in this process. Galectins are mammalian lectins expressed by various cell types including eosinophils. Intracellularly, they can regulate biological processes such as cell motility. Extracellularly, galectins interact with β-galactosides in cell surface-expressed glycans to regulate cellular responses like production of inflammatory mediators, cell adhesion, migration, and apoptosis. Eosinophils express galectins intracellularly or on the cell surface where they interact with cell surface glycoconjugate receptors. Depending on the type (galectin-1, -3, etc.) and location (extracellular or intracellular, endogenous or exogenously delivered), galectins differentially regulate eosinophil recruitment, activation, and apoptosis and thus exert a pro- or anti-inflammatory outcome. Here, we have reviewed information pertaining to galectins (galectin-1, -3 -9, and -10) that are expressed by eosinophils themselves and/or other cells that play a role in eosinophil recruitment and function in the context of allergic asthma and their potential use as disease biomarkers or therapeutic targets for immunomodulation.

Keywords: eosinophils, cell trafficking, galectins, airway recruitment, allergic airway inflammation, asthma

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INTRODUCTION

Eosinophils are the predominant granulocytic leukocytes present in allergic airways, and eosinophilia is the hallmark of airway inflammation in asthma (1–3). Although constituting only a small fraction of circulating white blood cells under healthy conditions, patients with allergic airway inflammation and asthma exhibit significantly higher numbers of eosinophils in peripheral blood (4). Mature

eosinophils are formed from progenitor stem cells in the bone marrow and released into circulation under the tight regulation of interleukin (IL)-5 (5). During disease conditions such as allergic asthma, they undergo proliferation and priming in response to specific stimuli (6). Indeed, peripheral blood eosinophils from allergic asthmatics have been shown to demonstrate spontaneously enhanced production of reactive oxygen species, increased chemotaxis, and diminished apoptosis relative to eosinophils from normal subjects (7). Once recruited to sites of inflammation, eosinophils exert their pathological and immunomodulatory effects. Eosinophils contain cationic granule proteins that have been shown to exert highly cytotoxic effects such as production of reactive oxygen species, desquamation, and lysis of airway epithelial cells, as well as synthesis of remodeling factors by epithelial cells [reviewed in Ref. (8)]. In addition, eosinophils are a source of various cytokines, chemokines, and growth factors that are either preformed or synthesized and secreted upon activation (9). Thus, identifying key players involved in selective eosinophil recruitment and understanding their role in supporting this event is critical for the identification of therapeutic targets.

The trafficking of primed mature eosinophils from the blood stream into inflamed tissues such as the lung is finely regulated by adhesion molecules, several cytokines, and chemokines with overlapping functions. We and others have demonstrated that eosinophil trafficking under conditions of flow involves a multistep paradigm, which includes initial tethering/rolling followed by activation-dependent firm adhesion and chemoattractant-induced transmigration into extravascular sites of inflammation [as reviewed in Ref. (10–12), see **Figure 1**]. Studies with mouse and/or human eosinophils have shown that rolling along the vascular endothelium is supported by L-selectin, very late antigen-4 (α4β1), and P-selectin glycoprotein ligand-1 (PSGL-1). On the endothelial side, in addition to the α4 ligand vascular cell adhesion molecule (VCAM)-1, eosinophil rolling under physiological conditions of flow is mediated by P-selectin, the ligand for PSGL-1 (13, 14). Apart from mediating inter-eosinophil interaction under physiological flow conditions (13), L-selectin has been shown to interact with endothelial ligands bearing sialyl-Lewis^x epitopes, such as CD34, to play a role in recruitment of eosinophils to allergic lungs in mice (15). Rolling of human eosinophils under conditions of physiologic blood flow in post-capillary venules does not appear to be dependent on E-selectin, albeit these cells can roll on immobilized E-selectin under sub-shear flow rates (16). While an overview of eosinophil trafficking is depicted in **Figure 1**, it must be noted that human and mouse eosinophils are likely to utilize different vascular adhesion molecules during cell trafficking due to species-specific differences in expression of these molecules in response to inflammatory stimuli. Activation-dependent stable adhesion of eosinophils to the vascular endothelium is mediated by α4β1/VCAM-1 and integrin αMβ2/intercellular adhesion molecule (ICAM)-1 interaction. Trans-endothelial migration is under the control of pro-inflammatory chemokines, predominantly eotaxins, and the eotaxin receptor, C–C chemokine receptor type 3 (CCR3). In addition to these key players, there are several other molecular events that support the dynamic recruitment of eosinophils into extravascular sites such as chemoattraction by molecules other than eotaxins [e.g., PAF, C–C motif chemokine ligand 5 (CCL5), C–C motif chemokine ligand 3 (17), C3a and C5a (18), and serotonin/5-hydroxytryptamine (19)] as well as release and activation of matrix metalloprotease-9, which enables eosinophil migration through the extracellular matrix (20–22). Of particular interest in our laboratory is the role

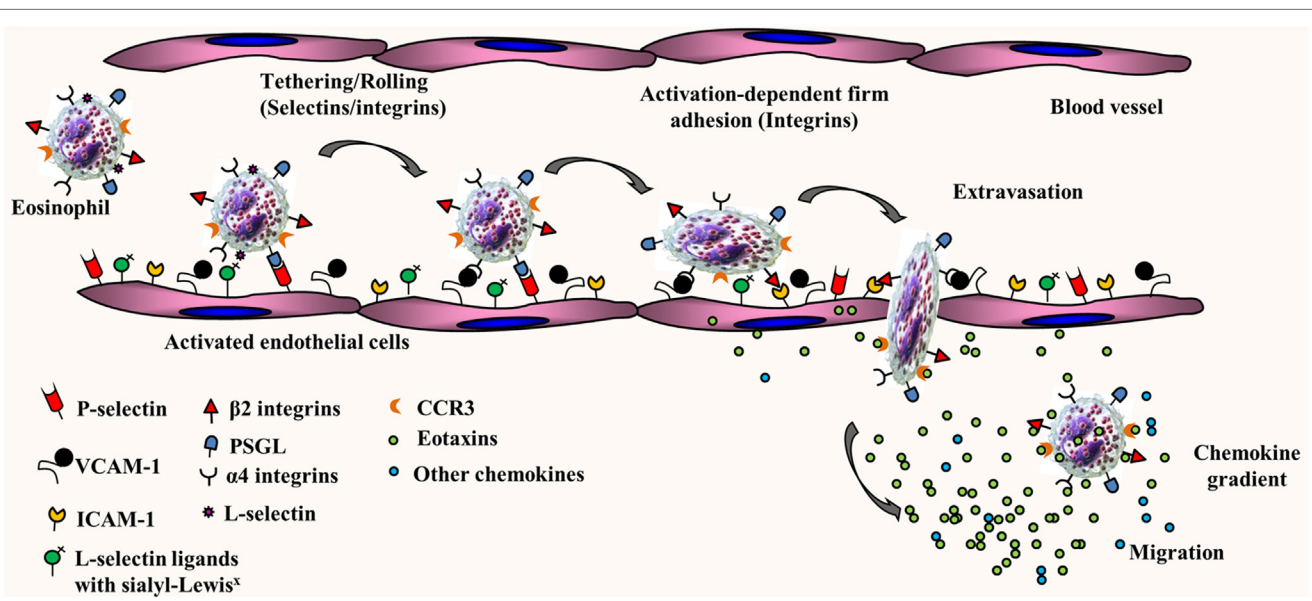

FIGURE 1 | Multistep paradigm of eosinophil trafficking under conditions of flow. Eosinophil rolling along the inflamed and activated vascular endothelium is supported by eosinophil-expressed L-selectin, α4β1, and P-selectin glycoprotein ligand-1 (PSGL-1) and endothelial-expressed vascular cell adhesion molecule (VCAM)-1 and P-selectin. Activation-dependent stable adhesion of eosinophils to the vascular endothelium is mediated by α4β1/VCAM-1 and αMβ2/intercellular adhesion molecule (ICAM)-1 interactions. Extravasation or trans-endothelial migration is under the control of chemokines (eotaxins and other chemokines).

played by cell surface-expressed complex carbohydrate structures such as heparan sulfate proteoglycans (23–25) as well as branched *N*-glycans (26) and their galectin ligands (27–30) in leukocyte/eosinophil–endothelial interactions during cell trafficking and migration/recruitment.

Galectins are a family of animal lectins that specifically bind to β-galactosides in cell surface glycoproteins and glycolipids or in free form (31, 32). Fifteen galectins have been identified in mammals to date and are classified into three groups based on their structure. The three groups are (i) the prototypic galectins (Gal-1, -2, -5, -7, -10, -11, -13, -14, and -15), which contain one carbohydrate recognition domain (CRD), (ii) the tandem-repeat galectins (Gal-4, -6, -8, -9, and -12), which contain two homologous CRDs within a single polypeptide, and (iii) chimeric galectins (Gal-3), which have a single CRD and a large amino-terminal domain. While many galectins exhibit wide tissue distribution, some are tissue specific such as Gal-7, which is predominantly expressed in stratified epithelium, and Gal-4, which is mostly expressed in mouse gastrointestinal tissue (33, 34). Galectins are largely present in the cytoplasm and nucleus within cells. However, despite the lack of a classical signal sequence for export, several galectins are also present extracellularly either interacting with cell surface glycans or in a soluble form (e.g., in bronchoalveolar lavage fluid and serum) (31). Glycoproteins on the cell surface (including adhesion receptors and cytokine receptors) containing branched *N*-glycans with *N*-acetyllactosamine residues function as epitopes for galectin binding, especially for Gal-1 and Gal-3 (35). Galectins form complexes (dimers or oligomers) that cross-link cell surface glycoprotein receptors to form dynamic lattices that can regulate responses and properties of these receptors (32). Depending on the type (e.g., Gal-1 or Gal-3), location and binding partners (intracellular/non-carbohydrate or extracellular/cell surface glycans), and concentration, galectins positively or negatively regulate various cellular events such as signal transduction, cell differentiation and maturation, production of cytokines and growth factors, trafficking and recruitment, and apoptosis during acute and chronic inflammation [as reviewed in Ref. (36, 37)]. Thus, galectins play a role in various aspects of health and disease such as normal development, innate immunity, inflammation, cardiovascular disease, obesity, type 2 diabetes, and cancer [as reviewed in Ref. (31, 34, 36, 38–40)]. This review is focused on galectins that are expressed by eosinophils themselves and/or other cells that play a role in eosinophil recruitment and function, i.e., Gal-1, -3, -9, and -10 in the context of allergic asthma.

Galectin-3

Gal-3 [same as IgE-binding protein (εBP), CBP35, or Mac-2] was first identified in rat basophilic leukemia cells (41). This lectin is a chimeric galectin containing a single CRD connected to a non-lectin domain rich in proline, glycine, and tyrosine residues with the ability to self-aggregate and thus function bivalently or multivalently (42). Gal-3 is expressed by most tissues, including the lung, all types of epithelia, and most inflammatory cells (mast cells, neutrophils, monocytes/macrophages, eosinophils, and T cells) (43). It is extensively characterized and has been shown to exert diverse functions depending on its location (extracellular or intracellular) that suggest a positive regulatory role for this lectin during inflammation. Extracellular Gal-3 affects cellular events ranging from cell activation [mast cells (44), neutrophils (45), macrophages (46), and lymphocytes (47)] to adhesion [neutrophils (48)], migration [macrophages (49)], and apoptosis [mast cells (50) and T cells (51)]. Intracellular Gal-3 has been shown to participate in pre-mRNA splicing (52), phagocytosis by macrophages (53), and exert antiapoptotic activity in T cells *via* interaction with Bcl-2 (54) as well as in peritoneal macrophages (55). Studies in Gal-3-deficient mice have provided strong evidence for the pro-inflammatory role of Gal-3 in various acute models of inflammation (55–58) including allergic disorders such as asthma (59) and atopic dermatitis (60).

Acute allergen exposure was shown to result in increased recruitment of Gal-3-expressing inflammatory cells (macrophages and eosinophils) to the airways and elevated levels of soluble Gal-3 in the bronchoalveolar lavage fluid of wild-type mice (59). On the other hand, allergen-challenged Gal-3-deficient mice exhibited significantly decreased airway eosinophil recruitment and an overall reduction in airway inflammation (decreased mucus secretion, airway hyperresponsiveness, and Th2 responses) relative to the wild-type mice. In support of this, studies from our laboratory showed that eosinophils from allergic subjects express elevated levels of Gal-3 on the cell surface and exhibit increased adhesive interactions (rolling and firm adhesion) on VCAM-1 compared to cells from normal subjects under conditions of flow in a Gal-3-dependent manner (27). Additionally, we showed that inflamed human endothelial cells express elevated levels of Gal-3 on the cell surface and that blockade of endothelial Gal-3 with specific antibodies inhibits eosinophil rolling and adhesion. At a molecular level, Gal-3 was found to interact with α4 integrin *via* its CRD and showed co-localized expression with α4 on the cell surface of eosinophils from allergic subjects. In addition, eosinophil-expressed Gal-3 interacted with endothelial Gal-3. Self-association to homodimerize or form multivalent complexes is a characteristic feature of Gal-3 (61). Since galectins do not contain a classical signal sequence or a transmembrane domain but are still present extracellularly, it is likely that eosinophil-derived Gal-3 is presented on the cell surface anchored to glycosylated residues on α4 *via* its CRD (based on the blockade of these interactions by lactose) after exiting the cell where it is then able to mediate eosinophil rolling and adhesion on VCAM-1 and Gal-3 as depicted in the schematic shown in **Figure 2**. Studies with total leukocytes from bone marrow of Gal-3-deficient mice further support these findings (28). While cells from wild-type mice demonstrated increased rolling on VCAM-1 and Gal-3 that was specifically inhibited by lactose, rolling of Gal-3-deficient cells on both these endothelial-expressed adhesion molecules was significantly lower and unaffected by lactose. Further, in a model of chronic asthma, there was significantly decreased eosinophil infiltration associated with an overall reduction in the development of a Th2 phenotype and diminished remodeling of the airways (reduced mucus secretion, subepithelial fibrosis, smooth muscle thickness, and peribronchial angiogenesis) in Gal-3-deficient mice compared to wild-type mice (28). In addition to integrin receptors, Gal-3 has been shown to bind to CD66b (CEACAM8), a single chain, highly glycosylated member of the Ig superfamily expressed exclusively on activated human

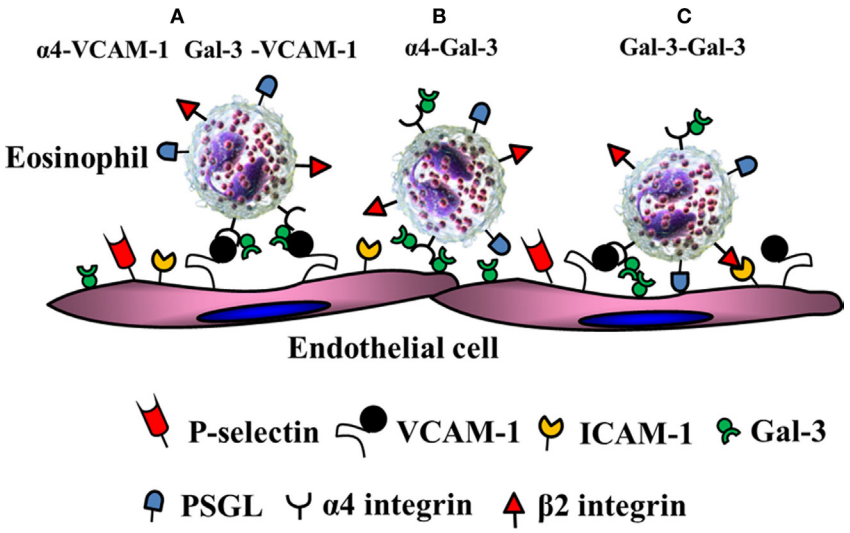

FIGURE 2 | Gal-3-mediated eosinophil–endothelial interactions during cell trafficking. Gal-3 is present on the surface of eosinophils bound to α4. **(A)** In addition to α4β1/vascular cell adhesion molecule (VCAM)-1 interactions, eosinophil-expressed Gal-3 can independently interact with EC-expressed VCAM-1. **(B)** Eosinophil-expressed α4 can interact with EC-expressed Gal-3. **(C)** Eosinophil-expressed Gal-3 can bind to EC-expressed Gal-3.

eosinophils and induce cell adhesion, superoxide production and degranulation (62).

The identification of novel procedures to culture mature primary murine eosinophils from bone marrow *in vitro* (63) has enabled further understanding of the role played by eosinophil-expressed galectins in cell trafficking and activation. Eosinophils cultured from bone marrow of Gal-3-deficient mice showed significantly less rolling on VCAM-1 under conditions of flow, which was also associated with decreased adhesion on ICAM-1 likely due to the inherently decreased expression levels of αM integrin (29), which is necessary for firm adhesion of eosinophils. Interestingly, αM integrin functions as a binding partner for extracellular Gal-3 in macrophages (64). While only hypothetical, it is possible that expression of Gal-3-binding integrin receptors on the cell surface may be regulated by intracellular Gal-3. Adherent Gal-3-deficient cells demonstrate limited ability to spread and form membrane protrusions, which are important cytoskeletal changes that enable stable adhesion and directed movement of cells toward a chemokine gradient (65). Consistent with this observation, Gal-3-deficient eosinophils show reduced migration toward eotaxin-1 despite normal levels of CCR3 (the eotaxin receptor) expression relative to wild-type eosinophils. Studies have shown that intracellularly, Gal-3 in fibroblasts is phosphorylated and that phosphorylation is required for localization of Gal-3 at the cell periphery [an event that has been shown to be required for cell motility (57)] as well as for secretion of Gal-3 (66). Inflammatory mediators such as eotaxin-1 induce secretion of Gal-3 by wild-type murine eosinophils into the culture supernatant (29), which might be one of the mechanisms by which intracellular Gal-3 is presented on the cell surface to promote cell trafficking *via* interaction with endothelial ligands. It is possible that regulation of eosinophil trafficking and migration by intracellular Gal-3 as well as its secretion during conditions of inflammation may involve phosphorylation.

As indicated earlier, galectins bind to β-galactoside epitopes found in *N*- and *O*-glycan modifications of glycoproteins and glycolipids (31, 32). Thus, galectin binding to glycoproteins is determined by the number of glycosylation sites and activity of various glycosyltransferase enzymes of the Golgi complex [reviewed in Ref. (32)]. UDP-*N*-acetylglucosamine:α-6-D-mannoside β1,6 *N*-acetylglucosaminyltransferase V (Mgat5) generates intermediate *N*-glycans that are further extended by the addition of *N*-acetyllactosamine units resulting in high affinity ligands for Gal-3 (67). Studies using mice deficient in this enzyme further confirmed the involvement of Gal-3 and the requirement for finely regulated *N*-glycosylation of surface glycoproteins in eosinophil trafficking and recruitment (26). *In vivo* allergen-challenged Mgat5-deficient mice demonstrated significantly decreased recruitment of eosinophils to the airways along with reduced Th2 cytokines and airway mucus production compared to their wild-type counterparts. *In vitro* eosinophils from Mgat5-deficient mice showed decreased rolling and adhesion on Gal-3 and VCAM-1. It is well known that *N*-glycosylation regulates the biological functions of integrins (clustering, adhesion, and migration) and that changes in glycosylation can affect these functions [as reviewed in Ref. (68)]. Thus, in the absence of Mgat5, not only are α4β1-VCAM-1 interactions likely to be affected but also the ability of Gal-3 to bind to α4, resulting in decreased eosinophil recruitment. Although it is currently not known whether expression of glycosyltransferases involved in generating galectin-specific ligands are elevated during allergic asthma, there is evidence for regulation of expression of glycosyltransferases (α1,3/4-fucosyltransferase and α2,3-sialyltransferases genes) in the lung by pro-inflammatory cytokines (IL-6, IL-8, and TNF-α) in diseases such as cystic fibrosis, which contributes to disease pathogenesis by increasing the number of sialyl-Lewis^x epitopes and thus favoring attachment of *P. aeruginosa* (69).

In contrast to the pro-inflammatory role ascribed to endogenous Gal-3, studies by other investigators have shown that gene therapy with Gal-3-encoding plasmid DNA can suppress eosinophil infiltration and normalize pulmonary function in acute as well as chronic settings of allergic asthma (70, 71) by negatively regulating gene expression of suppressors of cytokine signaling 1 and 3, which play an important role in controlling the Th1–Th2 balance (72). Further, these studies suggest that administration of exogenous Gal-3 could potentially serve as a therapeutic tool for allergic asthma. Differences in the effects noted with exogenous Gal-3 delivery versus endogenous Gal-3 expression may be multifactorial such as the cells in which the protein is expressed endogenously during allergic asthma versus those targeted by the delivery of exogenous Gal-3, the concentration at which Gal-3 is present in the local milieu, as well as the activity and mode of action of exogenous Gal-3 delivered into the lungs *via* plasmid versus that of endogenously expressed Gal-3 in the lung. While glycans serve as the predominant ligands for galectins on the cell surface, there is growing evidence that intracellular galectins interact with non-glycan partners to exert their effects (31, 73). This may be yet another reason for the divergent effect noted with Gal-3 delivered into the lungs *via* plasmid. Currently, there are no therapeutic agents/inhibitors targeting endogenous Gal-3 that are commercially available for treatment. Development of selective inhibitors is complicated by the weak nature of the protein–carbohydrate interactions and the extensive sequence homology in the CRD of galectins (74). However, selective Gal-3 antagonists/inhibitors that are effective in attenuating lung fibrosis have been developed that are currently in preclinical or phase I testing (http://glycomimetics.com/galectin-inhibitors/; http://galecto.com/products/galectin-3-inhibitors/).

Galectin-1

Gal-1 was first identified in electric eels (75). This lectin is a "prototypic" galectin containing a single CRD that can occur as a monomer or a non-covalent homodimer and is found in virtually all adult tissue including lung, liver, brain, kidney, spleen and striated muscle (76). It is expressed by polymorphonuclear cells, macrophages, dendritic cells, activated T cells, stromal cells, endothelial cells, epithelial cells (76, 77) as well as eosinophils (30). Gal-1 is present both inside (nucleus, cytoplasm, and inner surface of plasma membrane) and outside cells (outer surface of cell membrane and extracellular matrix) and as such has intracellular as well as extracellular functions that play a profound role in resolving acute and chronic inflammation by affecting processes such as immune cell adhesion, migration, activation, signaling, proliferation, differentiation, and apoptosis [as reviewed in Ref. (36, 76)]. Increasing evidence from multiple chronic inflammatory disease models such as arthritis, hepatitis, encephalomyelitis, colitis, and nephritis supports the critical anti-inflammatory role of exogenous or endogenous Gal-1 in limiting or resolving inflammation (77). The resolving effects of Gal-1 have also been reported in models of acute inflammation where neutrophil adhesion and transmigration across the inflamed endothelium (78), as well as neutrophil extravasation and mast cell degranulation at sites of inflammation (79) was suppressed. Until recently, little was known regarding the potential role of Gal-1 in allergic asthma. By far the most investigated and well-established role of Gal-1 that may be relevant to allergic inflammation and asthma is the maintenance of T cell homeostasis by virtue of its ability to induce apoptosis of activated T cells and thus control or regulate a strong ongoing immune response [as reviewed in Ref. (36, 80)]. Other known Gal-1 effects that could potentially have a beneficial effect during chronic asthma include induction of IL-10 production by T cells (81, 82) [required for regulatory T cell (Treg)-mediated inhibition of airway inflammation in asthma], supporting inhibitory function of Tregs (83), and suppression of inflammatory cytokine [TNFα and interferon γ] release by T cells (84).

Recent studies from our laboratory demonstrate that allergen-challenged mice deficient in Gal-1 develop more severe airway inflammation (significantly higher eosinophil and T cell infiltration, TNFα level in the lung, and an increased propensity to develop airway hyperresponsiveness) relative to wild-type mice (30). At a cellular level, Gal-1 was found to exert divergent effects on murine bone marrow-derived eosinophils that were *N*-glycan-mediated. At lower concentrations (≤0.25 μM), Gal-1 promoted eosinophil adhesion to VCAM-1 and caused redistribution of α4 integrin to the cell periphery associated with cell clustering/aggregation but inhibited their ability to migrate toward eotaxin-1. While our studies demonstrate that Gal-1 (and Gal-3, described earlier in this review (27)) bind/interact with the α4 subunit of integrin α4β1, they do not rule out the possibility that these galectins can also interact with the β1 subunit. Indeed, previous studies have shown that Gal-1 and Gal-3 can bind to the β1 subunit in other integrin receptors (31, 85). Binding of these galectins to α4 on eosinophils may influence the activation state (resting to active) or conformation of the receptor [as shown in the case of Gal-1 binding to αIIbβ3 in platelets (86)] and enhance cell–cell interaction through receptor clustering/redistribution or bridging through self-association (interaction of α4-bound Gal-1/3 with endothelial-expressed Gal-1/3) (27). Gal-1 binding to integrin receptors (α5β1) has also been shown to affect downstream signaling events, albeit in tumor cells lines (87). Although exposure of eosinophils to Gal-1 leads to reduced activation of ERK1/2 (30), it is unclear at this time whether this effect is dependent on Gal-1-α4 binding. Consistent with the ability of Gal-1 to cause eosinophil aggregation and inhibit eosinophil migration, our studies showed that allergen-challenged wild-type mice had significantly more eosinophils adherent on the endothelium of the blood vessels in lungs but fewer eosinophils in the lung tissue in contrast to allergen-challenged Gal-1-deficient mice that exhibited fewer eosinophils adherent on the endothelium and more eosinophils in the lung tissue. The highlights of our findings pertaining to the role of Gal-1 in eosinophil trafficking are depicted by the schematic shown in **Figure 3**. Independent of the inhibitory effect of extracellular Gal-1 on cell migration, Gal-1-deficient eosinophils (derived from the bone marrow of Gal-1-deficient mice) showed inherently increased ability to recruit to sites of inflammation *in vivo* relative to wild-type cells, suggestive of a role for intracellular Gal-1 as well in regulating migration. At concentrations (≥1 μM), Gal-1 induced apoptosis in eosinophils and disrupted the cellular actin cytoskeleton leading to decreased levels of F-actin. In a previous study with

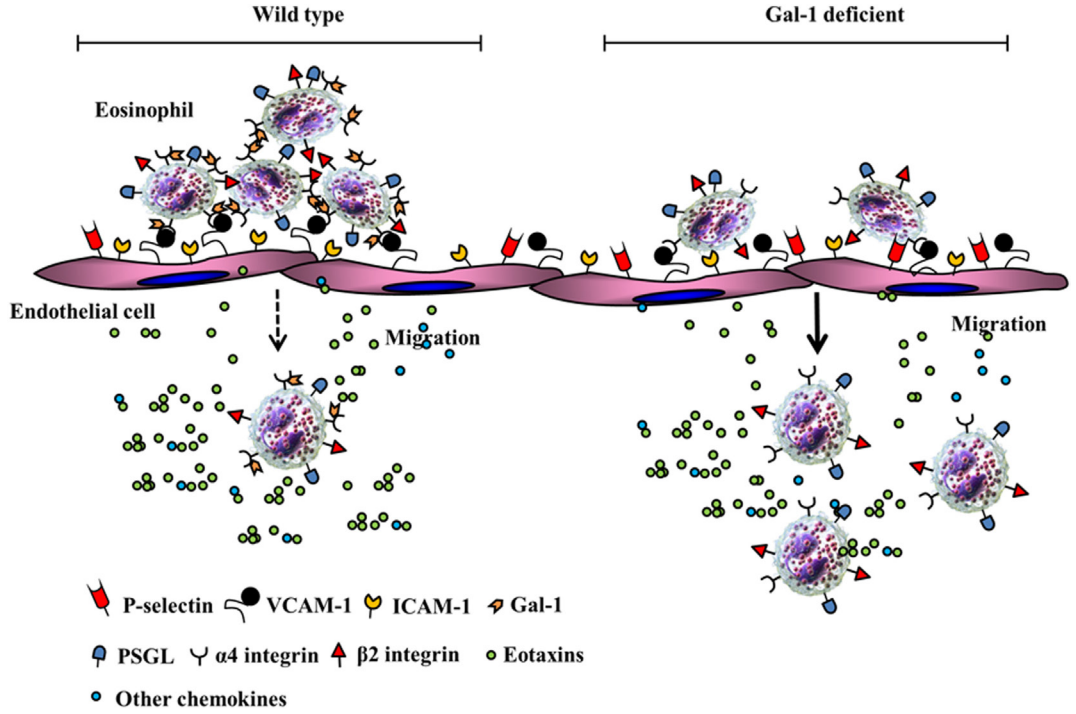

FIGURE 3 | Potential role of Gal-1-α4 interactions in limiting eosinophil trafficking and recruitment. In a wild-type setting, Gal-1 interacts with α4, causes cell clustering, increased adhesion in inflamed blood vessels resulting in restricted chemokine-induced migration. In the absence of Gal-1, eosinophils exhibit decreased adhesion in inflamed blood vessels and increased chemokine-induced migration to extravascular sites of inflammation.

human peripheral blood eosinophils, immobilized Gal-1 (coated on plastic supports) marginally increased cell adhesion but strongly inhibited migration (relative to P-selectin) and altered actin polymerization/depolymerization dynamics resulting in a prevalence of glomerular actin (i.e., decreased polymerization) (88). Remodeling of the actin cytoskeleton and coordinated polymerization/depolymerization of actin in response to extracellular signals are critical for cellular activities such as cell motility and active processes including cell adhesion and migration (89). The inhibitory effects of Gal-1 on eosinophil migration are likely to be caused by the ability of this lectin to target the actin cytoskeleton as noted in studies by us and others (described above). While allergen exposure causes infiltration of the airways with Gal-1-expressing inflammatory cells and increased soluble Gal-1 in extracellular spaces in the lungs of wild-type mice, this lectin appears to play an essential regulatory role during disease progression by limiting eosinophil recruitment to allergic airways and promoting eosinophil apoptosis, thus suppressing airway inflammation (30). This is further supported by the development of more severe allergic airway inflammation in mice in the absence of Gal-1. Studies have shown that cells (predominantly macrophages) in the sputum of asthmatic patients express lower levels of Gal-1 on the surface relative to cells from healthy donors (90) and propose that decreased Gal-1 levels may contribute to the exacerbated asthmatic immune responses.

The use of Gal-1 as a potential therapeutic (immunosuppressive) agent in Th1- and Th17-mediated inflammatory responses has been investigated in various disease models (91–93). While there are no studies using Gal-1 as a therapeutic agent for allergic asthma, it has been examined in other models of allergic inflammation. Administration of recombinant Gal-1 to mice with IgE-mediated allergic conjunctivitis showed divided results; on the one hand, Gal-1 treatment led to resolution of clinical signs of conjunctivitis and reduced Th2 cytokines (IL-4 and IL-13) and chemokines (eotaxin and CCL5) but resulted in eosinophilia in the conjunctiva with increased Gal-1 expression in the epithelium of the bulbar conjunctiva relative to untreated mice (94). In a model of oral-intestinal allergy syndrome, challenge of mice sensitized to peanut extracts along with Gal-1 showed reduced intestinal allergic inflammation (lower levels of serum histamine, peanut extract-specific IgE and IL-4 with decreased mast cell and eosinophil recruitment in oral and intestinal mucosa) compared to mice sensitized to peanut extracts alone by restoring IL-10 expression in the intestine (95). While findings from these studies are optimistic, it is evident that a better understanding of the functions of extracellular/exogenous versus intracellular/endogenous Gal-1 and its binding partners in these milieus is essential to its utility as a therapeutic agent. Although Gal-1 therapy approaches are being explored, there are challenges to effective therapy, which include stability (monomer–dimer equilibrium, oxidized versus reduced forms) and delivery of intact protein to the site of inflammation (96). In this context, a Gal-1 chimeric molecule with enhanced stability has been developed and shown to alleviate T-cell dependent inflammation in a mouse model of contact hypersensitivity (97). Nonetheless, identifying pathways to induce Gal-1 synthesis and/or favor its biological activity (as

in the latter study) might enable exploitation of its pro-resolving function to suppress allergic asthma.

Galectin-9

Gal-9 was first cloned in 1997 (98) and subsequently isolated from mouse embryonic kidney (99). Like Gal-3, this lectin is widely expressed in many tissues including the lung (99, 100) as well as by immune cells [T cells, B cells, and monocytes (101), eosinophils (102), and dendritic cells (103)]. Gal-9, previously known as ecalectin, belongs to the "tandem-repeat" family of galectins, which contain two conserved CRDs connected by a short peptide domain of varying length (31, 98). Depending on the length of the peptide domain linking the CRDs, three isoforms of Gal-9 have been identified (Gal-9L, Gal-9M, and Gal-9S). Studies by Matsumoto and coworkers in the late 1990s demonstrated that human T cell-derived Gal-9 functions as a potent and specific chemoattractant for human eosinophils *in vitro* and for murine eosinophils when administered intraperitoneally (100). Additionally, antigen stimulation of T cells was found to upregulate Gal-9 expression and release by these cells (104). The CRDs of Gal-9 exhibit high affinity for branched complex *N*-glycans with *N*-acetyllactosamine residues and both CRDs interact with the same or similar ligands on the cell surface of eosinophils to mediate chemotactic activity, with the length of the peptide domain linking the CRDs not being a determinant of this activity (105, 106). Gal-9-induced eosinophil chemotaxis was not mediated *via* binding to the IL-5 receptor or the eotaxin receptor CCR3 (107). Gal-9 has also been shown to activate eosinophils by inducing cell aggregation and superoxide production, but not degranulation (107). Interestingly, differential effects were noted with respect to eosinophil survival. While Gal-9 was shown to prolong survival of normal eosinophils in culture at lower concentrations ($\leq$10 nM) (107), proapoptotic activity was observed when cells were cultured with this lectin at a higher concentration (30 nM) under similar conditions (102). Additionally, Gal-9 suppressed apoptosis of eosinophils from eosinophilic patients but enhanced apoptosis of eosinophils from normal volunteers.

Consistent with its *in vitro* role as an eosinophil chemoattractant, elevated expression of endogenous Gal-9 in the lungs correlated with increased eosinophil recruitment/accumulation in animal models of allergic asthma in guinea pigs (108) and mice (109). Additionally, increased Gal-9 expression associated with increased eosinophil accumulation has also been reported in the nasal polyps of patients with nasal congestion and rhinorrhea (110) and in patients with acute eosinophilic pneumonia (111). While these studies indicate that elevated expression of endogenous Gal-9 may contribute to the pathogenesis of allergic asthma, administration of exogenous Gal-9 has been shown to have the opposite effect resulting in attenuation of Th2-mediated asthma. In a mouse model of allergen-induced airway inflammation, administration of exogenous Gal-9 inhibited airway inflammation by binding to CD44 and preventing CD44-hyaluronic acid interaction, an event that is essential for leukocyte adhesion and migration to the lung (112). Exogenously administered Gal-9 was also shown to suppress airway resistance and eosinophil recruitment in a guinea pig model of allergen-induced airway inflammation (113). Additionally, a suppressive effect was noted on passive cutaneous anaphylaxis in mice, suggestive of a stabilizing effect on mast cells. *In vitro* studies showed that Gal-9 specifically bound to IgE preventing IgE–antigen complex formation and mast cell degranulation (113). Gal-9 has also been shown to induce apoptosis of activated eosinophils, but not non-activated eosinophils, suggesting a potential regulatory function by Gal-9 for activated eosinophils at the site of inflammation (111).

In recent years, the immunoregulatory role of Gal-9 has been widely investigated. In a model of *Ascaris suum*-induced eosinophilic pneumonia, Gal-9-deficient mice exhibited higher numbers of eosinophils and Th2 cells relative to wild-type mice. Interestingly, levels of Foxp3$^+$ Tregs were lower. Additionally, administration of exogenous Gal-9 to *A. suum*-exposed wild-type mice prevented eosinophilic inflammation of the lung and increased release of endogenous Gal-9, suggesting an immunoregulatory role for Gal-9 in Th2-mediated eosinophilic inflammation (114). In support of this, cells (macrophages) from sputum of asthmatic patients were found to express lower levels of Gal-9 on the surface than cells from healthy donors, which might be responsible for the exacerbated immune response (90). Studies by Wu et al. have shown that induced Tregs (iTreg) express high levels of Gal-9 and that exogenous Gal-9 plays an important role in maintaining the stability and function of iTreg *via* direct interaction with CD44 (115). Most recently, in a study designed to test the efficacy of Gal-9 as an adjuvant to allergen-specific sublingual immunotherapy in a mouse model of chronic asthma, administration of Gal-9 was found to inhibit eosinophilic airway inflammation, airway hyperresponsiveness, and allergen-specific IgE while inducing transforming growth factor β-1 production as well as the number of CD4$^+$CD25$^+$Foxp3^{high} Tregs in the BALF, thus suggesting that using Gal-9 as an adjuvant to sublingual immunotherapy may be a more effective treatment option (116). Another interesting finding is that exogenously added Gal-9 suppresses Th17 cell development and expands Foxp3$^+$ Tregs from naïve CD4 T cells in an IL-2-dependent manner *in vitro* under "Th17-skewing" conditions (117). This is of importance in the context of those forms of asthma where neutrophils contribute to airway inflammation more than eosinophils (118).

OTHER EOSINOPHIL-EXPRESSED GALECTINS

Gal-10, also known as Charcot–Leyden crystal protein, is a mannose binding, prototypic (i.e., contains one CRD) galectin strongly expressed in human, but not mouse, eosinophils (119). This lectin has the ability to form bipyramidal hexagonal crystals and was identified in the sputum of asthmatics as early as 1872 (120). Several studies have shown that it is associated with eosinophilic inflammation seen in diseases of the airways, and more recently the gastrointestinal tract. Sputum specimens from patients with acute asthma and patients with certain respiratory diseases associated with bronchopulmonary infection have been shown to contain elevated levels of Gal-10 (121). Gal-10 was found to be present in nasal lavage fluid from patients with seasonal allergic rhinitis during allergy season but not before allergy season (122). Overexpression of Gal-10 mRNA was noted in peripheral

blood of patients with aspirin-induced asthma compared to patients with aspirin-tolerant asthma despite similarity in other parameters associated with severe asthma in these two groups (i.e., age, peripheral eosinophilia, inhaled corticosteroid use, etc.) (123). More recently, studies have shown that Gal-10 is released in the nasal lavage fluid and expressed at high levels in nasal polyp tissue of patients with aspirin-sensitive respiratory disease relative to aspirin-tolerant asthmatics (124). Genetic variation in the promoter region of the Charcot–Leyden crystal/Gal-10 gene was found to be associated with allergic rhinitis suggesting the possibility that Gal-10 gene transcription may be altered in these individual (125). In another study, Gal-10 concentration in the sputum strongly correlated to the number of eosinophils in the sputum and accurately identified sputum eosinophilia in patients with asthma (126). Apart from its association with eosinophilic disorders of the airways, a direct correlation between Gal-10 protein expression, eosinophil recruitment, and extent of tissue damage has been shown in gut biopsies from patients with celiac disease (127). Along these lines, eosinophils from children with eosinophilic esophagitis had higher levels of Gal-10 mRNA compared to eosinophils from healthy controls (128). Gal-14 is the ovine ortholog of Gal-10, which is expressed specifically by eosinophils and released into the lungs after challenge with house dust mite allergen (129). Because it was found to be released by activated eosinophils and was abundantly present in mucus scrapings from the lung and intestinal tract of sheep after challenge with an allergen or a parasite, this galectin is believed to be secreted by eosinophils at epithelial surfaces and play a role in promoting cell adhesion and changing mucus properties during allergies or parasitic infections *in vivo* (130).

Although Gal-10 has long been considered as an eosinophil-specific protein, later studies showed that Gal-10 is constitutively expressed in the cytoplasm of human $CD25^+$ Tregs and is necessary for limiting cell proliferation and Treg-cell-mediated suppression of cocultured $CD4^+$ T cells (131). However, many unanswered questions remain regarding the mechanism of suppression, intracellular binding partners of Gal-10, participation of mannose residues, etc. In the context of allergic disease, Gal-10 was recently shown to be associated with atopic dermatitis (132). Serum Gal-10 levels were higher in patients with atopic dermatitis relative to healthy controls, positively correlating with disease severity. Further, Gal-10 was overexpressed in circulating $CD3^+$ T cells and IL-22-producing $CD4^+$ T cells from atopic dermatitis patients as well as in the skin of chronic atopic dermatitis patients. Overall, the functions of Gal-10 are still elusive; however, the consistent finding that it is associated with eosinophilic inflammatory disorders indicates its potential role as a biomarker for eosinophilic inflammation.

CONCLUDING REMARKS

Eosinophils play a critical role in mediating inflammatory processes in asthmatic lungs by virtue of their ability to release pro-inflammatory cytokines, chemokines, and growth factors that promote development of the hallmark features of asthma. The recruitment of primed mature eosinophils from the blood stream to sites of inflammation in the lung is a multistep paradigm involving initial rolling in the lumen of the blood vessels followed by activation-dependent firm adhesion to the vessel wall and chemoattractant-induced transmigration across the vascular endothelium to extravascular sites, a process driven by cell adhesion molecules (integrins and selectins), chemokines (eotaxin and other chemoattractants), and metalloproteases. In recent years, studies have indicated a regulatory role for galectins in eosinophil trafficking, migration, and activation and thus impact the pathogenesis of allergic asthma. As detailed in this review, Gal-3 plays a pro-inflammatory role in allergic asthma by promoting eosinophil trafficking and migration, while Gal-1 exerts an anti-inflammatory effect due to its ability to limit eosinophil migration and induce apoptosis, wherein decreased expression or absence of this galectin results in increased eosinophilia and exacerbated asthmatic immune responses. The role of Gal-9 in asthma is more intricate; on the one hand, this galectin functions as a chemoattractant for eosinophils and activates these cells but also appears to exert a regulatory function by inducing apoptosis of only activated eosinophils. In disease models, absence of Gal-9 results in increased eosinophilia and Th2 cells but low $Foxp3^+$ Tregs while administration of Gal-9 causes an attenuated asthmatic response attributable to induction of endogenous Gal-9 and direct interaction of Gal-9 with CD44 (limiting leukocyte adhesion and migration and promoting stability and function of iTregs).

Studies at a cellular level and in animal models clearly indicate a role for Gal-1, -3, and 9 in regulating (positively or negatively) eosinophil recruitment and the pathogenesis of allergic asthma; however, further studies to elucidate expression patterns of these galectins in relation to different phenotypes and endotypes of allergic asthma in humans are necessary to define whether they can serve as disease biomarkers or therapeutic targets for pharmacological modulation. Currently, there are no therapeutic agents that are commercially available for targeting these galectins endogenously for treatment of asthma. However, considerable progress has been made with respect to Gal-3. Gal-3 has been suggested as a reliable biomarker to predict the modulation of airway remodeling in severe asthma patients before they begin omalizumab therapy (133) and selective Gal-3 antagonists/inhibitors that are effective in attenuating lung fibrosis have been developed that are currently in preclinical or phase I testing. The final outcome of an immune response is often determined by the fine balance of pro- and anti-inflammatory signals. Development of novel forms of Gal-1 and Gal-9 that are stable and can be used for immunotherapy or innovative approaches to induce synthesis or enhance biological activity of these anti-inflammatory galectins may pave the way for future clinical strategies in management of allergic asthma.

AUTHOR CONTRIBUTIONS

All the authors listed contributed to the review in close collaboration.

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Prostaglandins and Their Receptors in Eosinophil Function and As Therapeutic Targets

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Of the known prostanoid receptors, human eosinophils express the prostaglandin D_2 (PGD_2) receptors DP1 [also D-type prostanoid (DP)] and DP2 (also chemoattractant receptor homologous molecule, expressed on Th2 cells), the prostaglandin E_2 receptors EP2 and EP4, and the prostacyclin (PGI_2) receptor IP. Prostanoids can bind to either one or multiple receptors, characteristically have a short half-life *in vivo*, and are quickly degraded into metabolites with altered affinity and specificity for a given receptor subtype. Prostanoid receptors signal mainly through G proteins and naturally activate signal transduction pathways according to the G protein subtype that they preferentially interact with. This can lead to the activation of sometimes opposing signaling pathways. In addition, prostanoid signaling is often cell-type specific and also the combination of expressed receptors can influence the outcome of the prostanoid impulse. Accordingly, it is assumed that eosinophils and their (patho-)physiological functions are governed by a sensitive prostanoid signaling network. In this review, we specifically focus on the functions of PGD_2, PGE_2, and PGI_2 and their receptors on eosinophils. We discuss their significance in allergic and non-allergic diseases and summarize potential targets for drug intervention.

Keywords: allergy, inflammation, respiratory and gastrointestinal tract, bone marrow, chemotaxis, endothelium

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THE PROSTANOID—EOSINOPHIL AXIS IN ALLERGIC DISEASES

Atopy is a genetically determined disorder, which results in characteristic inflammatory responses to *per se* innocuous antigens. Atopic diseases can manifest in different tissues as allergic rhinitis, conjunctivitis, bronchial asthma, dermatitis, or food allergies, and are associated with a major reduction in quality of life and life expectancy. In addition, some diseases, such as intrinsic asthma, aspirin sensitivity, nasal polyposis, adenoid hyperplasia, or chronic idiopathic urticaria, share several clinical and pathophysiological aspects of allergy, but with less clear ties to allergens. The basic concept of atopic reactions is grounded in an inadequate activation of immune cells by both specific and non-specific stimuli, with a shift toward the type-2 spectrum of inflammatory mediators, such as interleukin (IL)-4, -5, -9, and -13 (1). In allergen-specific IgE-mediated hypersensitivity reactions mast cells release preformed and newly synthesized mediators [histamine, leukotriene C_4, prostaglandin (PG) D_2, TNFα, and many others] (2). This is the pivotal step in the inflammatory cascade as it initiates the early phase of an allergic reaction. On the one hand, these mediators provoke symptoms such as sneezing, nasal congestion, rhinorrhea, wheezing, skin rash, etc., on the other hand, they trigger the infiltration of innate and adaptive immune cells, which favors the development

of the late phase response that is characterized by symptoms such as bronchoconstriction, mucus hypersecretion, edema, pain, heat, and erythema.

Eosinophils are regarded as crucial effector cells in chronic allergic inflammation. Activated eosinophils release an array of cytotoxic and pro-inflammatory mediators promoting mucosal damage in chronic asthma and allergic inflammation. The tissue damage repeatedly initiates repair mechanisms that can lead to imbalance of epithelial-to-mesenchymal transition (3, 4). Consequently, eosinophils also play a role in airway remodeling and angiogenesis in chronically inflamed tissue, and hence contribute to the progression of the disease (5, 6). Consequently, eosinophil-deficient mice are protected against allergen-induced pulmonary inflammation and airway hyperresponsiveness (7, 8). The pathogenic role of eosinophils was eventually highlighted in a pivotal study showing that patients whose treatment is adjusted according to sputum eosinophil counts have significantly fewer severe asthma exacerbations than patients on standard management therapy (9). Therefore, eosinophils are currently considered a major therapeutic target in allergic diseases, such as conjunctivitis, rhinosinusitis, asthma, and atopic dermatitis, but they might also play pathogenic roles in several other diseases, such as eosinophilic esophagitis and gastroenteritis, pancreatitis, colitis ulcerosa, hypereosinophilic syndrome, renal disease, and cancer (10–19).

Importantly, the role of eosinophils in murine models of allergic airway inflammation is discussed controversially. IL-5 transgenic mice show pronounced eosinophilia and intrinsic airway hyperreactivity whereas the latter is abolished when $CD4^+$ cells are depleted in these mice (20). However, it has also been observed that IL-5 transgenic mice are protected from airway hyperreactivity, and eosinophils isolated from BAL of OVA-challenged IL-5 transgenic mice do not release superoxides when activated with physiological stimuli (eotaxin, IL-5, PAF, or IgG) (21), which is in sharp contrast to human eosinophils. Therefore, the role of mouse vs. human eosinophils might differ in the pathophysiology of allergic diseases.

Human eosinophils express a distinct pattern of prostanoid receptors, comprising the receptors for PGD_2, DP1 [also D-type prostanoid (DP)] (22) and DP2 [also chemoattractant receptor homologous molecule expressed on Th2 cells (CRTH2)] (23), the prostaglandin E_2 receptors EP2 and EP4 (24), and the PGI_2 (prostacyclin) receptor IP (25). When activated, these seven-transmembrane receptors couple to G proteins, which initiate further intracellular signaling events and are eventually eliciting a cellular response. Depending on the G protein subtypes involved, this can lead to the activation of opposing signaling pathways (26–29). For instance, the DP2 receptor couples to $G\alpha_i$ and $G\alpha_q$ causing eosinophil shape change and migration, while the IP receptor inhibits these eosinophil responses, likely through $G\alpha_s$. In the mouse, eosinophils express DP1 and DP2 (30). EP2 is expressed on murine eosinophils since the EP2 agonist butaprost inhibits eosinophil trafficking, and in OVA-sensitized mice, the infiltrating leukocytes after allergen challenge were immunohistologically stained EP2 positive (31). The expression of EP1, EP3, EP4, and IP remains elusive; however, IP-deficient OVA-sesitized mice show less eosinophils in the brochoalveolar lavage and airway inflammation after allergen challenge as compared to wild type mice (32, 33).

PROSTAGLANDIN D_2 (PGD_2)

Prostaglandin D_2 is the principal ligand for two receptors, DP1 and DP2 (34), of which both are expressed on the surface of eosinophils (35). At micromolar concentrations, PGD_2 is also an agonist of the thromboxane receptor, TP, which mediates the direct bronchoconstrictor effect of PGD_2 (36). Moreover, a major metabolite of PGD_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2 is a potent agonist of peroxisome proliferator-activated receptor (PPAR)-γ, which is also expressed by eosinophils (37). PGD_2 had been known to stimulate eosinophil locomotion for some time (38, 39), but it was only in 2001 that the DP2 receptor was found to mediate this effect (22, 40, 41). Also, DP2 activation by PGD_2 or DP2-selective ligands triggers Ca^{2+} flux, CD11b upregulation, respiratory burst, and release of eosinophil cationic protein (22, 40–42). Eosinophil responses to DP2 activation seem to depend on $G\alpha_q$ proteins, exemplified by the lack of effect of pertussis toxin on PGD_2-induced eosinophil shape change, which—however—is abrogated by phospholipase C inhibition (43). However, PGD_2-induced chemotaxis was abrogated by pretreatment of eosinophils with pertussis toxin (unpublished observation). In addition to directly stimulating eosinophil migration, we also observed that PGD_2 is capable of priming eosinophils for other chemoattractants like eotaxin, 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE), or complement factor C5a, an effect that is likewise mediated by the DP2 receptor (42, 44). Conversely, eosinophil migration toward PGD_2 is impaired by eotaxin or 5-oxo-ETE in a pathway depending on phosphoinositide 3-kinase as well as p38 mitogen-activated protein kinase (44). The subcellular signaling cascades that mediate the priming effect of PGD_2 are not yet understood, while the priming effect of the PGD_2 metabolite 15-deoxy-$\Delta^{12,14}$-PGJ_2 seems to involve PPAR-γ (45). Thus, it appears that a hierarchy exists among eosinophil chemoattractants: PGD_2 might be regarded as an initial chemoattractant, since its potency is sustained also in whole blood and primes eosinophils for other chemoattractants; however, eotaxin seems to be an end-point chemoattractant, as it has reduced efficacy in blood as compared to isolated eosinophils, and effectively downmodulates eosinophil migration toward other chemoattractants (44).

Besides PGD_2, DP2 is also activated by the PGD_2 metabolites 13,14-dihydro-15-keto- (DK-) PGD_2, PGJ_2, Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (42, 46, 47). Considering that PGD_2 is as short-lived molecule and rapidly degraded into metabolites (48), it is interesting that the PGD_2 actions on eosinophils are maintained through metabolites binding to DP2. Moreover, one of the major metabolites of the thromboxane pathway, 11-dehydro-TXB_2, and even the common precursor of all prostanoids, PGH_2, are also potent DP2 agonists (49, 50). Similarly, PGF2α has been found to activate eosinophils through DP2 (51).

In human disease, DP2 on peripheral blood eosinophils is upregulated in allergic dermatitis and rhinitis patients (52, 53), but it is diminished in active ulcerative colitis (26).

Although PGD_2 binds to DP1 with similar affinity as to DP2 (34), the exact function of this receptor in immune cells has not

been fully elucidated yet, and both pro- and anti-inflammatory effects have been reported (29). For instance, DP1 mediates the PGD_2-induced expression of the airway mucin MUC5B in human nasal epithelial cells (54) and stimulates mucus production *in vitro* (55) but inhibits the functions of platelet, neutrophils, basophils, and dendritic cells (56–62). Unlike DP2, which is preferentially expressed on immune cells, such as eosinophils, basophils, macrophages, mast cells, a subset of Th2 lymphocytes and group 2 innate lymphoid cells (23, 40, 63–66), DP1 is more widely expressed, including the vasculature, the central nervous system, the retina, and the lungs (55, 67–69).

DP1-deficient mice were shown to be protected from development of allergic lung inflammation in terms of airway hyperresponsiveness, reduced numbers of BAL eosinophils, and BAL levels of IL-4, IL-5, and IL-13 (70). In contrast, intratracheal administration of DP1 agonist BW245c protected mice from airway hyperresponsiveness and lung eosinophilia in a OVA models of experimental asthma, thereby counteracting DP2-mediated proinflammatory responses (30, 71). DP1 activation has also been linked to inhibition of dendritic cell function (60) and to reduce inflammation in an IL-10-dependent mechanism (71). DP1, but not DP2, expression in lung tissue (mRNA) is upregulated upon OVA challenge (72). More recently, in guinea pigs, PGD_2 aerosols were shown to induce the activation of sensory nerves and cough *via* DP1 receptor activation. Interestingly, DK-PGD_2 modulated the sensory nerve activity by inhibiting the response to capsaicin (73).

In eosinophils, the DP1 receptor transmits antiapoptotic signals by PGD_2 (22), but has been found to limit DP2-mediated CD11b upregulation (41). At micromolar concentrations, however, PGD_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 drive eosinophils into apoptosis in a nuclear factor κB-dependent manner (74). Regarding other eosinophil responses, there is growing literature reporting cooperative signaling of DP1 and DP2 receptors. In guinea pigs, both DP1 and DP2 activation can stimulate the mobilization of eosinophils from the bone marrow (75). Moreover, DP1-dependent eosinophil responses such as migration and production of reactive oxygen species are—to some extent—co-mediated by DP1 (75, 76). On the molecular level, we have shown that DP1 activation is substantially involved in DP2-triggered Ca^{2+} signaling in a heterologous expression system and in human peripheral blood eosinophils and, therefore, might be an important regulator of DP2-mediated pro-inflammatory signaling (35). Cooperative signaling of the two receptors also converges in the PGD_2-induced synthesis of leukotriene C4 synthesis in eosinophils. Only a simultaneous activation of DP1 and DP2 led to a sufficient response while the activation of either one or the other receptor did not equal the full PGD_2 response (77). This finding does not only substantiate the significance of PGD_2 in stimulating the synthesis of LTC_4 but also highlights the cooperative function of the two PGD_2 receptors (**Figure 1**).

TARGETING PGD_2 SIGNALING IN EOSINOPHILIC DISEASES

DP2 Receptor Antagonists

Blood and tissue eosinophilia is a key feature of allergy and asthma. It correlates with the severity of the disease on the one hand, and levels of PGD_2 on the other hand (78). Exogenously applied PGD_2 and DP2 agonists provoke peripheral blood eosinophilia and infiltration of eosinophils into the conjunctiva, lung, nose, and skin in animal models (30, 38, 79–82), whereas pharmacological blockade of DP2 can ameliorate models of atopic dermatitis, asthma, rhinitis, and conjunctivitis (83–88). Interestingly, DP2-deficient

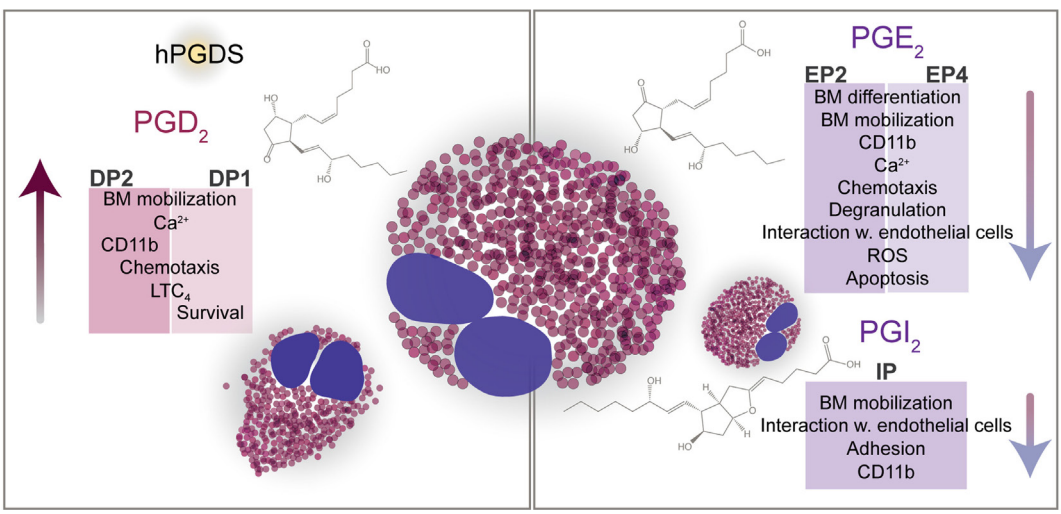

FIGURE 1 | PGD_2, PGE_2, and PGI_2 direct the functions of eosinophils. Eosinophils express receptors for PGD_2 (DP1, DP2), PGE_2 (EP2, EP4), and PGI_2 (IP). *Via* DP2, PGD_2 attracts eosinophils to the site of inflammation, enhances eosinophil mobilization from the bone marrow, and upregulates CD11b expression. In line with the chemotactic response, PGD_2-mediated activation of eosinophils results in increased size and altered cell shape. DP1 and DP2 cooperatively regulate the synthesis of LTC_4. DP1 has been shown to enhance the DP2-mediated Ca^{2+} response and to prolong the survival of eosinophils *in vitro*. Counteracting pro-inflammatory mechanisms PGE_2 and PGI_2 suppress the activation of eosinophils and hence dampen pro-inflammatory signals. Despite the negative regulation of eosinophil effector and chemotactic functions by PGE_2 and PGI_2, PGE_2 was shown to decrease eosinophil apoptosis *in vitro*.

mice develop a normal chronic allergic inflammatory response to allergen challenge after sensitization and challenge, while the acute inflammatory response and eosinophil infiltration in the skin are abrogated (89).

The effects of the DP2 antagonist timapiprant (OC-459) was studied in a large patient cohort ($n = 482$) of mild-to-moderate persistent asthma. In this randomized, double-blind placebo-controlled study, the DP2 antagonist was given over 12 weeks with overall beneficial effects on lung function. A *post hoc* analysis revealed that the greatest improvement of lung function by timapiprant was observed in patients with active eosinophilia (≥250/µl peripheral blood) and—even more pronounced—in younger patients (90). This applies also for the humanized murine IL-5 antibody mepolizumab, which is most effective and only given in asthma patients with severe eosinophilic airway inflammation (91). In eosinophil esophagitis, timapiprant significantly reduced the esophageal eosinophil load and induced some clinical improvement (92). Timapiprant also successfully reduced nasal and ocular symptoms in allergic subjects exposed to grass pollen (93).

Fevipiprant (QAW039) is another DP2 antagonist, but as compared to timapiprant, it has slower dissociating properties and is, therefore, a candidate compound with potentially improved efficacy (94). In 170 patients with uncontrolled asthma, however, fevipiprant administered once daily did not meet the overall expected primary clinical end point (increase in FEV_1), but led to an improvement of clinical symptoms in a sub-cohort with severe asthma ($FEV_1 < 70\%$), leading to a significant improvement in FEV_1 and the asthma control questionnaire score, in addition to being well tolerated by the patients (95). It has to be considered, however, that *post hoc* analyses like these performed with fevipirant and timapiprant (90) need to be interpreted with caution. Importantly, fevipirant reduced eosinophilic airway inflammation in a separate, small trial comprising 61 patients with persistent moderate-to-severe asthma, uncontrolled by inhaled corticosteroids and elevated sputum eosinophil counts (96).

Several other DP2 antagonists have been subject to clinical trials in asthma or even COPD, but showed little efficacy and are discussed elsewhere (97).

DP1 Receptor Antagonists

Based on *in situ* hybridization and immunohistochemistry, DP1 mRNA and DP2 protein expression were detectable in eosinophils in nasal polyp tissue of allergic rhinitis patients; in contrast, only DP1 but not DP2 was observed in nasal tissue of healthy subjects (67).

A pivotal study using DP1-knockout mice suggested that DP1 plays an important role in the OVA-induced asthma model. DP1-deficient mice not only showed markedly reduced eosinophils in BAL fluid but also did not develop airway hyperresponsiveness (70). In a rat model of OVA-induced pulmonary inflammation, DP1 expression was upregulated in the lungs while bronchial hyperresponsiveness and immune cell infiltration was diminished by the DP1 antagonist S-5751 (98). In an OVA-induced allergic rhinitis model in guinea pigs, S-5751 inhibited late phase responses such as infiltration of eosinophils and mucosal plasma exudation (99). A newly developed DP1 antagonist (S-555739, asapiprant) showed improved affinity and bioavailability, and reversed antigen- and PGD_2-induced nasal congestion and airway hyperresponsiveness in guinea pigs and sheep, respectively, along with significantly decreased eosinophils and other inflammatory cells in nasal lavage fluid (100). A phase II clinical trial in the USA (NCT01651871) and a phase III clinical trial (JapicCTI-132046) in Japan are underway testing asapiprant in seasonal allergic rhinitis. The results are yet to be announced. Previously, another DP1 antagonist, laropiprant (MK-0524), was shown to prevent nasal congestion induced by PGD_2 in healthy subjects (101) but failed in phase II trials in allergic rhinitis and asthma (102). Similarly, the dual DP1/DP2 antagonist vidupiprant (AMG 853) provided no benefit as an add-on to inhaled corticosteroid therapy in moderate-to-severe asthma (103).

Inhibition of PGD_2 Synthases—HPGDS and Lipocaline Prostaglandin D_2 Synthase (LPGDS)

In mammals, two isoforms of PGD_2 synthases are expressed: the lipocaline type (LPGDS), which is highly abundant in the central nervous system and the hematopoietic type (HPGDS), which is mainly expressed in mast cells, but also can be found in macrophages and Th2 lymphocytes (**Table 1**). Additionally, resident eosinophils themselves might be a late source of PGD_2 at the site of allergic inflammation acting in an autocrine manner to attract and activate further eosinophils (104, 105). An interesting novel link between PGD_2 and eosinophils is the recent discovery of pro-eosinophilic, so-called pathogenic effector (pe)Th2 lymphocytes, which highly express IL-5 and IL-13, and can be found at elevated levels in eosinophilic patients suffering from atopic dermatitis and eosinophilic gastrointestinal disease. These cells express not only DP2 but also HPGDS (106).

Both PGD synthases are regarded as promising drug targets in a variety of diseases, such as allergic inflammation, mastocytosis, asthma and chronic obstructive pulmonary disease, metabolic disorders, muscular dystrophy, Alzheimer's disease, or spinal cord injury (127), stimulating the development of several selective inhibitors (128–136). Transgenic mice overexpressing LPGDS show exaggerated eosinophilic pulmonary inflammation (72), which was reversed by AT-56, a LPGDS inhibitor (129). In contrast, eosinophil numbers in OVA-induced pulmonary inflammation are not significantly increased in transgenic mice overexpressing HPGDS, but the HPGDS inhibitor HQL-79 abrogated eosinophilic pulmonary inflammation in OVA-challenged mice (128). HPGDS in healthy nasal mucosa is expressed only in mast cells, but in allergic rhinitis and nasal polyps also in infiltrating inflammatory cells including eosinophils (67, 137). In a guinea-pig model of allergic inflammation, the HPGDS inhibitor TAS-204 prevented OVA-induced nasal obstruction and eosinophil infiltration (132).

Activation of PPAR-γ

In an OVA-induced allergic model, 15-deoxy-$\Delta^{12,14}$-PGJ_2 and the PPAR-γ agonist rosiglitazone abrogated peritoneal accumulation of eosinophils and eosinophil proliferation in bone marrow (138). Similarly, several studies have shown that synthetic PPAR-γ

TABLE 1 | PGD_2 release and expression of hematopoietic prostaglandin D_2 synthase (HPGDS) and lipocaline prostaglandin D_2 synthase (LPGDS) in human cells.

Cell type	HPGDS	LPGDS	PGD_2 release
Astrocytes	Mohri et al. (68)		
Basophils	Tanaka et al. (107); Dahlin et al. (108)		
Dendritic cells	Shimura et al. (109)		Shimura et al. (109)
Endothelial cells		Taba et al. (110)	Taba et al. (110), Camacho et al. (111)
Eosinophils	Luna-Gomes et al. (112)		Luna-Gomes et al. (112)
Epithelial cells (choroid plexus)		Blödorn et al. (113)	
Bronchial epithelial cells (HBEC)			Jakiela et al. (114) (mass spectrometry)
ILC2	Björklund et al. (115)		
Keratinocytes			Kanda et al. (116)
Langerhans cells (epidermal)	Shimura et al. (109)		
Macrophages	Jandl et al. (65)		Tajima et al. (117)
Mast cell progenitors	Dahlin et al. (108)		
Mast cells	Nantel et al. (67)		Schleimer et al. (118); Lewis et al. (119)
Megakaryoblastic cells (CKM, Dami cells)	Mahmed et al. (120); Suzuki et al. (121)		
Microglia	Mohri et al. (68)		
Myocardial cells		Eguchi et al. (122)	
Oligodendrocytes		Mohri et al. (68); Kagitani-Shimono et al. (123)	
Osteoarthritic chondrocytes		Zayed et al. (124)	Zayed et al. (124)
Smooth muscle cells (arteriosclerotic plaques)		Eguchi et al. (122)	
Th2 subsets	Mitson-Salazar et al. (106); Tanaka et al. (107); Wang et al. (125); Nagata et al. (126)		

agonists are beneficial in mouse models of allergic pulmonary inflammation and rhinitis (139, 140). Pioglitazone was tested in patients with mild asthma but did not reproduce the results from animal studies (141).

PROSTAGLANDIN E_2

Infiltration of eosinophils along with other proinflammatory parameters in OVA-induced asthma model was found to be markedly enhanced in COX-1 and COX-2 knockout mice (142) and after pharmacological blockade of these enzymes (143). Conversely, inhaled PGE_2 reduced airway inflammation, hyperresponsiveness, and eosinophil counts in BAL fluid of asthmatic patients (144). These findings suggested a possible inhibitory effect of PGs on eosinophils.

In airways, PGE_2 is released by epithelial-, endothelial-, and smooth muscle cells, macrophages, and fibroblasts, and potently counteracts the pro-inflammatory actions of PGD_2. PGE_2 has bronchodilator functions and reduces airway hyperresponsiveness *via* activation of EP2 receptors (145). Recently, we found that PGE_2 promotes the endothelial barrier by EP4 receptors expressed on the endothelium and protects against thrombin-induced junctional disruption (146).

Early studies indicated that PGE_2 inhibits the release of eosinophil cationic protein (39) and homotypic aggregation of eosinophils (147) that is mediated by the β2-integrin CD18 (148). Of the known PGE_2 receptors (EP1, EP2, EP3, and EP4), eosinophils express mRNA for EP2 and EP4 (24). Accordingly, we found both EP2 and EP4 protein in eosinophils using flow cytometry and Western blot, respectively (27, 31). By directly addressing the significance of PGE_2 in eosinophil function, we could show that PGE_2 acts to suppress eosinophil responses such as chemotaxis and degranulation, which seemed to be mediated by both EP2 and EP4 receptors (27, 31). On the subcellular level, EP4 receptor activation resulted in blockade of intracellular Ca^{2+} release, cytoskeletal reorganization, and production of reactive oxygen species (27). EP4 agonist treatment inhibited CD11b upregulation, activation, and clustering of β2 integrins, and L-selectin shedding of eosinophils, which were all abolished using an EP4 antagonist (149). We could delineate the underlying signaling pathways to involve phosphoinositide 3-kinase, phosphoinositide-dependent kinase 1, and protein kinase C but not the cyclic AMP/protein kinase A pathway (27, 150). Likewise, the PGE_2—EP4 axis acted inhibitory on the interaction of eosinophils with endothelial cells, including adhesion and transmigration (149). In contrast, mobilization of eosinophils from guinea pig bone marrow was mediated by the EP2 receptor (31). Previously, *in vitro* eosinophilopoiesis stimulated by IL-5 was also observed to be under negative control of PGE_2 in normal and OVA-sensitized mice by selectively inducing apoptosis in developing eosinophils (151, 152). Unexpectedly, PGE_2 has been found to be antiapoptotic for peripheral blood eosinophils (153, 154), which might be linked to elevated PGE_2 levels in airways of asthmatic patients (155), and even more in non-asthmatic eosinophilic bronchitis (156). Another study, however, found an inverse relationship between sputum eosinophil counts and PGE_2 levels (157). Nevertheless, activation of the EP2 receptor inhibited the allergen-induced increase of eosinophils in the bronchoalveolar lavage fluid of OVA-sensitized mice (31).

Hence, the activation of EP2/EP4 receptors can be protective against the accumulation and activation of eosinophils in the affected tissue, and is therefore considered as a potential treatment strategy in allergy (**Figure 1**).

PROSTAGLANDIN I_2

Parts of the immune-suppressive effects of PGE_2 are shared by PGI_2 (prostacyclin). In contrast to EP2/EP4 signaling, the activation of PGI_2 receptors (IP) is mediated by intracellular cAMP, thereby inhibiting eosinophil functions. PGI_2 and the stable PGI_2 mimetic iloprost negatively regulate the trafficking of guinea pig bone marrow eosinophils *via* IP receptor activation (158). In experimental asthma in mice, iloprost attenuates dendritic cell function and the concomitant allergen-specific Th2 response and inhibits eosinophilia in lung tissue (159). After repeated allergen challenge, endogenous PGI_2 abrogates airway remodeling (32).

In an *in vitro* study using human eosinophils and endothelial cells, we found that endothelium-derived PGI_2 is an important modulator of eosinophil–endothelial interaction and might have a bearing on eosinophil accumulation at sites of allergic reaction. Moreover, PGI_2 promotes the barrier function of lung endothelial cells and limits eosinophil adhesion and transendothelial migration (25). Our data might hence explain previous findings that deletion of IP receptors in mice augments the eosinophilic infiltrate in allergic responses of the lung and skin and enhances airway remodeling (32, 33).

THE PROSTANOID—EOSINOPHIL AXIS IN NON-ALLERGIC DISEASES

Aspirin-Exacerbated Respiratory Disease (AERD)

Also referred to as aspirin intolerance or Samter's triad, AERD is a chronic inflammatory state of the airways resulting in rhinosinusitis, nasal polyps, and asthma. In some patients, these symptoms are accompanied by skin rash such as urticaria or angioedema, while in others the skin manifestations are prevailing. These symptoms are aggravated after intake of aspirin (acetylsalicylic acid) or any other non-selective COX inhibitor, occasionally culminating in massive anaphylactoid reactions or even death. In contrast, selective COX-2 inhibitors are mostly tolerated. A comprehensive overview on clinical presentations and pathobiologic mechanisms is provided elsewhere (160–162). In brief, an imbalance of anti-inflammatory PGE_2 and proinflammatory LTC_4 exists in these patients at baseline, which is further enhanced after intake of COX inhibitors, which alludes into activation of mast cells, eosinophils, and several other immune cells. In addition to mast cells, LTC_4 biosynthesis in eosinophils is upregulated in AERD patients. Similarly, both cell types express more HPGDS and release excessive levels of PGD_2 in this condition (163). Urinary levels of a stable PGD_2 metabolite were found to be twofold higher in patients with AERD relative to those in control subjects and—most remarkably—increased further upon aspirin exposure. This correlated with reductions in blood eosinophil counts and lung function, and clinical symptoms such as nasal congestion (164). Aspirin-induced secretion of PGD_2 was abrogated after successful aspirin desensitization therapy (165). Aspirin by itself was found to activate blood eosinophils in terms of Ca^{2+} flux, degranulation, and CD11b upregulation, the latter being more pronounced in AERD patients (166, 167). These effects were reversed by PGE_2. We observed that the expression of the EP4 receptor in blood eosinophils tended to be reduced in AERD patients, and inhibition of eosinophil chemotaxis by PGE_2 or an EP4 agonist was less pronounced in AERD patients as compared to healthy controls (168). Single nucleotide polymorphisms of the *ptger2* and *ptger4* were detected in aspirin-intolerant Korean patients, predicting lower EP2 and EP4 receptor expression levels (169, 170). A single nucleotide polymorphism in the DP2 gene *crth2* was also observed to correlate with increased levels of the eosinophil chemoattractant, eotaxin-2 in Korean AERD patients (171). Similarly, the prevalence of a *crth2* single nucleotide polymorphism was found to be increased in a female Japanese AERD patient cohort (172). These findings suggest that targeting PGE_2 and PGD_2 receptors might provide potential novel treatment options for AERD. Whether these genetic alterations specifically contribute to AERDS pathophysiology is still unclear, as similar finding have also been made for allergic disease and asthma (173).

Miscellaneous

Eosinophil infiltration into tumor-surrounding areas is observed in various types of cancer (174). The presence of tumor-associated tissue eosinophils (TATEs) seems to beneficially influence the prognosis of oral squamous cell carcinoma and other types of cancer. Davoine et al. have shown that eosinophil lysates inhibit the growth of the oral squamous carcinoma cells line (SCC-9) *in vitro* and correlates with the amount of released eosinophil peroxidase. Inhibition of HPGDS by HQL-79 in oral squamous cell carcinoma abrogated the migration of eosinophils toward the tumor cells. These results suggest an antitumor activity of PGD_2 *via* the activation of release of eosinophil peroxidase from, or by cytolysis of, eosinophils (175). By using HPGDS-deficient mice, Murata et al. have shown that mast cell-derived PGD_2 is an antiangiogenic factor in lung carcinoma (176). Therefore, stimulating the HPGDS/PGD_2 axis could be a beneficial strategy in cancer, with TATEs serving as an additional biomarker.

Eosinophils have been shown to play a significant role in inflammatory bowel disease, ulcerative colitis, and Crohn's disease (13, 177, 178). We have shown in experimental Crohn's disease that eosinophils contribute to intestinal inflammation *via* activation of DP2. Timapiprant inhibited the recruitment of eosinophils into the colon, reduced intestinal inflammation, and decreases cytokine levels (TNFα, IL-1β, IL-6) in mice. In Crohn's patients, PGD_2 and Δ^{12}-PGJ_2 levels were increased as compared to control individuals (179). In a subsequent study, increased expression of LPGDS in myenteric and submucosal neurons, and enhanced PGD_2 release, was observed in tissue samples from colon of patients with active Crohn's disease (180). In ulcerative colitis, we observed opposing effects of DP1 and DP2 as blockade of DP2 improved, whereas a DP1 antagonist worsened, inflammation in a mouse model of colitis (26). In ulcerative colitis patients, DP2 expression was downregulated on peripheral blood eosinophils, while DP1 was upregulated, and both findings correlated with disease activity. Biopsies of colitis patients revealed an increase of DP2-positive cells in the colonic mucosa and high DP2 protein content. Both PGD_2 and PGE_2 levels were elevated in serum of colitis patients (26). Eosinophils and macrophages

were suggested to be the main source of PGE_2 in colitis (181). Current literature suggests that, like in allergy, PGE_2 through its EP4 receptor opposes the pro-inflammatory action of PGD_2 in inflammatory bowel disease and plays a protective role in mouse models of colitis (182–184). In contrast, a large body of evidence supports EP4 receptors to predominantly mediate the overall pro-tumorigenic action of PGE_2 (185). Whether inhibition of eosinophil function is involved in the anti-inflammatory and pro-tumorigenic roles of the EP4 receptor in the gut has not been investigated yet.

CONCLUDING REMARKS

Accumulating data suggest that the DP2 receptor is an important activator of eosinophils, as it does not only respond to its cognate ligand, PGD_2, but also to most of its metabolites, and even unrelated prostanoid species. PGD_2 is generated by a large variety of immune cells under different conditions. Among other leukocytes, eosinophils are probably the most important DP2-bearing cells. Thus, it is believed that DP2, and to some extent also DP1, crucially contribute to various pathologies that involve eosinophils, and provide novel therapeutic approaches to conditions such as asthma, allergic rhinitis, conjunctivitis, esophagitis and skin disease, nasal polyposis, aspirin-intolerance, Crohn's disease, and certain types of cancer. In contrast, PGE_2 transmits inhibitory signals onto eosinophils through EP2 and EP4 receptors, and is thus a natural antipode to its isomer, PGD_2. For instance, HPGDS expression is enhanced, while microsomal PGE_2 synthase is decreased in chronic rhinosinusitis that results in eosinophilic inflammation favoring polyp formation (186). In asthma patients, a decrease of PGE_2 as compared to other prostanoids including PGD_2 correlates with airway obstruction (187). Similar findings are typical for AERD. An imbalance of PGD_2/PGE_2 secretion might hence potentially underlie and/or sustain the abovementioned, eosinophilic pathologies, and might constitute novel therapeutic targets.

AUTHOR CONTRIBUTIONS

MP, ES, and AH wrote and edited the review.

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REVIEW
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Cysteinyl Leukotrienes in Eosinophil Biology: Functional Roles and Therapeutic Perspectives in Eosinophilic Disorders

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Cysteinyl leukotrienes (cysLTs), LTC4, and its extracellular metabolites, LTD4 and LTE4, have varied and multiple roles in mediating eosinophilic disorders including host defense against parasitic helminthes and allergic inflammation, especially in the lung and in asthma. CysLTs are known to act through at least 2 receptors termed cysLT1 receptor (CysLT1R) and cysLT2 receptor (CysLT2R). Eosinophils contain a dominant population of cytoplasmic crystalloid granules that store various preformed proteins. Human eosinophils are sources of cysLTs and are known to express the two known cysLTs receptors (CysLTRs). CysLTs can have varied functions on eosinophils, ranging from intracrine regulators of secretion of granule-derived proteins to paracrine/autocrine roles in eosinophil chemotaxis, differentiation, and survival. Lately, it has been recognized the expression of CysLTRs in the membranes of eosinophil granules. Moreover, cysLTs have been shown to evoke secretion from isolated cell-free eosinophil granules operating through their receptors expressed on granule membranes. In this work, we review the functional roles of cysLTs in eosinophil biology. We review cysLTs biosynthesis, their receptors, and argue the intracrine and paracrine/autocrine responses induced by cysLTs in eosinophils and in isolated free extracellular eosinophil granules. We also examine and speculate on the therapeutic relevance of targeting CysLTRs in the treatment of eosinophilic disorders.

Keywords: eosinophils, leukotrienes, granules, cytokine, cysleukotrienes

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INTRODUCTION

Lipid mediators such as leukotrienes (LTs) possess multiple cell targets and immunologic functions in different pathological and physiological conditions. LT biosynthesis is initiated throughout the activation of cells, when arachidonic acid (AA) is released from the membrane phospholipids by a calcium-dependent cytosolic phospholipase A2 (1, 2). Free AA is metabolized enzymatically to eicosanoids through at least two major pathways, namely cyclooxygenase (COX) and lipoxygenase (LO) pathways. In the COX pathway, AA is metabolized to prostaglandin H2, which is further metabolized to prostaglandins and thromboxanes by particular prostaglandin and thromboxane synthases. In the LO pathway, AA is metabolized to 8-, 12- and 15-hydroperoxyeicosatetraenoic (HPETE) acids by 12- and 15-LO or to 5-HPETE by 5-LO and 5-lipoxygenase-activating protein (FLAP). FLAP presents AA to 5-LO, which catalyzes the formation of 5-HPETE (1–3). 5-HPETE forms LTA4, which is unstable and rapidly metabolized either to produce LTB4 by the act of LTA4 hydrolase (LTA4-H) or to generate LTC4 by the action of LTC4 synthase (LTC4-S). LTC4 is further enzymatically converted to LTD4 and LTE4 (1, 2) (**Figure 1**).

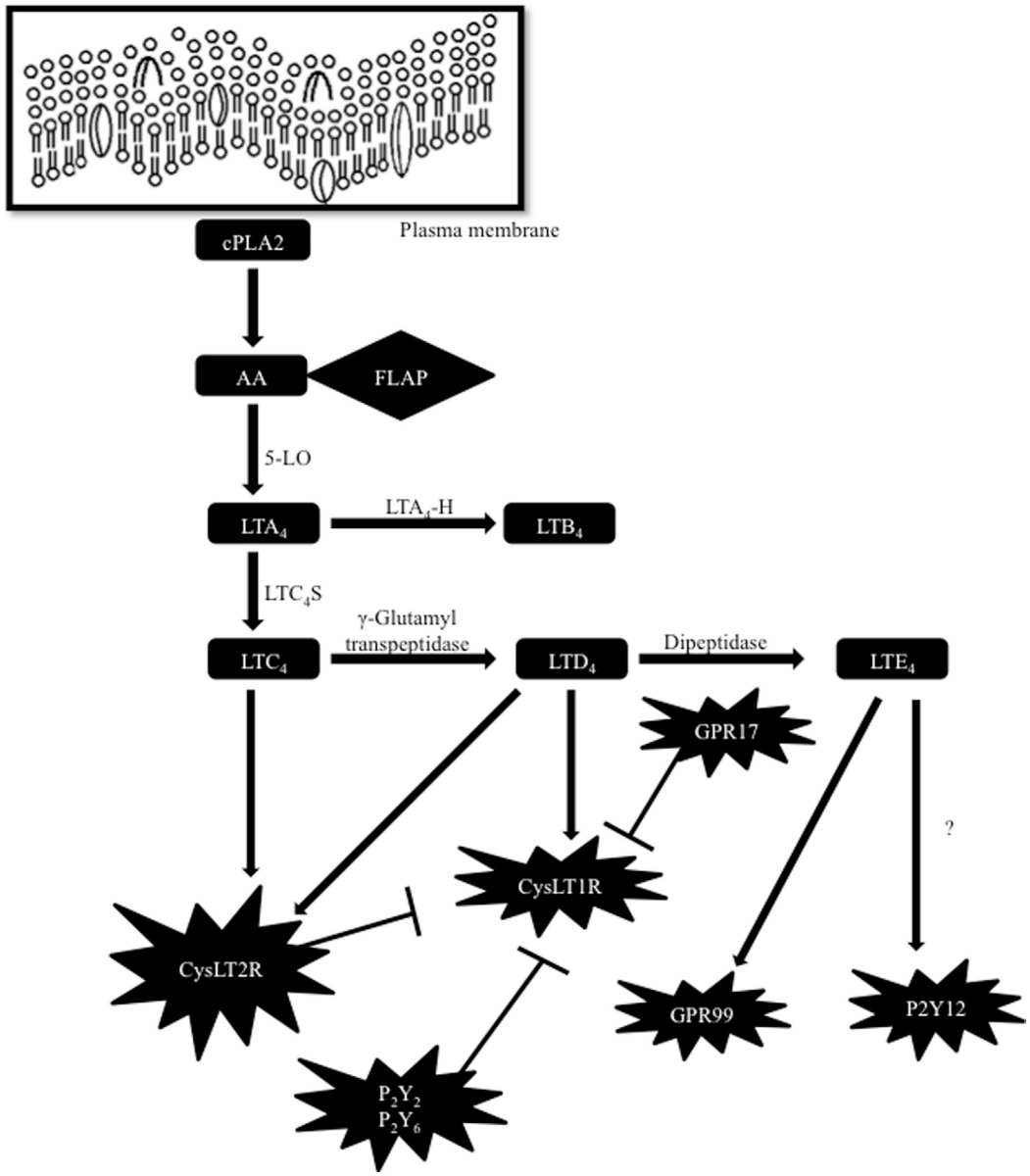

FIGURE 1 | Biosynthetic pathway of cysteinyl leukotrienes (cysLTs) and cross regulation of their receptors. Arachidonic acid (AA) is released from the plasma membrane by a cytosolic phospholipase A2 (cPLA2). To form cysLTs, 5-lipoxygenase-activating protein (FLAP) presents AA to 5-lipoxygenase (5-LO) leading to the formation of leukotriene (LT) A4. LTA4 is rapidly metabolized either to produce LTB4 by the act of LTA4 hydrolase (LTA4-H) or to generate LTC4 by the action of LTC4 synthase (LTC4-S). LTC4 is further enzymatically transformed to LTD4 and LTE4. CysLT2R or GPR17 and PKC-dependent phosphorylation by P2Y receptors inhibit CysLT1R function. P2Y12 receptor (P2Y12R) was primarily identified as a LTE4 ligand, but other studies have suggested that LTE4 does not activate intracellular signaling by acting through P2Y12R. More recently, GPR99 has been suggested as a new receptor sensitive to LTE4.

Although the biosynthesis of the cysteinyl leukotrienes (cysLTs) mainly occurs in cell-specific compartments, such as the nuclear envelope (4) and specific intracellular organelles called lipid bodies (5) (cytoplasmic organelles rich in lipids that have functions in lipid mediator production), other alternate routes have also been observed in different cells. In eosinophils, basophils, mast cells, and macrophages, LTC4S conjugates LTA4 to reduced glutathione, forming LTC4. Once formed, LTC4 is transported extracellularly *via* the ATP-binding (ABC) proteins and then metabolized to LTD4 and LTE4 by γ-glutamyl transpeptidases and dipeptidases, respectively (2). This process is named cysLTs transcellular biosynthesis and also occurs in other cells, such as endothelial cells, platelets, and even neuronal and glial cells. These cells lack the enzymes to produce LTA4, but they use the LTA4 from the surrounding neutrophils and produce LTC4 [for review, see Ref. (1)].

LTC4, LTD4, and LTE4 are the main ligands for the G-protein-coupled receptors (GPCRs) cysteinyl leukotrienes

type 1 (CysLT1R) and type 2 (CysLT2R) receptors. The rank of order is LTD4 > LTC4 > LTE4 by means of their affinity toward CysLT1R (6), whereas CysLT2R binds LTC4 and LTD4 with an affinity one-log less than CysLT1R (binding rank order LTD4 = LTC4 > LTE4) (7). CysLT1R, a high-affinity receptor for LTD4, is expressed in bronchial smooth muscle and substantially in eosinophils, macrophages, and mast cells and is the target of antagonists (montelukast, zafirlukast, and pranlukast) (6). CysLT2R is resistant to montelukast, and is expressed both on cells that also express CysLT1R (e.g., leukocytes) and other tissues.

Different studies have proposed the existence of another cysLT receptor (CysLTR), since several of the cell functions evoked by cysLTs are not well explained by the current knowledge of CysLTRs (8–14). For example, studies performed in mice and humans suggested that LTE4, known as the weakest CysLTRs activator, has biological effects that cannot be elucidated based on its currently accepted affinity to CysLT1R and CysLT2R (11–13). In fact, the purinergic P2Y12 receptor (P2Y12R) has been suggested as a different receptor responsive to LTE4 based on *in vitro* and *in vivo* studies (15, 16). In contrast, different investigations have suggested that cysLTs, including LTE4, do not trigger P2Y12R-mediated intracellular signaling. So, another receptor sensitive to LTE4 has yet to be recognized (17). More recently, a potential new receptor for LTE4 was identified and reported as an oxyglutarate receptor named GPR99 (18) (**Figure 1**). Current knowledge of CysLT1R and CysLT2R also reveal that CysLTR functions have many non-canonical modulation pathways. Now it is known that CysLT1R can be regulated by indirect or direct physical interactions with other GPCRs. For instance, protein kinase C activation by the purinergic P2Y2 and P2Y6 receptors can induce phosphorylation and desensitization of CysLT1R, when these receptors are coexpressed in cell lines, without causing CysLT1R internalization (19). Moreover, in human mast cells, CysLT1R and CysLT2R heterodimerize (20), limiting the levels of membrane expression of CysLT1R as well as its functional signaling capacity. GPR17, a GPCR homologous to CysLT1R and CysLT2R, was first characterized as a dual-specific receptor for cysLTs and uracil nucleotides (21). Nevertheless, in further studies, it was revealed that GPR17 operates as a negative regulator of CysLTR1 activation induced by LTD4 and distinctly reduces binding of LTD4 in cells that express both classes of receptors (22) (**Figure 1**). Thus, more investigations are needed in order to better understand the many unpredictable responses obtained in the studies with cysLTs. Potentially, many other direct or indirect interactions, that are still unknown, may exist among CysLTRs and other GPCRs.

Currently, eosinophils are defined as multifunctional cells that have long been related to allergy and host parasite responses. They are immunomodulatory cells that contribute both in innate and adaptive immune responses *via* the selective secretion of different cytokines and other mediators. CysLTs and CysLTRs have significant roles in allergic conditions and are valuable pharmacological therapeutic targets for the control of asthma and other eosinophilic diseases [for review, see Ref. (23)]. Human eosinophils are main producers of cysLTs and express both CysLT1R and CysLT2R on their cell plasma membranes (2, 24). Among other GPCRs capable of potentially responding to cysLTs or interacting with CysLTRs, it is now recognized that eosinophils express the P2Y2R, P2Y6R [for review, see Ref. (25)], P2Y12R (26), and the GPR99 (27). However, the functional roles of these receptors as regulators of CysLTRs in eosinophils are still not known. The expression of GPR17 in eosinophils has not been identified so far.

Mature human eosinophils are easily differentiated by the abundant presence of secretory granules termed crystalloid, secondary, or even specific granules (28). Eosinophils are also characterized by a vesicular system and lipid bodies, in which various lipid mediators are synthesized. Within eosinophils, synthesis of LTC4 (but not LTD4 or LTE4) occurs at perinuclear membranes and in cytoplasmic lipid bodies (24, 29). Eosinophil crystalloid granules present a unique morphology with a central crystalline core compartment surrounded by a matrix, which is delimited by a trilaminar membrane. These granules express different receptors in their wrapping trilaminar membrane and store a large number of preformed proteins such as cytotoxic cationic proteins and many cytokines and chemokines. Human eosinophils synthesize and store cationic proteins such as eosinophil peroxidase, eosinophil cationic protein (ECP), eosinophil granule major basic protein, and eosinophil-derived neurotoxin (EDN). They also biosynthesize, store, and selectively secrete growth factors, enzymes, chemokines (such as eotaxin and RANTES), and over more than three dozen cytokines in response to different stimuli (28, 30–35). Piecemeal degranulation (PMD), a process by which granule contents are selectively mobilized into vesicles that arise from the granules and fuse with the plasma membrane to extracellularly release their cargo, is the major mechanism of intact eosinophil granule protein secretion (32, 36). A different mechanism of human eosinophil "degranulation" is known as cytolysis, which involves damage of eosinophil cell membrane integrity, release, and deposition of cell-free membrane-bound crystalloid granules to the extracellular microenvironment. Even though PMD is recognized as the main mechanism operating during eosinophil protein secretion, cytolysis has been considered the main mechanism underlying the release and tissue deposition of intact, membrane-bound free eosinophil granules observed in different eosinophilic diseases. Exocytosis, whereby the entire granules fuse with the plasma membrane releasing their content extracellularly, has been considered a more unusual mechanism of eosinophil secretion and it is not usually observed *in vivo* (35, 37).

FUNCTIONAL ROLES OF cysLTs IN EOSINOPHIL BIOLOGY

Over the last years, a number of mediators (cytokines, chemokines, growth factors, alarmins, and lipid mediators) involved in the regulation of eosinophil recruitment, degranulation, survival, and other functions have been identified. A rising bulk of data has revealed essential roles of cysLTs in regulating different eosinophil functions.

It has been reported that cysLTs display eosinophilotactic activity *in vitro via* CysLT1R (38–40) (**Figure 2**). These studies revealed that LTD4 may act as a potent and selective

eosinophilotactic factor at physiological concentrations (38) and in directly increasing Mac-1 expression in a mechanism dependent on CysLT1R (39). Further data also show that LTD4 induced eosinophil transendothelial migration across human umbilical vein endothelial cells in a Pranlukast (a CysLT1R antagonist) dependent manner (40). *In vivo*, involvement of cysLTs in eosinophil influx was firstly demonstrated in guinea pigs in the 90s (41), and further in humans (42). Subsequently, these finding were reinforced by the effects of CysLT1R antagonists in inhibiting eosinophil recruitment in airway allergic inflammation (43, 44). Recently, roles for LTC4 in mediating eosinophil trafficking from lungs to paratracheal lymph nodes in experimental allergic asthma were described (45).

Regarding eosinophil secretory functions, published data show that LTD4 induced eosinophil ROS generation and EDN release. Pranlukast significantly inhibited EDN release, although the inhibitory effect on ROS generation was partial (40). In a different study, cysLTs induced the release of IL-4 from human cord blood progenitor derived-eosinophils in a dose- and time-dependent manner (46) (**Figure 2**).

CysLT1R antagonists also appear to play a role in limiting IL-5-responsive eosinophilopoeisis, since cysLTs and IL-5 act together at several stages of eosinophil differentiation and maturation during upper airway allergic inflammation (47). In addition, cysLTs also appear to enhance the *in vitro* survival of human eosinophils by activation of CysLT1R (48, 49) (**Figure 2**). Though it has been demonstrated that eosinophils isolated from asthmatic patients can have their apoptosis postponed by cysLTs, controversial data suggest that the cysLTs, despite raising intracellular calcium, are unable to prolong survival of eosinophils isolated from normal individuals or mildly atopic patients (50).

It is well established that eosinophils are major sources of cysLTs (24). Beyond their functions as paracrine mediators, cysLTs are now also known to exhibit autocrine and likewise intracrine effects. Lee and colleagues provided evidence for the involvement of an autocrine cysLT pathway that is involved in eosinophil survival in response to GM-CSF (48). Interestingly, it is also described that LTC4 can be synthesized in different intracellular compartments (nuclear membrane or lipid bodies) and may function as intracrine regulators of selective granule protein secretion (5, 51, 52). In 2002, Bandeira-Melo and colleagues (51) demonstrated that eotaxin (CCL11) stimulates human eosinophil to secrete IL-4 by PMD in a lipid body-generated LTC4-dependent mechanism. The authors also showed that 5-LO blockers inhibited the IL-4 secretion. In this way, the intracellular-formed LTC4 would function as an intracrine signaling molecule, mediating CCR3-induced IL-4 release (**Figure 3**). Exogenous LTC4 and LTD4 at low concentrations induced IL-4 release (but not RANTES) only after membrane permeabilization. Inhibitors of the CysLT1R and CysLT2R did not block LTC4-elicited IL-4 release suggesting that LTC4, *via* an intracellular CysLTR distinct from CysLT1R and CysLT2R, may also function as an intracrine mediator capable to trigger cytokine secretion *via* PMD. Another work that explored leukotrienes as possible intracrine mediators of eosinophils' PMD is a study published by Tedla and colleagues (52). The authors showed that the cross-linking of immobilized antibodies and CD9 and leukocyte immunoglobulin-like receptor 7 (LIR7) stimulates human eosinophil to secrete IL-12 (but not IL-4) by PMD and to generate LTC4 at perinuclear regions (52). However, pretreatment of eosinophils with two different inhibitors of 5-LO did not inhibit this selective release of IL-12 (52). These findings indicate that CD9- or LIR7-induced selective

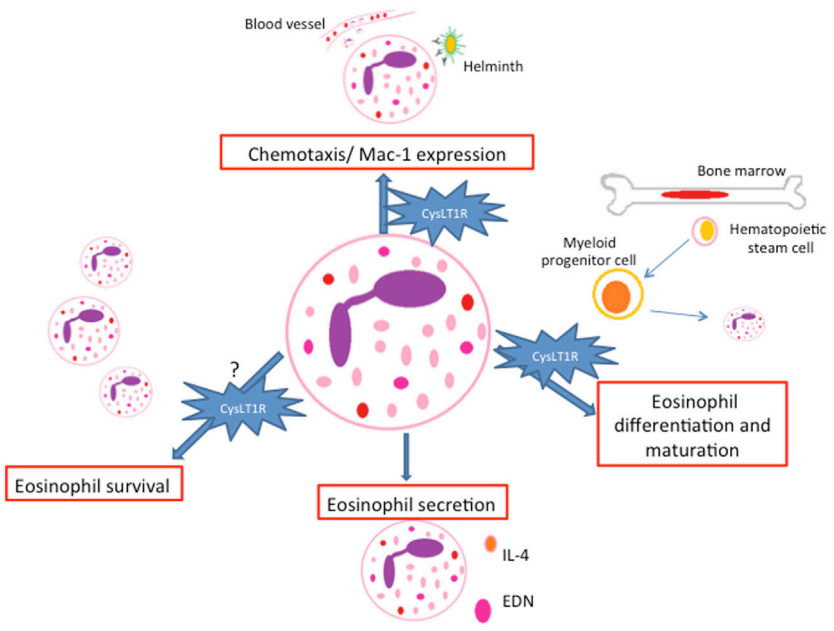

FIGURE 2 | Paracrine/autocrine responses evoked by cysteinyl leukotrienes (cysLTs) in eosinophils. CysLTs mediate different eosinophil functions such as chemotaxis, eosinophil differentiation and maturation, survival and protein secretion, most of them *via* the CysLT1R. EDN, eosinophil-derived neurotoxin.

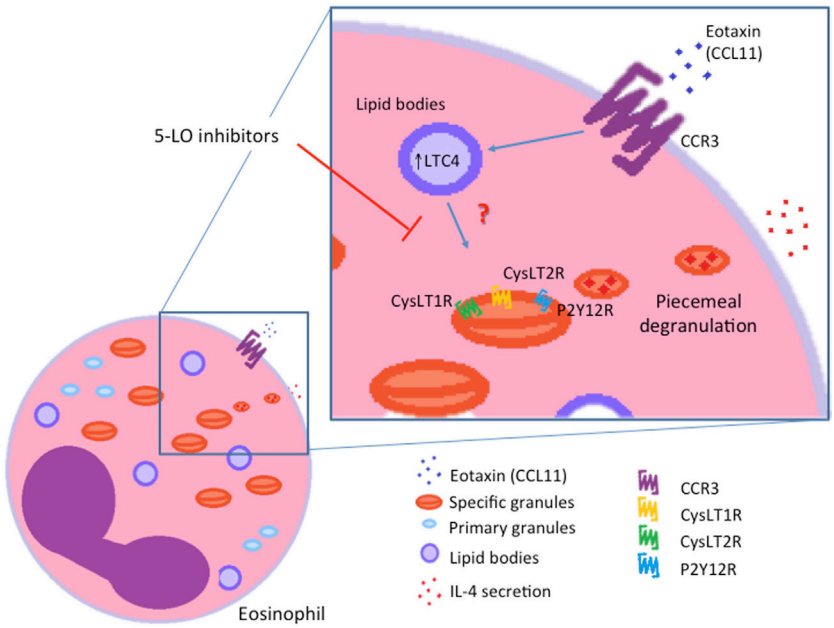

FIGURE 3 | Intracrine actions of cysteinyl leukotriene (cysLTs). IL-4 release induced by eotaxin (CCL11) is dependent on the intracrine action of lipid body-generated LTC4. Inhibitors of 5-lypoxigenase (5-LO) blocked intracellular LTC4 production and consequently IL-4 release from eosinophils. CysLT1R, cysLT1 receptor; CysLT2R, cysLT2 receptor; P2Y12R, purinergic P2Y12 receptor.

IL-12 release is not dependent on 5-LO action. Based on these studies, it has been suggested that lipid body-generated LTC4 may relate less to paracrine mediator formation and more to intracrine signaling functions. However, more studies are still needed in order to better clarify this point.

Although eosinophils express different GPCRs capable of potentially responding to cysLTs or interacting with CysLTRs, little is known about the intracellular distribution of these receptors in eosinophils. Recently, the expression of cysLT-responsive receptors has been recognized on the delimiting trilaminar membrane of intracellular crystalloid eosinophil granules. These receptors function mediating cysLT-evoked secretion from cell-free eosinophil granules protein content (26, 53). However, whether these receptors have roles when these granules are in the cytoplasmic microenvironment it is not known.

FUNCTIONAL ROLES OF cysLTs IN CELL-FREE FUNCTIONAL EXTRACELLULAR EOSINOPHIL GRANULES

Intracrine roles for cysLTs have been reported; however, the possible mechanisms that can elucidate the intracellular activities of cysLTs remain unknown (51, 54, 55). Recently, our group demonstrated that free eosinophil granules express CysLT1R and CysLT2R and the P2Y12R on their membranes (26). In addition, formerly, it was demonstrated that eosinophil granules are enriched sites of various cytokine and chemokine receptors (31, 53, 56); and that these granules, upon extrusion from eosinophils, responded to CCL11 and IFN-γ, through their granule membrane-expressed receptors. The activation of the receptors triggered signaling pathways within granules that promote protein secretion (53, 57). Isolated free eosinophil granules stimulated with cysLTs secrete ECP, but not chemokines or cytokines. CysLT1R or P2Y12R blockage inhibited the eosinophil granule ECP secretion. The capacity of both CysLT1R and the P2Y12R antagonists to similarly inhibit ECP secretion elicited by cysLTs, including LTE4, might suggest functional heterodimerization or cross regulation of CysLT1R with other GPCRs. However, so far this is not clearly defined. Remarkably, the dose response to the three cysLTs differed. LTC4 and LTE4 induced ECP release only at subnanomolar concentrations, which was coherent with the GPCRs' typical high-dose inhibition. Interestingly, LTD4 induced ECP secretion at low and high concentrations. At intermediate concentrations, LTD4 was unable to promote granule ECP secretion. As mentioned earlier, whether dimerization or cross regulation of GPCRs are involved in this response remains to be elucidated. However, considering the variable results of studies with cysLTs, what is certain is that there are pieces to this puzzle that are still missing. These studies highlight the ability of cysLTs to evoke isolated free granule secretory functions. Moreover, for granules functioning as cytoplasmic organelles, these studies reveal new mechanisms by which LTC4 and extracellularly formed LTD4 and LTE4 (after cellular uptake) may operate as intracrine signaling molecules capable to induce eosinophil granule protein secretion. Nevertheless, this is no evidence that the CysLTRs or the P2Y12R present on the trilaminar granule membranes participate in the intracrine cysLTs' actions reported earlier (51) (**Figure 3**). So far, more studies are needed in order

to elucidate whether the eosinophil granule membrane-expressed receptors mediate intracrine actions of cysLTs.

TARGETING CysLTRs IN THE TREATMENT OF EOSINOPHILIC DISORDERS: CONCLUDING REMARKS AND QUESTIONS FOR THE FUTURE

Among eosinophilic disorders, the CysLT1R blockers (zafirlukast, montelukast, and pranlukast) are mainly used in the management of some chronic respiratory diseases, particularly allergic rhinitis and bronchial asthma. In fact, in the management of asthma, the current clinical data are in favor of their use as an add-on or alternative therapy to inhaled corticosteroids (58, 59). Clinical trials evaluating zafirlukast, montelukast, and pranlukast have shown a decrease of eosinophil count in blood and airways of asthmatic patients (60, 61). However, other studies (62, 63) suggest that the development of dual CysLT1/2R antagonists might bring additional advantages to the asthma treatment over the current used CysLT1R blockers. In fact, patients with chronic persistent asthma presented superior improvement in lung function when treated with a cysLT synthesis inhibitor compared to a CysLT1R antagonist (62). However, recently, a clinical study with a dual CysLT1/2R blocker, the compound ONO-6950, in non-smoking subjects with mild allergic asthma, showed no additional benefits of this therapeutic strategy to the treatment of asthma (64).

Besides the two classic receptors for cysLTs (CysLT1R and CysLT2R), there remain important questions regarding the potential clinical implications of novel receptors for cysLTs or the cross regulation of CysLT1R. Current knowledge is the only beginning to understand the molecular pharmacology of the receptors sensitive to CysLTRs, their capacity to cross regulate or to signalize as dimers. So far, physiological and pharmacological reports have shown a great complexity and functional variation of the cysLT system. Important questions remain about the regulation of the CysLT1R by other GPCRs and its potential clinical relevance. For instance, considering that CysLT2R and GPR17 negatively regulate CysLT1R function, is it likely that functional diversification in each receptor could impact clinical relevance to CysLT1R antagonists? Other intriguing analysis can be performed regarding the purinergic receptors versus their sensitivity to CysLT1R antagonists, and their capacity to induce desensitization of the CysLT1R. Is it thinkable that the capacity of CysLT1R antagonists to inhibit these presumed negative regulators of the CysLT1R could (i) impair the benefits of their use, (ii) reduce the potential therapeutic benefit for some patients, and (iii) explain some of the heterogeneity of response to these agents? Moreover, considering the increased sensitivity of asthmatic patients to LTE4, and the fact that LTE4 has a long half-life and is abundantly found in asthmatic patients, would GPR99 blockers be in the horizon as an innovation for asthma treatment? To end, are cell-free secretory extracellular eosinophil granules new therapeutic targets beyond intact eosinophils for all these antagonists? So far, these and other questions remain unanswered.

AUTHOR CONTRIBUTIONS

GT-S, IG, and JN conducted a review of the literature. GT-S and JN wrote the manuscript.

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Eosinophil Activation Status in Separate Compartments and Association with Asthma

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Asthma is frequently characterized by eosinophil-rich airway inflammation. Airway eosinophilia is associated with asthma exacerbations and likely plays a part in airway remodeling. Eosinophil recruitment from the bloodstream depends on circulating eosinophils becoming activated, which leads to eosinophil arrest on activated endothelium, extravasation, and continued movement through the bronchial tissue by interaction with the extracellular matrix (ECM). Circulating eosinophils can exist at different activation levels, which include non-activated or pre-activated (sensitized or "primed"). Further, the bloodstream may lack pre-activated cells, due to such eosinophils having arrested on endothelium or extravasated into tissue. Increased expression, and in some instances, decreased expression of cell-surface proteins, including CD44, CD45, CD45R0, CD48, CD137, neuropeptide S receptor, cytokine receptors, Fc receptors, and integrins (receptors mediating cell adhesion and migration by interacting with ligands on other cells or in the ECM), and activated states of integrins or Fc receptors on blood eosinophils have been reported to correlate with aspects of asthma. A subset of these proteins has been reported to respond to intervention, e.g., with anti-interleukin (IL)-5. How these surface proteins and the activation state of the eosinophil respond to other interventions, e.g., with anti-IL-4 receptor alpha or anti-IL-13, is unknown. Eosinophil surface proteins suggested to be biomarkers of activation, particularly integrins, and reports on correlations between eosinophil activation and aspects of asthma are described in this review. Intermediate activation of beta1 and beta2 integrins on circulating eosinophils correlates with decreased pulmonary function, airway inflammation, or airway lumen eosinophils in non-severe asthma. The correlation does not appear in severe asthma, likely due to a higher degree of extravasation of pre-activated eosinophils in more severe disease. Bronchoalveolar lavage (BAL) eosinophils have highly activated integrins and other changes in surface proteins compared to blood eosinophils. The activation state of eosinophils in lung tissue, although likely very important in asthma,

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Abbreviations: ADAM, a disintegrin and metalloproteinase; BAL, bronchoalveolar lavage; βc, common β chain (of IL-3, IL-5, and GM-CSF receptors); C, complement; CD, cluster of differentiation; ECM, extracellular matrix; Fc, fragment, crystallizable (of immunoglobulin); FENO, fraction of exhaled nitric oxide; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; GLP, glucagon-like peptide; GM-CSF, granulocyte macrophage-colony stimulating factor; HLA, human leukocyte antigen; ICAM, intercellular adhesion molecule; IGSF, immunoglobulin superfamily member; IL, interleukin; ILA, induced by lymphocyte activation; LAMP, lysosome-associated membrane protein; LPS, lipopolysaccharide; mAb, monoclonal antibody; PC_{20}, provocative concentration of methacholine or histamine producing a 20% fall in FEV_1; PD-L, programmed death ligand; PSGL, P-selectin glycoprotein ligand; R, receptor; ROC, receiver–operator characteristic; TNFRSF, tumor necrosis factor receptor superfamily member; VCAM, vascular cell adhesion molecule.

is largely unknown. However, some recent articles, mainly on mice but partly on human cells, indicate that tissue eosinophils may have a surface phenotype(s) different from that of sputum or BAL eosinophils.

Keywords: eosinophils, activation, asthma, integrins, interleukin-5

INTRODUCTION

Asthma is often characterized by eosinophil-rich airway inflammation (1–8). Such eosinophilic inflammation is associated with exacerbations and appears to participate in airway remodeling (1, 8–14). Eosinophil recruitment from the bloodstream depends on circulating blood eosinophils becoming activated, which leads to eosinophil arrest on activated endothelium, extravasation, and continued movement through the bronchial tissue and lumen by interaction with the extracellular matrix (ECM) (2, 8, 15–17). Circulating eosinophils can exist in different states, including non-activated, or pre-activated or "primed" (8, 18). Moreover, the bloodstream may lack pre-activated cells, due to such eosinophils having extravasated (8). Increased expression, and in some instances, decreased expression of cell-surface proteins and activated states of integrins or Fc receptors on blood eosinophils have been reported to correlate with asthma (8, 19). Some of these proteins have been reported to respond to intervention (19).

This review will discuss eosinophil surface proteins proposed to be biomarkers of eosinophil activation and evidence for associations between the activation status of eosinophils and aspects of asthma. The search strategy is described in the notes for **Table 1**. Further, this review will discuss a subset of these proteins that appears to be downregulated or less activated on circulating eosinophils in severe asthma or after whole-lung antigen challenge. Finally, it will discuss how some eosinophil surface proteins respond to pharmaceutical intervention. A model of eosinophil activation status, focusing on integrins, in the circulation and the airway will also be presented.

EOSINOPHIL SURFACE PROTEINS ALTERED AFTER ANTIGEN CHALLENGE, IN THE AIRWAY, OR IN ASTHMA

Upregulation or downregulation of eosinophil surface proteins and activated conformations of integrins and Fc receptors have been proposed to be biomarkers of eosinophil activation, in many cases due to the reaction of eosinophils to various stimuli *in vitro* (2, 8, 17–19, 58–61). Many, but not all, i.e., not all that change in response to *in vitro* stimulation, of these surface proteins have been reported to be altered on blood eosinophils after whole-lung or segmental lung antigen challenge, or on bronchoalveolar lavage (BAL), or sputum eosinophils (**Table 1**). In addition, the surface proteins may be altered on blood eosinophils in asthma or in a manner that correlates with features of asthma (**Table 1**) (19). Segmental and whole-lung antigen challenge are models of allergic airway inflammation (62) and asthma exacerbation (63), respectively. Up- or downregulation in **Table 1** refers to changed or different protein expression of a cell surface protein, which usually has been determined by flow cytometry. Further, alterations are listed independently of what the mechanism may be, e.g., translocation to the surface from intracellular granules or the effect of increased transcription or protein synthesis and may consist of an alteration in mean or median expression on all eosinophils or an alteration in the percentage of expressing eosinophils (8, 19). Some references have studied purified cells, while others have used whole blood, BAL, or sputum cells. An unfractionated sample is beneficial in that just a small volume or number of cells is needed and that isolated cells *in vitro* may be different and more activated than cells *in vivo* (8, 19, 64, 65). Regarding more detailed information about individual proteins, please see Ref. (8).

Several proteins, including CD45, CD45R0, CD48, CD137, IL-17 receptor (R) A and B, α_L integrin, and some of the Fc receptors, are increased or decreased on circulating eosinophils in asthma compared to normal, non-allergic healthy individuals (**Table 1**) (8, 19). One specific example is that IL-17R and B (subunits of IL-25R) are increased in patients with non-severe allergic asthma but not in non-asthmatic patients with atopy (8, 19, 49). In the case of some proteins, reports are conflicting. For instance, some workers reported FcγRIII (CD16) to be increased on blood eosinophils in allergic asthma (or allergic rhinitis) (46) (**Table 1**), while other authors reported no alterations in airway allergies when compared to control subjects (8, 19, 66).

The expression level of a particular protein may not only be an effect of the eosinophil having been exposed to cytokines or other stimuli but may also partly result from actions of regulatory factors *in vivo*. One recent example of such a factor that may regulate eosinophil activation is glucagon-like peptide (GLP)-1, a member of the incretin family of hormones, which regulates glucose metabolism (47). A GLP-1 analog inhibited upregulation of α_M integrin and CD69 *in vitro* in response to lipopolysaccharide (47). Further, expression of GLP-1 receptor was lower on blood eosinophils in patients with allergic asthma than in normal controls (**Table 1**). The lower level of GLP-1R in asthma than in healthy subjects indicates that the eosinophil response to activating stimuli may be more regulated by GLP-1 in healthy persons and that eosinophil activation may be more easily achieved in asthma than in health. Further, Mitchell and colleagues suggest that GLP-1 agonists may have additional indications in treating patients with concomitant type 2 diabetes mellitus and asthma (47).

ASSOCIATIONS WITH ASPECTS OF ASTHMA

Expression and activation of some proteins have been found to be associated with clinical findings of asthma (**Table 1**) (8, 17, 19). Activated β_1 integrin, specifically the intermediate-activity conformation recognized by monoclonal antibody (mAb) N29, on blood eosinophils correlates inversely with lung function in non-severe asthma (52, 54), or directly with the magnitude

of the late-phase reaction in mild allergic asthma (36) or with exhaled NO [fraction of exhaled nitric oxide (FENO)], which reports airway inflammation, after inhaled corticosteroid (ICS) withdrawal (54). In addition, by receiver–operator characteristic (ROC) analysis, β_1 integrin activation, assessed with N29, predicts lowered pulmonary function in mild asthmatic patients (54). Intermediate-activity β_2, reported by the antibody KIM-127, is associated with the percentage of BAL eosinophils in patients with mild allergic asthma (34). Finally, activation of FcγRII (CD32) correlates with FENO in asthma (18).

TABLE 1 | Eosinophil surface proteins altered after antigen challenge or in the airway, or associated with asthma or aspects of asthma.

Protein	Observation	Reference
CD35 (CR1)	Downregulated in bronchoalveolar lavage (BAL)	(20)
CD44	Upregulated after segmental lung antigen challenge, in BAL, or in sputum	(21, 22)
CD45	Upregulated in asthma	(23)
CD45R0	Upregulated in asthma or mild-moderate asthma	(23, 24)
CD48	Upregulated in moderate asthma	(25, 26)
CD58	Upregulated in BAL	(27)
CD63 (lysosome-associated membrane protein 3)	Upregulated in BAL or sputum	(27, 28)
CD66b (CEACAM8)	Upregulated in sputum	(28)
CD66e (CEACAM5)	Upregulated after segmental lung antigen challenge or in BAL	(22)
CD67	Upregulated in BAL	(27)
CD69	Upregulated after whole-lung antigen challenge, in BAL, or in sputum	(28–32)
CD137 (tumor necrosis factor receptor superfamily member 9, induced by lymphocyte activation, 4-1BB)	Upregulated in asthma	(33)
CD274 (programmed death ligand 1)	Upregulated in sputum	(28)
α_L integrin (CD11a)	Upregulated in asthma or after segmental lung antigen challenge	(34, 35)
α_M integrin (CD11b)	Upregulated after segmental lung antigen challenge, in BAL, or in sputum; Correlates inversely with PC_{20}	(20, 27, 28, 34, 36–40)
α_X integrin (CD11c)	Upregulated in BAL or sputum	(27, 37)
α_D integrin	Upregulated in BAL	(34, 36, 41, 42)
β_2 integrin (CD18)	Upregulated after segmental lung antigen challenge or in BAL	(34, 36)
Aminopeptidase N (CD13)	Upregulated in BAL	(43)
βc (CD131)	Downregulated in BAL	(44)
FcαRI (CD89)	Upregulated in asthma	(45)
FcγRIII (CD16)	Upregulated in allergic asthma or after whole-lung antigen challenge	(46)
Glucagon-like peptide-1R	Downregulated in allergic asthma	(47)
Granulocyte monocyte-colony stimulating factorRα (CD116)	Upregulated in BAL	(44, 48)
HLA-DR	Upregulated in BAL or sputum	(20, 37)
Intercellular adhesion molecule-1 (CD54)	Upregulated in BAL or sputum	(27, 37)
Interleukin (IL)-2Rα (CD25)	Upregulated in BAL	(22)
IL-3Rα (CD123)	Upregulated after segmental lung antigen challenge or in BAL	(22, 48)
IL-5Rα (CD125)	Downregulated in BAL	(44, 48)
IL-17RA	Upregulated in mild allergic asthma	(49)
IL-17RB	Upregulated in mild allergic asthma	(49)
L-selectin (CD62L)	Downregulated in BAL or sputum	(27, 28, 38, 40, 50)
Neuropeptide S R	Upregulated in severe asthma	(51)
P-selectin glycoprotein ligand-1 (CD162)	Upregulated after segmental lung antigen challenge or (48 h) after whole-lung antigen challenge	(34, 52)
Semaphorin 7A (CD108)	Upregulated in BAL	(53)
Activated α_M integrin	Highly activated conformation [reported by monoclonal antibody (mAb) CBRM1/5] in BAL or sputum	(28, 34, 41)
Activated β_1 integrin (CD29)	Partially activated conformation (reported by mAb N29) increased in all or non-severe asthma, or after segmental antigen challenge	(34, 36, 52, 54, 55)
	Correlates negatively with forced expiratory volume in 1 s (FEV_1) after or during withdrawal of inhaled corticosteroid (ICS) in non-severe asthma and predicts decreased FEV_1 according to receiver–operator characteristic analysis	
	Correlates with fraction of exhaled nitric oxide (FENO) upon withdrawal of ICS in non-severe asthma	
	Correlates negatively with FEV_1/forced vital capacity in younger non-severe asthmatic patients or in phenotype clusters 1–2 (mild–moderate allergic asthma)	
	At 48 h, post-segmental lung antigen challenge correlates with decrease in FEV_1 during the late phase post-whole-lung antigen challenge in mild allergic asthma	
	Highly activated conformation (reported by mAbs HUTS-21 and 9EG7) in BAL	

(*Continued*)

TABLE 1 | Continued

Protein	Observation	Reference
Activated β_2 integrin	Partially activated conformation (reported by mAb KIM-127) correlates with BAL eosinophil percentage in mild allergic asthma	(34, 36)
	Highly activated conformation (reported by mAb24) in BAL	
Activated FcγRII	Activated conformation (reported by mAb A17 or A27) increased in mild asthma, after whole-lung antigen challenge (in dual responders), or in BAL	(18, 56, 57)
	Correlates with FENO in asthma	

Observations refer to expression level, usually determined by flow cytometry, and are, if not indicated otherwise, on human blood eosinophils. For abbreviations, please see list immediately after abstract.
The search strategy used Pubmed (https://www.ncbi.nlm.nih.gov/pubmed?db=PubMed) with various combinations of terms including "eosinophils" and "activation" and "asthma" and "state" or "status" or "biomarker" or "review" (the combination of only "eosinophils" and "activation" and "asthma" resulted in an unmanageable large number of publications). Publications covering years until 2017 were examined, with primary publications covering the years 2014–2017 being especially examined. Primary references covering years until 2013 were partly obtained from published review articles.

DOWNREGULATION IN SEVERE ASTHMA OR AFTER ANTIGEN CHALLENGE

Some surface proteins on blood eosinophils are downregulated in more severe or uncontrolled asthma compared to less severe disease (**Table 2**). These include CD44, a hyaluronan receptor, and CD48, whose levels are lower in poorly controlled or severe asthma compared to well-controlled or moderate disease (21, 25). Similarly, activated β_1 integrin, reported by mAb N29 (see above), is increased in non-severe, but not in severe, asthma compared to healthy control subjects (52).

A possible explanation for this phenomenon is a high degree of ongoing extravasation of the most activated eosinophils, i.e., those with the highest level of CD44, CD48, and β_1 integrin activation, in severe asthma. This is consistent with a role for CD44 in the movement of eosinophils to the airway in mice after antigen challenge (8, 68). Also, CD44, like P-selectin glycoprotein ligand (PSGL)-1, relocalizes on blood eosinophils after stimulation with IL-5 or related cytokines, when the eosinophil changes shape and polarizes, and becomes concentrated at one end of the eosinophil in the nucleopod, which is a specialized uropod next to the nucleus (69). Such clustering of CD44 and other surface molecules may stimulate arrest and extravasation of eosinophils (8, 69). Similarly, uropod elongation, at the rear of a moving cell, is considered a crucial step in other leukocytes, including neutrophils and lymphocytes, before extravasation (70). Further, there is greater lung endothelial expression of vascular cell adhesion molecule (VCAM)-1, the ligand for $\alpha_4\beta_1$ integrin, in severe asthma, as observed in bronchial biopsies (71), which is compatible with efficient extravasation of eosinophils with activated $\alpha_4\beta_1$ integrin. The fraction of eosinophils that does not adhere to VCAM-1 *in vitro* has decreased β_1 activation, as reported by N29 (64), which also provides support for the scenario in which the eosinophils with a higher degree of $\alpha_4\beta_1$ activation are the ones that preferentially adhere to VCAM-1 (8, 17, 64). Finally, N29 reactivity, surface-associated P-selectin, and level of PSGL-1 decrease transiently after whole-lung antigen challenge in patients with mild allergic asthma (**Table 2**) (52). P-selectin activates eosinophil β_1 integrin and induces the N29 epitope *in vitro* (64) and is associated with N29 reactivity *in vivo* (52). P-selectin is not synthesized by eosinophils (67). The P-selectin bound to the eosinophil surface is likely derived from activated platelets associated with the eosinophils; a proportion (variable among different subjects) of eosinophils both in whole blood samples and purified eosinophils stain positively for the platelet marker α_{IIb} integrin (CD41) and P-selectin by flow cytometry or immunofluorescence microscopy (52, 64). The role of platelets, platelet activation, and platelet–eosinophil complexes in eosinophil recruitment and eosinophilic inflammation is the focus of another review within this Frontiers in Medicine Research Topic of "Pathogenic Advances and Therapeutic Perspectives for Eosinophilic Inflammation" and is described in more detail there (Shah S, Page CP, and Pitchford S: "Platelet–eosinophil interactions as a potential therapeutic target in allergic inflammation and asthma," submitted). Overall, the observations described above support the scenario that the most activated eosinophils; i.e., in this case, the cells with the highest degree of bound P-selectin, the highest level of the P-selectin counter-receptor PSGL-1, and activated $\alpha_4\beta_1$; extravasate, for instance, after whole-lung antigen challenge or in severe asthma.

In addition to possible extravasation of the most activated eosinophils in severe asthma and after whole-lung antigen

TABLE 2 | Eosinophil surface proteins downregulated in severe or poorly controlled asthma, or transiently after whole-lung antigen challenge.

Protein	Observation	Reference
CD44	Downregulated in poorly controlled compared to well-controlled asthma	(21)
CD48	Downregulated in severe compared to moderate asthma	(25)
P-selectin (CD62P)	Decreased transiently after whole-lung antigen challenge	(52)
P-selectin glycoprotein ligand-1 (CD162)	Decreased transiently after whole-lung antigen challenge	(52)
Activated β_1 integrin	Intermediate-activity state (recognized by monoclonal antibody N29) increased in non-severe but not severe asthma	(52)
	Decreased transiently after whole-lung antigen challenge	

Observations refer to expression level, determined by flow cytometry, and are on human blood eosinophils.
P-selectin is not synthesized by eosinophils (67) but can be associated with the eosinophil surface and is likely derived from activated platelets (52, 64).

challenge, it may also be, at least under some circumstances, that it is the most "activatable" cells that extravasate and the least "activatable" ones that remain in the circulation. High percentage of sputum eosinophils in asthmatic patients was found to be associated with low or no upregulation of α_M integrin or activation of FcγRII (CD32) on blood eosinophils in response to formylmethionine-leucyl-phenylalanine (fMLF) *in vitro*, whereas low sputum eosinophil count was associated with great α_M upregulation and CD32 activation in response to fMLF (72). These results indicate that the responsiveness of circulating eosinophils to a chemoattractant is lower in subjects with high sputum eosinophilia. This is possibly because the most responsive cells are continuously extravasating. An alternative or additional explanation may be that in patients with low sputum eosinophilia, the circulating eosinophils are not activated and are able to mount a great response to fMLF. On the other hand, in subjects with high sputum eosinophilia, the blood cells may already be at least partly activated (i.e., α_M already upregulated and CD32 altered to an activated conformation). In such a situation, fMLF may not achieve, or may achieve only little, further activation *in vitro*.

RESPONSE TO INTERVENTION

The expression or activation state of some proteins changes after pharmaceutical administration, e.g., with mepolizumab, an antibody against IL-5 (**Table 3**) (19). Anti-IL-5 mepolizumab causes decreased β_2 integrin, but not β_1 integrin, activation of blood eosinophils (**Figure 1**) (34). This indicates that the intermediate β_2 activation state on circulating eosinophils is the result of exposure to IL-5 *in vivo* and is consistent with *in vitro* data that IL-5 causes β_2 but not β_1 activation, whereas P-selectin activates β_1 but not β_2 (64). The differential response to anti-IL-5 demonstrates that pharmaceutical intervention may inhibit one aspect of eosinophil activation but not another. Further, comparing blood eosinophils after segmental lung antigen challenge pre- and post-mepolizumab demonstrated that anti-IL-5 caused decreased levels of α_L, α_M, and β_2 integrins as well as PSGL-1 (34), indicating that the upregulation of these proteins that occurs on blood eosinophils after segmental lung antigen challenge is IL-5-dependent. Finally, in contrast to the situation with blood eosinophils, anti-IL-5 did not affect the activation state of $\alpha_M\beta_2$ and the level of α_L, α_M, and β_2 on BAL eosinophils (34), indicating that the activation status of airway lumen eosinophils is independent of IL-5. This is consistent with the finding that BAL eosinophils have downregulated or no IL-5 receptor (**Table 1**) (44, 48), whereas they, in contrast, have upregulated IL-3 and granulocyte monocyte-colony stimulating factor (GM-CSF) receptors (**Table 1**) (22, 44, 48).

Whereas anti-IL-5 causes a decrease in blood eosinophil count (9, 10, 34), administration of anti-IL-13 or anti-IL-4 receptor α causes an increase in blood eosinophils (11, 73, 74). This observation is consistent with a scenario in which IL-13- or IL-4-induced factors, including VCAM-1, periostin, and eotaxins, promote eosinophil extravasation and trafficking (75–78). It would be interesting to know whether the circulating eosinophils after anti-IL-13/IL-4Rα treatment have become more or less activated, or not altered, but the surface phenotype of blood eosinophils after anti-IL13/IL-4Rα has not yet been reported. On one hand, one may imagine that they should become more activated, since ongoing extravasation of activated cells presumably has decreased, so activated cells instead may be expected to accumulate in the circulation. On the other hand, IL-13 or IL-4

TABLE 3 | Eosinophil surface proteins reported to respond to intervention in asthma.

Protein	Observation
α_L integrin (CD11a)	Decreased by anti-interleukin (IL)-5 (mepolizumab) after segmental lung antigen challenge
α_M integrin (CD11b)	Decreased by anti-IL-5 (mepolizumab) after segmental lung antigen challenge
β_2 integrin (CD18)	Decreased by anti-IL-5 (mepolizumab) after segmental lung antigen challenge
P-selectin glycoprotein ligand-1 (CD162)	Decreased by anti-IL-5 (mepolizumab) after segmental lung antigen challenge
Activated β_2 integrin	Intermediate-activity state (recognized by monoclonal antibody KIM-127) decreased by anti-IL-5 (mepolizumab)

Observations refer to expression level, determined by flow cytometry, and are on blood eosinophils in mild allergic asthma (34).

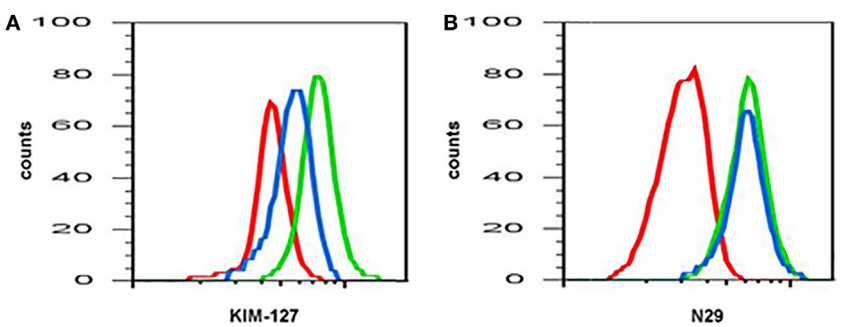

FIGURE 1 | Anti-interleukin (IL)-5 (mepolizumab) decreases β_2 but not β_1 integrin activation on blood eosinophils. Reactivity of monoclonal antibody **(A)** KIM-127 (to the intermediate-activity state of β_2 integrin), and **(B)** N29 (to the intermediate-activity state of β_1 integrin) on blood eosinophils before (green) and after (blue) anti-IL-5 mepolizumab administration. Red, isotype control. A representative subject with mild allergic asthma from Ref. (34).

themselves may stimulate some aspects of eosinophil activation, e.g., they have been reported to upregulate CD69 (8), indicating that anti-IL-13/IL-4Rα may lead to lower activation of blood eosinophils, or that at least some aspects of eosinophil activation may be decreased.

Recently, a mathematical modeling approach was taken in order to understand the effect of anti-IL therapy on eosinophil activation and dynamics (79). The mathematical model of Karelina and colleagues predicts a rapid decrease in total and activated eosinophil counts in blood and airways after anti-IL-5 mepolizumab administration. The decrease in blood eosinophils in the model is consistent with the literature, whereas the model appears to predict a greater proportional decrease in airway eosinophil counts than what actually happens after mepolizumab (9, 10, 34). The decrease in blood eosinophil activation in the model is consistent with the observed decrease in β_2 integrin activation (see here above and **Figure 1**) (34). However, the decrease in activated eosinophils in the airway in the model is not consistent with the observed lack of effect on integrin activation and expression levels on BAL eosinophils after mepolizumab (34). This disagreement between the model and observations may be because the model assumes that IL-5 is involved in the activation of airway eosinophils, whereas in reality, it may not be. Other factors such as IL-3 may be essential or compensate for IL-5 to stimulate and maintain airway eosinophil activation (see above and more below). Further, the model correctly predicts an increase in total eosinophils in blood and a decrease in the airway for anti-IL-13 therapies. Finally, it also predicts an increase in the number of activated eosinophils in blood after anti-IL-13, something which has not been reported but would be interesting to investigate, as mentioned above.

A MODEL FOR EOSINOPHIL ACTIVATION STATES IN THE CIRCULATION AND THE AIRWAY IN ASTHMA

The results from the studies on integrins discussed above suggest that there is variation in the activation status of circulating eosinophils among different individuals (8). Healthy persons and some patients with asthma display inactive β_1 integrins, patients with non-severe asthma have partially activated β_1 and β_2 integrins to different degrees, and patients with severe asthma have inactive or less activated β_1 integrins (8). The latter occurs likely due to arrest and extravasation of activated eosinophils (see model in **Figure 2** and references in the figure legend). Similarly, a scenario for FcγRII (CD32) activation on blood eosinophils has been described, where the degree of activation initially is elevated along with a higher level of systemic inflammation and then lower at the greatest degree of systemic inflammation (8, 18).

Airway lumen eosinophils, as sampled during BAL, have highly activated and upregulated $\alpha_M\beta_2$ and highly activated β_1 (34, 36), downregulated or no IL-5 receptor (44, 48), as well as upregulated IL-3 receptor (22, 48) and upregulated and highly activated FcγRII (CD32) (56). As the integrin activation state and levels on BAL eosinophils are not affected by anti-IL-5 (see above in Section "Response to Intervention") and BAL eosinophils lack IL-5-receptor, the airway lumen eosinophil phenotype is presumably the result of and maintained by other stimuli than IL-5, e.g., the related cytokines IL-3 and/or GM-CSF. IL-3 is the most likely responsible factor, since it, compared to IL-5, causes a higher degree of prolonged upregulation and activation of $\alpha_M\beta_2$ and CD32 (81).

The activation status of the lung tissue eosinophil in asthma is largely unknown. As depicted in **Figure 2**, eosinophils in lung tissue likely are adherent to or migrating in the ECM, e.g., by interacting with the ECM protein periostin, which is upregulated and associated with eosinophil recruitment to the airway in type 2 immunity-high asthma (82–86). Eosinophil adhesion to and motility on periostin is mediated by $\alpha_M\beta_2$ integrin and stimulated by nanogram per milliliter IL-5 (75, 80), which induces the high-activity conformation of $\alpha_M\beta_2$ (41, 64). Thus, assuming that tissue eosinophils interact with periostin, they likely have highly activated $\alpha_M\beta_2$. Whether tissue eosinophils express (like blood eosinophils) or lack (like BAL eosinophils) IL-5R, and in the latter case are stimulated and maintained active by GM-CSF or IL-3, appears uncertain and would be very interesting to determine.

Some recent very interesting articles studied mouse lung tissue eosinophils and partly also human lung tissue eosinophils (87, 88). Abdala Valencia and colleagues reported that, after antigen challenge, mouse lung tissue eosinophils shifted from a surface phenotype with intermediate expression of Siglec-F and no or very low α_X integrin (CD11c) to a Siglec-F-high/CD11c-low phenotype, and that BAL eosinophils were of the latter phenotype (87). Mesnil and others found that mouse steady-state pulmonary resident eosinophils were IL-5-independent and expressed an intermediate level of Siglec-F (in consistency with the first tissue phenotype in the Abdala Valencia publication), high L-selectin (CD62L), and low CD101 (immunoglobulin superfamily member 2) (88). After antigen challenge, these resident tissue cells were accompanied by newly recruited inflammatory tissue eosinophils, which were IL-5-dependent, Siglec-F-high, CD62L-low, and CD101-high (88, 89). In addition, Mesnil and colleagues determined that parenchymal resident eosinophils found in non-asthmatic human lungs had a CD62L-high, IL-3R-low phenotype, which was distinct from the phenotype of asthmatic sputum eosinophils, being CD62L-low/IL-3R-high (88). Their description of sputum eosinophils is consistent with earlier findings on sputum and BAL eosinophils (**Table 1**). Thus, both in humans and mice, lung tissue eosinophils may be of two phenotypes, one resident phenotype unrelated to asthma and different from the asthmatic airway lumen eosinophil phenotype, and one inflammatory phenotype recruited in asthma and similar or more similar to the airway lumen phenotype. A more detailed description of the tissue-resident eosinophils is given in another review within this Research Topic (Marichal T, Mesnil C, and Bureau F: "Homeostatic eosinophils: characteristics and functions," submitted). As indicated above, a more complete description of the inflammatory lung tissue eosinophil phenotype in asthma and a comparison to the blood and airway lumen phenotypes in asthma are warranted, e.g., to answer questions about integrin activation status of the lung tissue eosinophils and whether they express IL-5R.

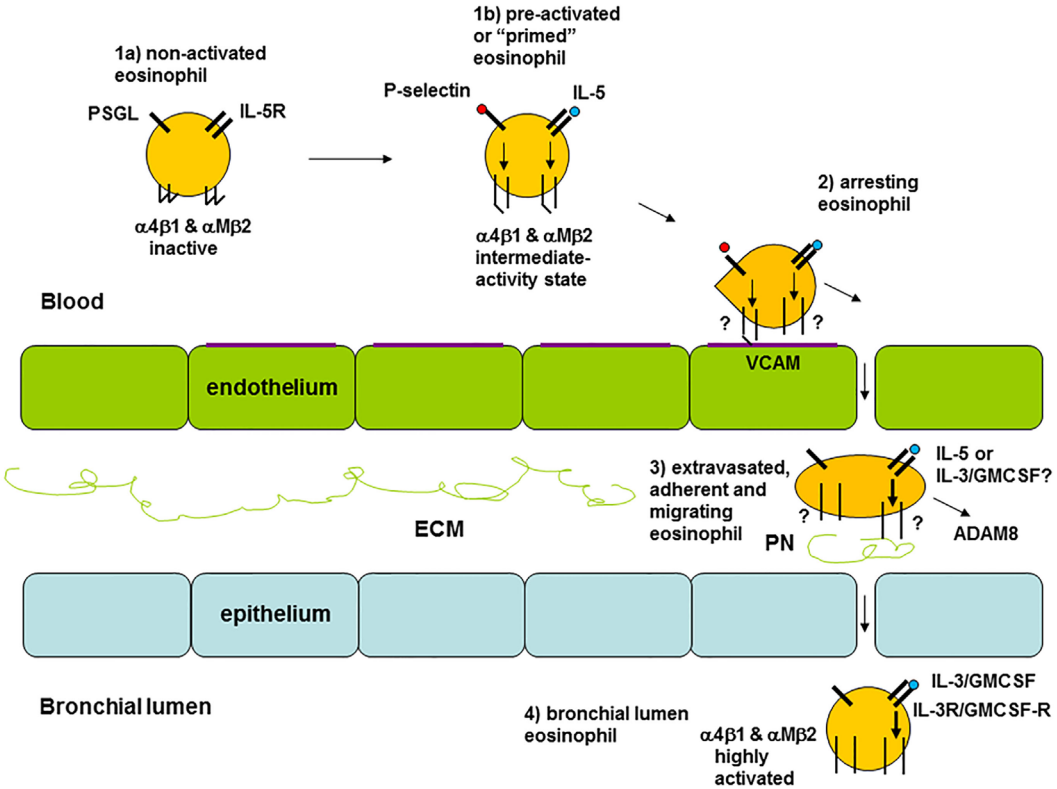

FIGURE 2 | Model of eosinophil activation states in asthma. (1a) Circulating non-activated eosinophil with $\alpha_4\beta_1$ and $\alpha_M\beta_2$ integrins in the inactive conformation or state, as found in normal subjects, some patients with non-severe asthma, or as observed in severe asthmatic patients likely because of a high degree of extravasation of activated eosinophils. (1b) Pre-activated or "primed," partly activated, circulating eosinophil with $\alpha_4\beta_1$ and $\alpha_M\beta_2$ in the intermediate-activity state, as a result of signaling triggered by P-selectin (likely derived from activated platelets, see the main text) and low concentration of interleukin (IL)-5, respectively, as found primarily in some subjects with non-severe asthma. (2) Eosinophil arresting on activated endothelium in asthma with $\alpha_4\beta_1$ and $\alpha_M\beta_2$ in unknown state, with $\alpha_4\beta_1$ primarily mediating arrest on vascular cell adhesion molecule (VCAM)-1 with a possible minor contribution of $\alpha_M\beta_2$. (3) Extravasated, adherent, and migrating tissue eosinophil in asthma with $\alpha_4\beta_1$ and $\alpha_M\beta_2$ likely in the high-activity state, with high-activity $\alpha_M\beta_2$, resulting from cytokine-triggered signaling, mediating interaction with the adhesive and pro-migratory extracellular matrix (ECM) protein periostin, and the eosinophil-releasing disintegrin and metalloproteinase (ADAM) 8 involved in PN degradation and cell migration. (4) Bronchial lumen highly activated eosinophil in asthma with $\alpha_4\beta_1$ and $\alpha_M\beta_2$ in the high-activity state and with downregulated or no IL-5 receptor, and with high-activity $\alpha_M\beta_2$ resulting from IL-3 and/or granulocyte monocyte-colony stimulating factor (GM-CSF)-triggered signaling. Modified and extended from Ref. (8) and also based on Ref. (34, 36, 44, 69, 80). Note: this model focuses on the activation states of integrins and on receptors for IL-5 family cytokines. It is not intended to be a full rendition of all possible factors involved in eosinophil recruitment. For instance, glycoproteins and glycans, including endothelial surface selectins and their role in eosinophil rolling, are covered in other reviews within this Research Topic (O'Sullivan JA, Carroll DJ, and Bochner BS: "Glycobiology of eosinophilic inflammation: contributions of siglecs, glycans, and other glycan-binding proteins," submitted; and Rao AP, Ge XN, and Sriramarao P: "Regulation of eosinophil recruitment and activation by galectins in allergic asthma," accepted). Further, chemokines and their receptors are the focus of yet another review (Larose M-C, Archambault A-S, Provost V, Laviolette M, and Flamand N: "Regulation of eosinophil and group 2 innate lymphoid cell trafficking in asthma," submitted).

CONCLUSION AND PERSPECTIVES

In this article, a number of proteins on the cell surface that have been suggested to mark eosinophil activation and are altered after antigen challenge or in the airway, or are associated with asthma or aspects of asthma, as well as a subset of these proteins that respond to intervention are reviewed. Partial β_1 integrin activation on blood eosinophils is associated with impaired pulmonary function or airway inflammation, and partial β_2 integrin activation is associated with airway eosinophilia in non-severe asthma. The associations do not occur in severe asthma, presumably due to greater extravasation of pre-activated eosinophils in severe disease. Airway lumen eosinophils have highly activated integrins and other changes in surface proteins compared to blood eosinophils. The activation state(s) of eosinophils in human lung tissue, although likely very important in asthma, is largely unknown but has begun to be studied.

The utility of the potential biomarkers of eosinophil activation in blood, a clinically accessible compartment, e.g., as correlators with or reporters of aspects of asthma, particularly severe asthma, needs to be explored further in translational and clinical studies. Although an occasional marker increases with asthma severity, some markers are downregulated in severe disease compared to non-severe disease. Possible reasons for the latter phenomenon are discussed above in the text. Still, since some of the markers respond to anti-IL-5, these or other IL-5-dependent markers may be predictors of response to intervention. The effect of other therapies, e.g., anti-IL-13/IL-4R, on surface markers of eosinophil activation has not been reported

but is a very interesting question. For instance, it may be interesting to examine whether potential alterations in eosinophil surface activation markers after various interventions may turn out to be associated with disease improvement, or possibly with decreased or increased risks for adverse eosinophil-related events. Finally, the potential relevance of these biomarkers in other eosinophilic and allergic diseases (19) also requires future exploration.

AUTHOR CONTRIBUTIONS

MJ conceived and designed, and interpreted the literature for this review; drafted and revised the manuscript, and approved the final version.

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Tissue Remodeling in Chronic Eosinophilic Esophageal Inflammation: Parallels in Asthma and Therapeutic Perspectives

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Chronic eosinophilic inflammation is associated with tissue remodeling and fibrosis in a number of chronic T-helper 2 (Th2)-mediated diseases including eosinophilic esophagitis (EoE) and asthma. Chronic inflammation results in dysregulated tissue healing, leading to fibrosis and end organ dysfunction, manifesting clinically as irreversible airway obstruction in asthma and as esophageal rigidity, strictures, narrowing, dysmotility, dysphagia, and food impactions in EoE. Current therapies for EoE and asthma center on reducing inflammation-driven tissue remodeling and fibrosis with corticosteroids, coupled with symptomatic control and allergen avoidance. Additional control of Th2 inflammation can be achieved in select asthma patients with biologic therapies such as anti-IL-5 and anti-IL-13 antibodies, which have also been trialed in EoE. Recent molecular analysis suggests an emerging role for structural cell dysfunction, either inherited or acquired, in the pathogenesis and progression of EoE and asthma tissue remodeling. In addition, new data suggest that inflammation-independent end organ rigidity can alter structural cell function. Herein, we review emerging data and concepts for the pathogenesis of tissue remodeling and fibrosis primarily in EoE and relevant pathogenetic parallels in asthma, focusing additionally on emerging disease-specific therapies and the ability of these therapies to reduce tissue remodeling in subsets of patients.

Keywords: eosinophilic esophagitis, asthma, inflammation, tissue remodeling, fibrosis, structural cell dysfunction, corticosteroid, biologic therapy

INTRODUCTION

Allergic inflammation has the capacity to recruit eosinophils to the site of inciting stimulus. Prolonged eosinophil infiltration can contribute to significant tissue injury, leading to maladaptive tissue remodeling and fibrosis. We will focus primarily on eosinophilic disorders associated with robust tissue remodeling, specifically eosinophilic esophagitis (EoE) and its relevant pathogenetic parallels in asthma.

CLINICAL FEATURES OF TISSUE REMODELING

The hypereosinophilic syndrome (HES)-associated tissue remodeling is arguably the most severe with cardiac damage leading to potential morbidity due to endomyocardial fibrosis. Asthma-associated airway remodeling occurs with epithelial denudation and goblet cell metaplasia, subepithelial fibrosis, angiogenesis, and smooth muscle hypertrophy (1). Remodeling is believed to be the mechanism to irreversible airway obstruction (2). EoE is an emerging chronic allergen-driven immune-mediated inflammatory disease that has been gaining recognition, with an increasing prevalence reaching 1 case per 1,000 persons (3–5). Chronic, unbridled inflammation in EoE leads to progressive esophageal fibrostenosis with rigidity and dysmotility with food impactions (6–9). Adult studies clearly demonstrate a natural history to stricture formation (6, 7). In both asthma and EoE, remodeling begins early in life, before the age of 6 years, and children with EoE can have histologic remodeling at as young as 2 years of age (2, 10).

Eosinophilic esophagitis is defined as a marked esophageal eosinophilic inflammation (≥15 eosinophils per high power field) that includes other inflammatory cells that likely contribute to remodeling such as mast cells, basophils, and adaptive as well as innate lymphoid cells (11–15). In the face of chronic antigen exposure and tissue damage, a progressive maladaptive esophageal tissue remodeling response causes clinical manifestations of dysphagia, food impactions, and, sometimes, spontaneous esophageal perforation (6, 16–20). In children, EoE often presents clinically as abdominal pain, nausea, vomiting, regurgitation, feeding difficulty, food aversion, weight loss, and failure to thrive; in adults, dysphagia and food impactions become more clinically prominent due to progression of esophageal dysfunction and fibrosis (13, 21). EoE severity has been associated with a lower body mass index, likely secondary to chronic nutritional deficit from recurrent dysphagia, food impaction, and food aversion (22). Although most EoE patients are well appearing, they often require a multimodal management approach that includes chronic medical treatment, dietary restriction, lifestyle changes, and repeated endoscopic diagnostic and therapeutic evaluations, creating a significant healthcare burden and impaired quality of life (18, 23–28).

In EoE, endoscopic features of remodeling vary between age groups. In children, features of esophageal pallor and furrows associate with histologic fibrosis and clinical dysphagia (29). In contrast, adult features of remodeling include concentric rings, narrowing, strictures, and the esophageal "pull" sign (30, 31). The narrowed and fibrostenotic esophagi are often the endoscopic features of adult EoE and can be intermittently observed in a subset of children (32). Functional readouts of esophageal rigidity include esophageal manometry and the novel application of the functional luminal imaging probe to assess esophageal rigidity and motility (33, 34). Indeed, esophageal rigidity predicts the risk of food impactions. Ultrasound studies in both adults and children show transmural esophageal thickening (35, 36). Similarly, CT scans of asthmatic airways demonstrate airway wall thickening even in children, while the HES heart can show increased cardiac muscle fibrosis with decreased chamber space (2).

Histologic Features of Remodeling

Asthmatic airways demonstrate subepithelial fibrosis, with increased trichrome staining. The asthmatic epithelium demonstrates defective epithelial barrier function and loss of junctional proteins, with goblet cell metaplasia (2, 37). Airway epithelial barrier function is thought to regulate asthma pathogenesis (38). Subepithelial angiogenesis accounts for airway wall edema, while thickened airway smooth muscle causes airway hyperreactivity. On the basis of the findings in remodeled asthmatic airways, our lab sought to understand whether esophageal biopsies from children with severe EoE had histologic findings akin to the remodeled asthmatic airway. Indeed, histopathologic analysis has shown extensive cellular and extracellular remodeling changes in EoE (13, 21, 39, 40). Remodeling is manifested in the epithelium as basal cell hyperplasia, dilated intercellular spaces, and desquamation; and in the subepithelium as fibrosis, angiogenesis, and smooth muscle hyperplasia (16, 21, 41). The loss of barrier function is a cardinal feature of the EoE esophagus with decreased expression of desmoglein-1 and filaggrin in addition to decreased E-cadherin and claudin-1 (42–44).

MOLECULAR MECHANISMS OF TISSUE REMODELING

Interleukins and Cytokines Involved in Remodeling

Current concepts of tissue remodeling have centralized on cellular and extracellular matrix responses to repetitive tissue injury and ineffective tissue regeneration in the context of chronic inflammation (1, 13, 21, 45). It appears that the mechanisms of remodeling are similar in asthma and EoE (**Table 1**). IL-4 and IL-13 play pivotal roles in asthma pathogenesis (1, 46). Progress in EoE pathogenesis to date has focused mainly on IL-13 (42, 47). Allergen-mediated induction of IL-4, IL-5, and IL-13 promotes a T-helper 2 (Th2) immune response, resulting in eosinophil recruitment and activation. In addition, profibrotic factors such as TGFβ1 appear to play an important role in the remodeling associated with these allergic diatheses (21).

IL-13 has emerged as a master regulator in EoE and drives the recruitment and activation of eosinophils *via* eotaxin-3/CCL26 and IL-5, further augmenting Th2 inflammation in the esophagus that can result in irreversible stricture formation (42, 47, 48). IL-13 contributes to the disruption of the epithelial barrier function, in part, *via* induction of calpain-14 that cleaves desmoglein-1 (49). Esophageal epithelial cells respond to IL-13 stimulation with STAT6-dependent expression of eotaxin-3/CCL26 that amplifies the chemotactic signals for further eosinophilic recruitment (47). IL-13 either alone or in combination with TGFβ1 can induce tissue fibroblasts to express periostin, further promoting eosinophil adhesion to fibronectin (50). IL-13 overexpression in an inducible transgenic murine model causes esophageal eosinophilia and stricture formation; turning off IL-13 overexpression to remove allergic inflammation reduces tissue eosinophilia but is unable

TABLE 1 | Eosinophilic esophagitis and asthma: summary.

	Eosinophilic esophagitis	Asthma
Clinical manifestations of dysregulated tissue remodeling	Esophageal narrowing, strictures, rigidity, dysmotility, dysphagia, food impactions	Irreversible airway obstruction, dyspnea, wheezing, oxygen desaturations
Relevant pathogenic cytokines	IL-5, IL-13, TGFβ1	IL-4, IL-5, IL-13, TGFβ1
Relevant pathogenic chemokines	CCL26	CCL11, CCL24, and CCL26
Cellular manifestations	Epithelial desquamation, basal zone hyperplasia, subepithelial fibrosis, angiogenesis, smooth muscle cell hypertrophy	Epithelial denudation, goblet cell metaplasia, subepithelial fibrosis, angiogenesis, smooth muscle hypertrophy
Tissue mastocytosis	Yes	Yes
Structural cell alterations	Myofibroblast formation, smooth muscle cell hypertrophy, epithelial barrier dysfunction	Myofibroblast formation, smooth muscle cell hypertrophy, epithelial barrier dysfunction

to reverse the established esophageal stricture (48). In addition, GATA-1-null eosinophil-deficient IL-13 transgenic mice are able to develop esophageal tissue remodeling as evidenced by esophageal epithelial thickness, collagen deposition, and cellular hyperplasia (51). In contrast to IL-5, IL-13-mediated esophageal dysmotility and dysfunction *via* collagen deposition, angiogenesis, and epithelial hyperplasia can occur independently of eosinophilic inflammation (48, 51). In murine models of asthma, airway structural remodeling has been shown to persist even after complete resolution of allergic inflammation (52, 53).

IL-5 is a major cytokine that regulates eosinophilopoeisis and the trafficking, survival, and activation of eosinophils (54). Arguably, the best evidence for the role of IL-5 in human asthma is the success of humanized, monoclonal anti-IL-5 antibodies in treating eosinophilic asthma although their ability to decrease remodeling in human tissues is not clear. Peripheral blood from patients with active EoE have increased frequency of circulating activated eosinophils and IL-5-expressing $CD4^+$ T cells, and peripheral blood mononuclear cells from EoE patients produce significantly more IL-5 compared to healthy controls when stimulated with house dust mites, ragweed, milk, *Aspergillus fumigatus*, or soy (55–59). Upregulated local expression of IL-5 promotes eosinophilic trafficking to the esophagus (60–62). Mice deficient in either IL-5 or eosinophils have diminished lamina propria collagen and fibronectin deposition in experimental EoE (62, 63). Esophageal strictures develop in IL-5-overexpressing transgenic mice, but not if these mice are also genetically deficient in eosinophils (48), demonstrating that the pro-remodeling effects of IL-5 are not intrinsic to this interleukin but, rather, through its capacity to recruit and activate inflammatory cells.

Eosinophils and Other Immune Cells in Tissue Remodeling

Tissue inflammation in EoE is patchy and can be transmural, with immune cell infiltration and structural changes extending from the epithelium to the underlying muscle layers allowing multiple tissue layers to be directly exposed to the damage induced by inflammatory cells (39, 64–66). Epithelial barrier disruption activates a program of IL-33, TSLP, and eotaxin-3/CCL26 expression in EoE that promotes Th2 immune activation and eosinophil infiltration (12, 67–71). Eotaxin-3/CCL26 is a potent chemoattractant for eosinophils that is highly upregulated in esophageal biopsies and sera of EoE patients (72, 73); plasma levels of eotaxin-1/CCL11 and eotaxin-2/CCL24 are not increased in active EoE (73). In comparison, epithelial levels of CCL24 and CCL26, but not CCL11, are elevated in severe asthma (1, 74). Asthmatic eosinophils migrate better in response to *ex vivo* stimulation with CCL26 than CCL11 or CCL24 (75); in addition, CCL26 stimulation of asthmatic eosinophils demonstrates a biphasic migration pattern that potentially contributes to eosinophil-dependent pathogenesis of persistent asthma. IL-33 and TSLP can activate the recently discovered Th2-promoting group 2 innate lymphocytes (ILC2), which are enriched in active EoE and may promote remodeling *via* the expression of IL-5 and IL-13 (14). Infiltrating eosinophils further drive EoE inflammation *via* a multitude of mechanisms including degranulation, inflammatory, and profibrotic cytokine secretion such as IL-4, IL-5, IL-13, GM-CSF, and TGFβ1, and eosinophil extracellular trap formation, which correlates with inflammatory features such as white exudates in active EoE (40, 69). GM-CSF blockade reduces basal cell hyperplasia and epithelial remodeling in experimental EoE (76). Other eosinophil blocking strategies are also successful in EoE animal models including antibody blockade with anti-Siglec-F (63, 77). Although eosinophils infiltrate densely in EoE, their complex interactions with non-immune cells such as epithelial cells, fibroblasts, and smooth muscle cells and other immune cells such as mast cells, ILC2, basophils, T cells, and invariant natural killer T cells likely dictate the histologic and clinical remodeling outcomes of the disease (5, 13, 14, 40, 78).

Eosinophilic esophagitis and asthma are also characterized by tissue mastocytosis, which contributes to esophageal and airway dysfunction. Murine models of EoE, which are deficient in mast cells, show that mast cells contribute to smooth muscle cell mass (11). Mast cells are also reservoirs for profibrotic factors such as TGFβ1, and decreases in mucosal mast cell numbers are likely one mechanism by which fibrosis improves following therapy (79). Similarly, other tryptase-positive cells such as basophils have been implicated in EoE, and blocking the TSLP receptor diminishes basophil-induced complications such as food impactions in experimental EoE (12).

Profibrotic Cytokines

Symptomatic EoE presents clinically as dysphagia, stemming from maladaptive esophageal tissue remodeling that results in fibrosis causing esophageal dysfunction and dysmotility.

Eosinophils and mast cells are significant sources of TGFβ1, as previously identified in the esophagus of EoE patients and in the lungs of asthmatic patients (16, 79, 80). Eosinophils and eosinophil-derived products increase extracellular matrix production of fibronectin and collagen I in primary human esophageal fibroblasts and muscle cells in a process dependent on TGFβ1 and p38 signaling (81). TGFβ1 expression is elevated in the epithelium and subepithelium of adult and pediatric EoE patients (16, 82). TGFβ1 signaling induces collagen deposition and production of fibronectin and other extracellular matrix proteins; and blockade of the canonical TGFβ1 signaling pathway, Smad2/3, decreases remodeling in an oral ova murine EoE model (83). Also invoking the canonical TGFβ1 pathway, there is an increased epithelial and subepithelial expression of nuclear Smad2/3 in pediatric EoE patients. In addition, eosinophil-derived products, secreted products from eosinophil-fibroblast/muscle cell co-cultures, TGFβ1, or IL-13 altered esophageal muscle contraction in a feline EoE model (81). In a cohort of pediatric EoE patients, fibrosis was associated with eosinophilic degranulation in the epithelium as measured by staining for eosinophilic major basic protein, whereas fibrosis was not associated with the degree of esophageal eosinophilia, the number of mast cells, or mast cell degranulation (67). Kita and colleagues proposed that detection of eosinophil degranulation might be a more accurate assessment of EoE severity, based on their observations of marked deposition of eosinophil-derived neurotoxin in adult EoE biopsies (84).

In addition to its profibrotic effects, TGFβ1 can alter tissue contractility. TGFβ1 activates tissue fibroblasts, resulting in myofibroblast differentiation that further contributes to extracellular matrix deposition and collagen contraction (85). In addition, TGFβ1 induces primary esophageal smooth muscle cell contraction, a mechanism dependent on the canonical Smad2/3 pathway and phospholamban, a sarcoendoplasmic reticulum protein that regulates calcium flux, which is upregulated in EoE biopsies (79, 85). It is interesting to speculate if esophageal phospholamban plays a role akin to asthmatic orosomucoid like 3, which is clearly implicated in the pathogenesis of asthma.

TGFβ1 also has significant effects on the epithelium. It breaks down epithelial barriers in asthma by decreasing the expression of adhesion molecules. In EoE, remodeling has been associated with epithelial–mesenchymal transition, a TGFβ1-regulated process (86, 87). TGFβ1 significantly induces plasminogen activator inhibitor 1 (PAI-1)/serpinE1 in esophageal epithelial cells. Epithelial PAI-1 reflects the severity of histologic fibrosis and is also required for TGFβ1-induced expression of phospholamban and α-smooth muscle actin (αSMA) in esophageal fibroblasts, suggesting that it is part of the pathway to esophageal myofibroblast accumulation (88). Children with genotype TT at the TGFβ1 promoter have significantly elevated numbers of TGFβ1-positive cells, increased mast cells (but not eosinophils), more severe epithelial remodeling, and, when food sensitized, worse fibrosis than children of non-TT genotype (89).

Fibrosis may also occur independently of TGFβ1, as other profibrotic molecules such as CCL18 and fibroblast growth factor-9 (FGF9) are elevated in EoE tissue biopsies (90, 91) and not all adult subjects have elevated TGFβ1 (82, 91). CCL18 is similarly elevated in the bronchoalveolar lavage and sera of asthmatic patients and preferentially attracts Th2 cells and basophils (92). Eosinophil-derived major basic protein induces FGF9 production that can contribute to the fibroproliferative response in EoE (90). To our knowledge, the role of FGF9 in asthma has not yet been described.

Mechanotransduction and Remodeling

There is accumulating evidence that mechanical signals ("mechanosignaling") alter the function of structural cells in the airway and esophagus in a manner that can be independent of, dependent on, or synergistic with, inflammation (93–95). Our recently published data demonstrate that rigid matrix alters the gene expression profile of primary human esophageal smooth muscle cells toward a pathogenic profile similar to that induced by TGFβ1 (95). EoE fibroblasts from children and adults had increased αSMA and traction force when cultured on a rigid matrix (94). Airway epithelial cells respond to physical parameters such as compressive forces mimicking those seen in an edematous airway with increased production of disease relevant inflammatory markers such as endothelin and TGFβ2 and decreasing expression of barrier proteins (93). In addition, compression forces increase fibroblast expression of collagens. Asthmatic bronchial fibroblasts exhibit higher elastic modulus than control cells; TGFβ1-induced differentiation of bronchial fibroblasts into myofibroblasts is enhanced by increasing matrix stiffness (96, 97). Airway smooth muscle cell contraction induces the release of more active TGFβ1 (98). Methacholine-induced bronchoconstriction in the absence of inflammation is sufficient to induce airway remodeling in asthmatic patients (99). Taken together, these compelling data invoke a shift in the thought paradigm from focus almost exclusively on inflammation to one with an integrated focus on the mechanosignaling coupled to inflammation. Indeed normalization of mechanosignaling is likely required to effectively reduce the propagation of inflammation and dysregulated structural cell gene expression. Currently, it is not clear what direct or indirect effects there are on inflammatory cells cultured either in an environment that is rigid or compressed. However, it is well accepted that cells such as mast cells respond to physical insults such as scratching.

CURRENT AND EMERGING THERAPEUTICS FOR ALLERGIC REMODELING

Currently, there are no FDA-approved drugs indicated for the treatment of EoE. Some of the current treatment strategies and their effects on airway and esophageal remodeling are summarized below (5, 18, 28, 100, 101).

Topical Corticosteroids

Topical esophageal corticosteroids constitute the most commonly utilized EoE therapy in children and adults. Similarly, inhaled corticosteroids are the most common agent used for persistent asthma. There has been relatively rapid accumulation of data for EoE since biopsies are procured regularly as part of disease monitoring. In contrast, airway biopsy is done in the context of

clinical trials. Short-term studies in children have demonstrated that topical corticosteroids decrease fibrosis, VCAM-1, epithelial remodeling, subepithelial TGFβ1, and nuclear Smad2/3-positive cells in the subset of patients who have resolution of epithelial eosinophils following therapy (102). As such, it appears that in "responder" children, remodeling is in flux and can be reversed or improved with short-term therapy. Such treatment-responsive remodeling likely constitutes a physiologic rather than a pathologic process. In contrast, children who are "non-responders" to therapy, as defined by persistent esophageal eosinophilia despite therapy, have continued subepithelial fibrosis, vascular activation, and TGFβ1-expressing cells. Topical fluticasone treatment downregulates mRNA expression of eotaxin-3 and decreases the degree of eosinophilic and lymphocytic tissue infiltration in EoE esophagi (47, 103, 104). In addition, topical fluticasone treatment of EoE patients reduces IL-13 mRNA expression and reverses expression of 98% of IL-13-induced EoE transcriptome to the levels of healthy controls (47). EoE esophageal mucosal integrity is improved with topical fluticasone, as seen with normalization of expression of desmoglein-1 and filaggrin (105, 106). Peripheral blood eosinophils isolated from adult corticosteroid-treated EoE patients sustain their activated phenotype (107), but exhibit decreased CD18 surface expression, with resultant diminished adherence of eosinophils to ICAM-1, ICAM-2, and endothelial cells (108). Budesonide treatment of adult EoE patients results in a statistically significant reduction in absolute blood eosinophil count and serum levels of CCL17, CCL18, CCL26, eosinophil-cationic protein, and mast cell tryptase. In addition, the absolute blood eosinophil count changes correlate with esophageal eosinophil density (109, 110).

Since EoE is a chronic disease, chronic therapy seems warranted. Studying a group of 32 children over a mean of 5 years (maximum of 10 years) treated with corticosteroids, Rajan and colleagues showed that children with EoE who persistently respond well to therapy have significantly less fibrosis and lower endoscopic scores than children who respond suboptimally to therapy (10). The clinical reasons for differences in response to therapy are not clear. However, it is possible that a "remodeling first-inflammation second" EoE phenotype is less responsive to steroid therapy. It is also possible that mechanical alterations in the esophagus, such as rigidity, change the structural and/or inflammatory cell response to interventions. This concept is echoed in the adult literature where the fibrostenotic, dysmotile esophagus is substantially more resistant to topical therapy with corticosteroids (9, 111). In terms of endoscopic and symptoms severity, topical corticosteroids can improve the diameter of the strictured adult EoE esophagus and decrease the rate of food impactions (8, 112).

In asthma, the effects of inhaled corticosteroids on remodeling and the best remodeling endpoint to follow are not entirely clear (2). This is likely due to the paucity of repeated human airway tissue for study and the complexity of the pulmonary structure as branching occurs. In a murine model of allergen-induced asthma, corticosteroids prevent myofibroblast accumulation and peribronchial collagen deposition and fibrosis (113). In addition, corticosteroids can improve a subset of gene transcripts in asthmatic airway fibroblasts (2). Combination treatments with inhaled corticosteroids and long-acting β2-adrenergic receptor agonists together have demonstrated superior prevention of asthma exacerbations (114). Systemic corticosteroids used during severe asthmatic exacerbations exhibit variable responsiveness, thought related to the underlying asthma heterogeneity, for example, corticosteroid-responsive type 2-high airway inflammation-driven "concordant disease" versus corticosteroid-resistant type 2-low "discordant disease" (115–119). Although inhaled steroids can improve epithelial shedding, this is not a consistent finding. Studies of the reticular basement membrane thickening demonstrate improvements, but whether improvement in basement membrane thickening corresponds to improvements in asthma complications such as difficult-to-treat airway hyperreactivity or irreversible airflow obstruction is not clear. Although there is not a paucity of human tissue for study in EoE, the most clinically meaningful endpoint of remodeling is still unclear, although the best targets are likely to be fibrosis and early-onset esophageal rigidity.

Efficacy of topical corticosteroid therapy is dependent on mucosal drug delivery and esophageal mucosal contact time (120). Swallowed aerosolized corticosteroid has variable delivery, with oral viscous corticosteroid preparation achieving superior esophageal mucosal delivery and treatment efficacy (120). Emerging non-proprietary and proprietary formulations of corticosteroid are expected to improve treatment options, drug bioavailability, and treatment efficacy (120–124). While corticosteroid treatment for EoE is effective, there exists a significant number of EoE patients who do not respond to topical corticosteroid treatments (122, 125). It has been proposed that topical corticosteroids are unable to penetrate the deeper esophageal layers where significant eosinophilic inflammation and tissue remodeling and fibrosis are likely to take place. Oftentimes, biopsies are limited to the superficial layers and may offer an incomplete picture of the histologic response. Targeting the fibrotic tissue may offer enhanced corticosteroid uptake. Currently, a clinical trial for EoE is examining the effect of losartan, an angiotensin II receptor blocker used clinically for hypertension that also exerts anti-fibrotic effect through suppression of active TGFβ1 levels (126). Losartan has been shown to inhibit collagen I synthesis, resulting in improved distribution and efficacy of antitumoral agents (126). Another potential beneficial effect of anti-fibrotic therapy might involve an indirect improvement of structural cell dysfunction by reducing tissue rigidity. This is based on the novel observation by Aceves and colleagues that a rigid matrix induces morphologic and transcriptional changes in esophageal smooth muscle cells with increased collagen deposition and cellular hypertrophy (95). Similar subsequent work by Muir et al. demonstrated the role of matrix stiffness in modifying TGFβ1signaling and contractility of primary esophageal fibroblasts (94). Taken together, targeting inflammation-dependent and inflammation-independent, rigidity-dependent pathways may represent novel strategies to modulate tissue remodeling and fibrosis in EoE and beyond.

Elimination Diets in EoE

In children, dietary modification to remove allergen-derived antigenic stimulation has been shown to reverse subepithelial fibrosis in EoE (127, 128). In addition, the combination of elimination

diet and topical corticosteroids can decrease fibrosis in children (127). The effect of elimination diet on adult remodeling is not as clear.

Biologic Therapy

Anti-IL-5 blockade with mepolizumab is safe and achieves significant reduction in circulating peripheral eosinophils and inflamed tissue eosinophilia (129–131). Even though IL-5 is a key regulatory cytokine of eosinophils that is upregulated in EoE, anti-IL-5 therapy using two different humanized monoclonal antibodies partially reduces tissue eosinophilia but does not alter esophageal fibrosis (132, 133). Although histologic or radiographic endpoints have not been systematically assessed in asthma, anti-IL-5 is effective in patients with severe, steroid refractory asthma and can be steroid sparing in patients with HES (134–136). In children with EoE, mepolizumab treatment decreases the numbers of tryptase-positive cells, IL-9-positive cells, and esophageal eosinophil–mast cell couplets (137).

Anti-IL-13 monoclonal antibody QAX576 significantly reduces esophageal eosinophilia and expression of EoE-related genes up to 6 months after treatment, but demonstrates only a trend for improved clinical symptoms (138). IL-13 blockade with a humanized monoclonal antibody RPC4046 significantly reduces esophageal eosinophilia and endoscopic features in EoE patients and also improves dysphagia; however, the effect is more prominent in steroid refractory EoE patients, suggesting that severe subjects may do well with anti-IL-13 therapy (139). This is consistent with the decrease in transcription of some remodeling genes including periostin for up to 6 months following treatment (138). Anti-IL-13 therapy also decreases markers of remodeling such as periostin and osteopontin in asthmatics, and subjects with higher serum periostin levels are more responsive to anti-IL-13 therapy (136, 140). Dupilumab, a blocker of both IL-4 and IL-13, may be of utility in asthma and EoE-associated remodeling (140–142).

CONCLUSION

Both EoE and asthma are diseases that involve robust tissue remodeling as part of the disease processes with resultant end organ dysfunction in a subset of subjects. In asthma, the clinical complication is irreversible airway obstruction. In EoE, it is stricture formation. One mechanism to this complication is prolonged, unbridled inflammation that can occur due to lack of therapeutic intervention or the failure of therapies to adequately control disease progression. The presumed inflammatory signals are from infiltrating cells that respond to alterations in structural cell physiology such as decreased barrier function and the onset of chemokine production. However, other signals such as mechanical changes in the airways due to airway rigidity and epithelial contraction during repeated rounds of bronchoconstriction drive structural cells such as epithelium to generate inflammatory signals that could propagate inflammation and be unresponsive to standard anti-inflammatory therapies such as corticosteroids.

In addition to these issues, a number of additional considerations should be made when assessing the Th2-associated remodeling. The first issue is what parameters reflect remodeling most reliably? The second is the issue of pathogenic versus physiologic remodeling. The use of physiologic markers such as esophageal strictures or fixed airway obstruction likely represents an endgame of chronic disease and will likely be difficult to control. For this reason, one goal should be to find early markers of remodeling and control them. Of course, remodeling is also a normal process of wound healing that is necessary and required. What is not clear is how the shift from physiologic to pathogenic remodeling occurs. Possible explanations include disease duration, chronic inflammation, and/or mechanical signals such as tissue rigidity. Understanding the molecular mechanisms and the clinical phenotypes of these processes will be essential to better control allergic tissue remodeling and its consequences.

AUTHOR CONTRIBUTIONS

QN and SA reviewed the literature and wrote the manuscript.

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REVIEW
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Pathogenic Effector Th2 Cells in Allergic Eosinophilic Inflammatory Disease

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There is an absolute requirement for Th2 cells in the pathogenesis of allergen-driven eosinophil-rich type 2 inflammation. Although Th2 cells are generally regarded as a homogeneous population, in the past decade there has been increasing evidence for a minority subpopulation of IL-5+ Th2 cells that have enhanced effector function. This IL-5+ Th2 subpopulation has been termed pathogenic effector Th2 (peTh2), as it exhibits greater effector function and disease association than conventional Th2 cells. peTh2 cells have a different expression profile, differentially express transcription factors, and preferentially use specific signaling pathways. As such, peTh2 cells are a potential target in the treatment of allergic eosinophilic inflammation. This review examines peTh2 cells, both in mouse models and human disease, with an emphasis on their role in the pathogenesis of allergic eosinophilic inflammation.

Keywords: Th2, CD161, CD294, chemoattractant receptor-homologous molecule expressed on Th2 cells positive, hematopoietic prostaglandin D synthase, interleukin-5, eosinophilic inflammation, eosinophilic gastrointestinal disease

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INTRODUCTION AND EARLY OBSERVATIONS

Since the first observation of cytokine heterogeneity of effector T helper (Th) cells (1), there have been attempts to examine the veracity of the Th1/Th2 paradigm and apply it to disease pathogenesis and treatment (1). Although initial investigations into this dichotomy in humans suggested a clearly laid out Th1–Th2 polarity (1), subsequent investigations were less clearly dichotomous (2). T cell biology is clonal, and as such, there are clear advantages to studying Th cell differentiation and cytokine expression at the single-cell level. The initial studies by Mossmann et al. (1) and the subsequent human investigations by Romagnani (3, 4) employed T cell clones, and although revolutionary at the time, had several technical limitations. T cell cloning is very labor intensive, limiting the number of clones (individual T cells) and patients who could be studied. More importantly, it is not clear that the cytokine phenotype of the resultant clone is the same as the original single T cell from which it was derived.

New scientific discovery is highly influenced by the development of new technology. During the mid-1990s intracellular cytokine staining was developed as a technique to interrogate the Th1/Th2 paradigm with greater fidelity and verisimilitude than possible with T cell cloning. Intracellular cytokine staining allows the examination of single-cell cytokine expression in thousands of individual cells, almost directly *ex vivo*. Initial publications clearly showed that although there was greater complexity in the Th1/Th2 paradigm than initially appreciated, the general paradigm was supported (5–7). Notably, in one of the authors' (CP) early papers, IL-4 and IL-5 expression patterns differed, indicating that IL-5-producing cells were a minority subpopulation within the larger

IL-4+ Th2 pool with a unique phenotype (CD27−, no IFN-γ coexpression) (5), which in a later review was hypothesized to be an IL-5+ Th2 subpopulation (8).

IL-5+ Th2 cell biology remained largely unexplored for the next 10 years, being relatively intractable to the available technology. The advent of polychromatic flow cytometry, and the ability to examine many phenotypic markers and cytokines within a single cell, facilitated subsequent murine and human investigations into IL-5+, pathogenic effector Th2 (peTh2) cell biology.

DEFINITIONS AND IDENTIFICATION OF peTh2 CELLS

Like many recently characterized cell populations, nomenclature has lagged behind the investigations of IL-5+ Th2 cells. Although the term "IL-5+ Th2" cells is probably the most commonly used descriptor, multiple other terms have been used. Clearly, a central distinguishing feature of these cells is their IL-5 expression. In our work in humans, we initially identified these cells by intracellular cytokine staining as IL-5+, IL-4+, IL-13+ relative to the IL-5− Th2 subpopulation that was IL-5−, IL-4+, IL-13+ (9). Subsequently, we demonstrated that the phenotypic markers hematopoietic prostaglandin D synthase (hPGDS) and CD161 individually identify IL-5+ Th2 cells (10). hPGDS in particular appears to be a more specific marker for pro-eosinophilic activity than IL-5 itself. To unify the various phenotypic descriptions, we have used the term peTh2, to emphasize the pathological role and enhanced effector function of these cells, which is due to more than simply IL-5 expression.

In parallel, Nakayama and colleagues have identified a murine peTh2 analog that they have termed "pathogenic memory" Th2, reviewed in Ref. (11). Their work has used an adoptive transfer memory model to generate IL-5+ Th2 and hence their terminology underscores the memory aspects of the model system. Luster and colleagues have identified a similar IL-5+ Th2 subpopulation during investigations to identify CCL8-responding T cells (12, 13). Lastly, Wambre and colleagues using class II tetramers have identified a subpopulation of human allergen-specific Th2 cells, which they have termed "Th2A" cells. Using both flow cytometric and transcriptome profiling, they characterize Th2A cells having an expression profile conforming to the various IL-5+ subpopulations noted above (14, 15). The continued development of peTh2 phenotypic markers that are amenable to immunohistochemcal detction, such as hPGDS, will facilitate understanding of their role in human disease. For the purposes of this review, we will use "peTh2" as a generic term referring to the various IL-5+ Th2 subpopulations described above. Conversely, the term "conventional" Th2 (cTh2) refers to a subpopulation of Th2 cells that is IL-5− or is negative for one of a variety of phenotypic markers associated with IL-5 expression, such as CD161, hPGDS, IL-17RB, or ST2.

MURINE STUDIES

Much of our understanding of peTh2 cells comes from the characterization of IL-5+ Th2 cells in mice. Immunological memory defines the adaptive immune system, and memory T cells can be subdivided into central memory (T_{cm}), effector memory (T_{em}), and resident memory (T_{rm}) populations, reviewed in Ref. (16, 17). Of these, T_{em} lack CD62L (L-selectin) and can express a variety of chemokine receptors for homing to peripheral tissues. Nakayama and colleagues categorized subsets of memory Th2 cells according to their expression of CD62L and the Th1-associated chemokine receptor CXCR3. While all memory Th2 subsets expressed IL-4 and IL-13, only the $CD62L^{low}$, $CXCR3^{low}$ Th2 subpopulation was enriched for IL-5. Depletion of $CD62L^{low}$, $CXCR3^{low}$ Th2 cells attenuated eosinophilic inflammation and airway hyperresponsiveness in a mouse model of allergic airway inflammation. These findings indicate that $CD62L^{low}$, $CXCR3^{low}$ cells have peTh2 function. Later findings by this group demonstrated that memory Th2 cell pathogenicity depends on the IL-33/ST2 axis (18), raising interesting questions about the conditions required for the development of these cells (see discussion below under Section "Relationship of peTh2 to Other T Cell Subsets").

Chemoattractant receptors mediate cell migration through lymphoid organs and peripheral tissues. A number of chemoattractant receptors have been associated with Th2 cells, including the prostaglandin D2 (PGD2) receptor CRTH2 (19), CCR3 (20), CCR4 (21), and CCR8 (22). Of these, Luster and colleagues (12) found that CCR8 defines an IL-5-enriched Th2 subset in both *in vitro*-differentiated and *ex vivo*-stimulated murine Th2 cells. CCR8 expression was associated with skin inflammation and tissue eosinophilia in a mouse model of chronic atopic dermatitis. In this model, CCL8-responsive CCR8+ Th2 cells showed increased proliferation and homing to allergen-sensitized skin. In line with these findings, the CCR8 ligand CCL8 was predominantly expressed in the skin and upregulated during allergic inflammation.

In summary, at least three studies in mice have characterized peTh2 cells as an IL-5-enriched subset of effector memory Th2 cells that have a distinct phenotype. These studies reveal a role for peTh2 cells in the pathogenesis of allergic inflammation. While mouse models have implicated peTh2 cells in allergic inflammation of the skin and airway, peTh2 cells have yet to be studied in murine models of allergic gut inflammation. Whether peTh2 cells are induced by or play a protective role in parasitic infection is unknown. Additional studies in models of allergic gut inflammation and parasite clearance will help further clarify the role of peTh2 cells in the type 2 immune response.

HUMAN STUDIES, ROLE IN HUMAN DISEASE

When human peTh2 cells were first identified, one obvious question was why had they not been previously described in the murine system? One reason for this unexpected delay in murine findings may be that most *in vivo* mouse experiments have a relatively short turn-around time that does not include sufficiently chronic antigen exposure to generate peTh2 cells in large numbers. In contrast, peTh2 may have been more easily identified in humans because of their role in diseases characterized by chronic antigenic exposure, including helminth infection

(5, 23), eosinophilic gastrointestinal disease (EGID) (24), allergic asthma (25), and atopic dermatitis (10).

Early clues to the existence of peTh2 cells were seen in the restriction of IL-5 expression to a minority subpopulation of Th2 cells (5, 6). Further, whereas IL-4 and IFN-γ were modestly coexpressed, IL-5 and IFN-γ demonstrated no coexpression (8), suggesting that the expression of IL-5 was accompanied by the silencing of IFN-γ. It was not until a decade later, when polychromatic flow cytometry was employed, that clear populations of IL-5+ (IL-4+, IL-5+) and IL-5− (IL-4+, IL-5−) Th2 cells could be routinely identified (24). Subsequently, Upadhyaya et al. developed reagents and techniques to examine all three Th2 cytokines and demonstrate two major human Th2 subpopulations: a minority IL-5+ Th2 (IL-5+, IL-4+, IL-13+) and majority IL-5− Th2 (IL-5−, IL-4+, IL-13+) subset (9).

Notably, during *in vitro* differentiation of Th2 cells from naïve CD4 cells, IL-4 and IL-13 expression is rapidly acquired, whereas the acquisition of expression of all three Th2 cytokines requires multiple rounds of antigenic exposure (9, 13, 26). *Ex vivo* peTh2 cells are CD45RO+, CD45RA−, CCR7−, CD62L−, and CD27−, consistent with their being highly differentiated CD4 T cells that have undergone repeated antigenic exposure. Such repeated antigenic exposure is typical of many allergens. For example, peanut allergen-specific IL-5+ Th2 cells were found in EGID, whereas in peanut anaphylaxis, the peanut-specific Th2 response was almost entirely IL-5− Th2. In EGID, patients typically do not have immediate type hypersensitivity and have chronic exposure to dietary peanut antigen; in contrast, in peanut anaphylaxis exposure to peanuts is rare. Conversely, in patients with peanut anaphylaxis undergoing peanut antigen oral immunotherapy, EGID has been a well-described adverse outcome (27), suggesting that chronic antigen exposure drives the differentiation of IL-5− into IL-5+ Th2 cells.

REGULATION OF Th2 GENE EXPRESSION IN peTh2 CELLS

The Th2 gene locus contains the genes for IL-4, IL-5, and IL-13 and is located on human chromosome 5q31 and mouse chromosome 11. The *IL4* and *IL13* genes are adjacent to each other, whereas *IL5* is 120 kb telomeric and in the opposite orientation. This gene arrangement, coupled with the finding that peTh2 cells are enriched for IL-5, suggests that epigenetic mechanisms may underlie peTh2 effector function. Histone modifications control chromatin structure and DNA accessibility to transcription factors; for example, H3K4 and H3K27 methylation marks are associated with gene activation and repression, respectively (28). In one study, peTh2 defined as $CD62L^{low}$, $CXCR3^{low}$ Th2 cells had increased H3K4me3 and decreased H3K27me3 binding to the *IL5* promoter, compared to other memory Th2 subsets (29). This same histone methylation pattern was seen in sorted human IL-5+ Th2 cells (9). These findings suggest that peTh2 cells are specifically licensed by an epigenetic program that results in the expression of *IL5*.

In addition to epigenetic regulation, current evidence suggests that peTh2 have a distinct transcriptional program. The Th2 master transcription factor GATA3 is required for both Th2 differentiation and for *IL5* and *IL13* expression (30). Interestingly, GATA3 increases with serial rounds of Th2 differentiation (9, 12) and is greatest in peTh2 cells (9, 29). Additionally, in peTh2 cells, GATA3 is preferentially associated with the *IL5* promoter, relative to cTh2 cells (9). Another regulator of peTh2 gene expression is the Th1-associated transcription factor eomesodermin. Eomesodermin is expressed at lower levels in $CD62L^{low}$, $CXCR3^{low}$ Th2 cells (peTh2) relative to other Th2 subpopulations (29). Through its interaction with GATA3, eomesodermin negatively regulates *IL5*, but not *IL4* or *IL13* expression by memory Th2 cells. In contrast to eomesodermin, T-bet expression is not differentially expressed in any specific Th2 subpopulation and knock-down of the T-bet gene (Tbx21) in Th2 cells did not affect Th2 cytokine expression. These data suggest a role for eomesodermin in inhibiting peTh2 development, in addition to its role in Th1 induction.

While several studies have shown that Th2 locus chromatin remodeling and Th2-associated transcription factors mediate peTh2 effector function, additional transcriptional mechanisms may also play a role. Wansley et al. recently found that the transcription factor retinoic acid receptor alpha (RARα) selectively regulates the proliferation and cytokine expression of IL-5+, but not IL-5−, human Th2 cells (31). This differential effect was attributed to a putative retinoic acid response element in the human *IL5* but not *IL4* or *IL13* promoters. Interestingly, vitamin A has been shown to promote the type 2 immune response *via* its metabolites binding RARα (32). These data suggest that vitamin A metabolites may amplify peTh2 effector function. In line with these findings, vitamin A supplementation correlated with disease severity in a murine model of asthma (33).

In summary, current evidence suggests that peTh2 cells have a unique epigenetic and transcriptional program underlying their effector function. The selective amplification of peTh2 cell activity by vitamin A metabolites raises the possibility that environmental factors can influence peTh2 cell responsiveness. Moving forward, the effect of diet on pathogenic type 2 inflammation may be a fruitful area of study.

RELATIONSHIP OF peTh2 TO OTHER T CELL SUBSETS

Several phenotypic and functional features distinguish peTh2 from cTh2 cells. Unlike cTh2 cells, peTh2 express hPGDS (10, 15). hPGDS is required for PGD2 production, and while mast cells are the dominant source of PGD2, we found that peTh2 cells produced PGD2 upon calcium ionophore stimulation (10). It is currently unknown whether and which physiological conditions induce PGD2 production by peTh2 cells. However, T cell receptor (TCR) stimulation failed to induce PGD2 in peTh2 (AMS, unpublished observations), raising the possibility that an innate stimulus drives hPGDS activity. Once produced, PGD2 binds to its receptor CRTH2, inducing Th2 cytokine production and chemotaxis of Th2 cells, type 2 innate lymphoid cells (ILCs), eosinophils, and basophils (34–36). Thus, peTh2 cells may propagate pathogenic type 2 inflammation *via* the hPGDS/PGD2/CRTH2 axis in both an autocrine and paracrine fashion.

Another difference between peTh2 and cTh2 cells lies in their effector function. Th2 cells have historically been identified by their expression of the Th2 cytokines IL-4, IL-13, and IL-5. When compared side-by-side, peTh2 cells not only express greater per-cell Th2 cytokines than their conventional counterparts but also have a distinct cytokine expression profile (9, 10). Indeed, several groups have found that IL-5 expression is restricted to peTh2 cells, whereas all Th2 subsets express IL-4 and IL-13 (9, 10, 13, 29). This differential cytokine expression is likely regulated by the epigenetic and transcriptional mechanisms outlined in the previous section and raises important questions about peTh2 development relative to cTh2 cells.

Several lines of evidence suggest that peTh2 are highly differentiated Th2 cells that arise from cTh2 cells after chronic antigen exposure. *In vitro*, Th2 differentiation can be induced by TCR stimulation of naïve T cells in Th2-polarizing conditions (26). While one round of differentiation induces cTh2 cells that express IL-4 and IL-13, *in vitro* generation of peTh2-like cells (that express IL-5, CCR8, and hPGDS in addition to IL-4 and IL-13) requires multiple rounds of differentiation (9, 10, 13). Notably, Paul and colleagues have demonstrated that the acquisition of ST2 expression and IL-33 responsiveness by Th2 cells (a peTh2 feature discussed below) similarly requires multiple rounds of *in vitro* differentiation (37). These findings are supported by *ex vivo* human studies in which peTh2 cells were uniformly CD27− (10), a pattern characteristic of highly differentiated memory effector T cells (38). Because Th differentiation is associated with chromatin remodeling at specific loci (39), the epigenetic signature of peTh2 cells (discussed in the previous section) further supports the notion that peTh2 are highly differentiated Th2 cells. Together, these studies suggest that peTh2 cell development and effector function require multiple rounds of differentiation that induce epigenetic modifications to Th2 cytokine loci. In support of this notion, Th2 cells that have undergone only two rounds of *in vitro* differentiation lack H3K4 methylation in the *IL5* promoter (40).

While peTh2-like cells can be generated *in vitro* through multiple rounds of Th2 differentiation, relatively little is known about the conditions required for their physiologic development *in vivo*. Recent studies, however, suggest that local inflammatory signals play a role. Thymic stromal lymphopoietin (TSLP) and IL-33 are epithelial-derived cytokines responsible for epithelial barrier maintenance (41, 42). In one study, TSLP-primed dendritic cells induced Th2 polarization and hPGDS expression (43). In another study, mice deficient in the IL-33R subunit ST2 failed to develop IL-5+ Th2 cells (44). Thus, local epithelial barrier disruption or pro-Th2 pathogen-associated molecular patterns that result in the release of TSLP and IL-33 may be an important pathway promoting peTh2 cell development.

Pathogenic effector Th2 can be further distinguished from cTh2 cells by their responsiveness to innate stimuli independent of canonical TCR activation. The innate and epithelial-derived cytokines IL-25, IL-33, and TSLP activate a type 2 immune response upon binding IL-17RB, the IL-33R complex, and the TSLPR complex, respectively, reviewed in Ref. (41, 42, 45). peTh2 cells not only express the receptors for but also produce Th2 cytokines upon stimulation by IL-25, IL-33, and TSLP (10, 12, 18, 37, 46). In one study, IL-33 induced H3K4 trimethylation and corresponding IL-5 production in memory Th2 cells *via* a p38/MAP kinase-dependent pathway (18). Whereas TCR stimulation induced IL-5 production by peTh2 only, IL-33 induced IL-5 production by all memory Th2 subsets. Together, these findings not only demonstrate that peTh2 cells have an innate-like program (see next section) but also implicate innate stimuli in the priming of peTh2 effector function.

The presence of peTh2 cells in the peripheral blood and at sites of allergic inflammation suggests that they have a pro-eosinophilic inflammatory chemotactic program (10, 12, 13). Indeed, peTh2 cells from subjects with EGID or atopic dermatitis expressed the Th2-associated chemokine receptor CCR3 and demonstrated enhanced chemotaxis to the CCR3 ligand eotaxin-1, whereas cTh2 cells did not (10). Furthermore, peTh2 cells from EGID and atopic dermatitis differentially expressed the gut and skin homing receptors α4β7 and CLA, respectively. In another study, peTh2 cells defined by their expression of CCR8 demonstrated increased homing to allergen-sensitized skin (12). In sum, peTh2 cells have an enhanced chemoattractant ligand and receptor program that facilitates their migration to sites of allergic inflammation.

The findings that peTh2 can be distinguished from cTh2 cells by their phenotype, enhanced effector function, innate responsiveness, and migratory capacity support a direct role for peTh2 cells in eosinophilic inflammation. This notion is further supported by the near perfect correlation of peTh2 cells with peripheral blood eosinophil counts in subjects with EGID and atopic dermatitis, suggesting that peTh2 cells drive eosinophilia in these diseases (10). peTh2 from these subjects have an activated phenotype and exhibit spontaneous proliferation relative to cTh2 cells. Together, these findings suggest that peTh2, and not cTh2 cells, mediate pathogenic type 2 inflammation. Whether peTh2 cells cause or result from chronic allergic inflammation, however, has yet to be formally investigated.

In summary, peTh2 are highly differentiated Th2 cells that likely develop from cTh2 cells through multiple rounds of Th2 polarization. Unlike cTh2 cells, peTh2 express IL-5 in addition to IL-4 and IL-13 and respond to innate stimuli including IL-25, IL-33, and TSLP. peTh2 have enhanced migratory function compared to cTh2 cells and localize to sites of allergic inflammation. Current evidence supports a model in which chronic antigen exposure at disrupted epithelial surfaces drive peTh2 cell differentiation, tissue trafficking, and consequent eosinophilic inflammation.

INNATE FUNCTION OF peTh2, SIMILARITIES AND DIFFERENCES BETWEEN peTh2 AND ILC2

Innate lymphoid cells are a recently characterized group of lymphocytes that lack the TCR but produce effector cytokines in patterns characteristic of Th cell subsets (47). While cTh2 cells require TCR stimulation for cytokine production, peTh2 can respond to stimuli independent of TCR activation, suggesting

that they have innate-like qualities. Indeed, peTh2 share several functional features with ILC2, including responsiveness to IL-25, IL-33, and TSLP (10, 12, 18, 37, 47–49). Interestingly, stimulation by these innate and epithelial-derived cytokines induced comparable levels of IL-5 and IL-13 in both cell types (10). In addition to IL-5 and IL-13, peTh2 and ILC2 can also express IL-9 (10, 48).

Underlying the functional similarities between peTh2 and ILC2 is a shared transcriptional program. The Th2 master transcription factor GATA3 and RORα drive ILC2 development and effector function (50). In addition to expressing high levels of GATA3 (10, 29), peTh2 expressed greater levels of RORα compared to other memory Th2 populations (29). A shared transcriptional program may also explain the phenotypic similarities of peTh2 and ILC2, as both cell types express CRTH2, hPGDS, and the C-type lectin CD161 (10, 51).

The similarities between ILC2 and peTh2 in their effector function, transcriptional program, and phenotype raise important questions regarding their respective roles in the type 2 immune response. ILC2 are predominantly tissue-resident innate effectors cells (47) and are increased at sites of allergic inflammation (51, 52). While peTh2 cells have largely been characterized *ex vivo* from peripheral blood, they have a tissue homing phenotype and have been shown to localize to sites of allergic inflammation (10, 12, 13). Thus, both cell types are implicated in local allergic inflammation. Our current understanding of peTh2 supports a model in which chronic allergen exposure and type 2 inflammation induces the differentiation of peTh2 cells that have innate function.

Few studies have directly compared ILC2 vs. peTh2 cells in pathogenic type 2 inflammation (10), but some inferences can be made regarding their timing in the immune response. peTh2 cells require multiple rounds of direct antigen stimulation for their development, whereas ILC2 do not. Therefore, primary responses are likely to be dominated by ILC2 cells, whereas after chronic antigen exposure, differentiation and clonal expansion of peTh2 increases their number and innate functionality. Future studies will help further define the relative roles of peTh2 vs. ILC2 in the development and maintenance of allergic inflammation. Intriguingly, because of the numerous similarities between peTh2 and ILC2 cells, many therapeutic approaches will target both cell populations.

THERAPEUTIC TARGETING OF peTh2

The localization of pro-eosinophilic function to peTh2 cells suggests their unique features may represent a therapeutic target. Indeed, it is likely that the current generation of anti-cytokine monoclonal therapeutics is largely exerting their effect through activity on peTh2 cells or their products (e.g., Th2 cytokines). Both the anti-IL-5 monoclonals mepolizumab and reslizumab as well as the anti-CD124 monoclonal dupilumab demonstrate the greatest clinical activity in patients with the highest baseline eosinophils counts, patients who are also expected to have the greatest numbers of peTh2 cells (53–55).

Given the high levels of GATA3 expression by peTh2 and the GATA3 requirement for *IL5* expression, it is likely that the investigational anti-GATA3 DNAzyme SB010 will directly affect peTh2 cells. Similar to the findings seen with the anti-cytokine monoclonals, SB010 had its greatest activity in subjects with the highest baseline eosinophil counts (56).

As antagonists of IL-25, IL-33, and TSLP advance in clinical development, the specific role of these innate pro-Th2 cytokines will become clearer. The p38 mitogen-activated protein kinase is a downstream mediator of IL-33/ST2 signaling (18). Inhibition of p38 kinase activity specifically inhibits IL-33-induced IL-5 expression, suggesting it may be a druggable target for clinical development.

TABLE 1 | Molecular features of pathogenic effector Th2 (peTh2) vs. conventional Th2 (cTh2).

Molecule	cTh2	peTh2	Reference
Cytokines			
IL-4	+++	++++	(9, 10, 13, 15, 29)
IL-5	+	++++	(9, 10, 13, 15, 29, 46)
IL-9	–	++	(10, 15)
IL-13	+++	++++	(9, 10, 13, 15, 29, 46)
IL-17	–	+	(10)
IFN-γ	+/–	–	(5, 9)
Cytokine receptors			
IL-17RB (IL-25R)	++	+++	(10, 15, 46)
IL-1RL1 (IL-33R, ST2)	++	+++	(10, 15, 18, 29, 46)
CRLF2 (TSLP-R)	++	++++	(10, 15)
Chemoattractant/homing receptors			
CCR3	+	+++	(10)
CCR4	+++	+++	(10)
CCR8	+	+++	(12, 13)
CXCR3	+	–	(10)
CRTH2	++	+++	(10)
Transcription factors			
GATA3	+++	++++	(9, 10, 12, 29)
T-bet	–	–	(11, 29)
Eomesodermin	+	–	(29)
Other			
Hematopoietic prostaglandin D synthase	+	++++	(10, 15)
CD27	++	–	(6, 9, 10, 15)
CD161	+	++++	(10, 15)

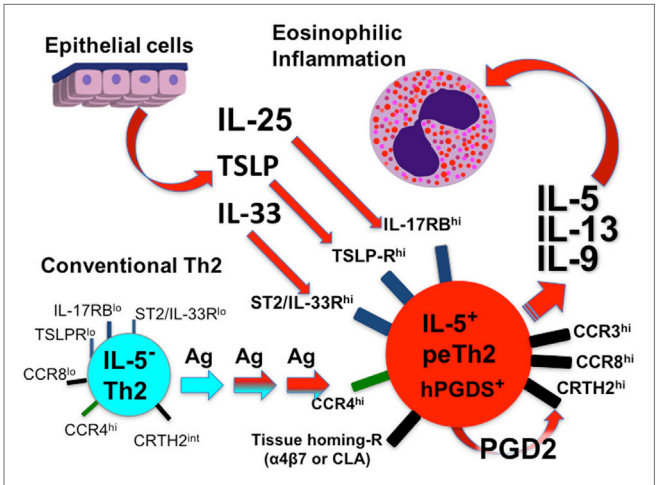

FIGURE 1 | Development of peTh2 enhances Th2 function thereby driving chronic allergic inflammation.

Pathogenic effector Th2 cells are notable for having both the biosynthetic machinery to synthesize PGD2 as well as the CRTH2 receptor to respond to PGD2. Although hPGDS inhibitors have been described in pre-clinical work, none has advanced thus far to clinical trials. In contrast, a number of CRTH2 inhibitors have been examined in clinical trials, the most promising being fevipiprant (57) and timapiprant (formerly OC000459) (58).

We have examined rapamycin as a potential anti-peTh2 drug. Notably, the rapamycin proliferation IC_{50} for peTh2 was shifted more than 2-fold vs. cTh2 and 100-fold vs. Th1 cells (59). Notably, the peTh2 IC50 was 0.1 nM, corresponding to serum concentrations <5% of that commonly used in transplant. peTh2 cells consistently demonstrated greater mechanistic target of rapamycin complex 1 (mTORC1) activity and greater susceptibility to mTORC1 inhibition than cTh2 or other CD4 T cell subsets. These data suggest that bioenergetic differences specific to the peTh2 subpopulation may allow their selective therapeutic targeting. Unfortunately, these promising *in vitro* findings were not translated in three EGID subjects who were treated with sirolimus for 8 weeks (CP, unpublished data).

CONCLUSION

Although many questions remain, the overwhelming evidence demonstrates two subpopulations of Th2 cells with distinct features (**Table 1**). Do peTh2 cells represent a separate Th2 subpopulation? If peTh2 cells are actually a distinct subpopulation, why does it matter? There is some evidence for a continuum of differentiation states between cTh2 and peTh2, which would argue against a clear dichotomy. However, under most conditions, the less differentiated cTh2 state is dominant, with peTh2 differentiation occurring only in specific pathological conditions (i.e., chronic antigen exposure). Thus, the acquisition of peTh2 function and pathology is a consequence of this chronic antigen exposure (**Figure 1**).

Given the importance of peTh2 cells in eosinophilic inflammatory diseases, investigational approaches that focus on peTh2 cells, rather than the entire Th2 compartment, are more likely to yield insights into these diseases. Similarly, therapeutic attempts to inhibit eosinophilic inflammation that focus on peTh2 cells will have greater potential for success.

AUTHOR CONTRIBUTIONS

AM-S and CP contributed equally to this work, including the conceptualization, writing, and editing.

AUTHOR'S NOTE

This work was performed as an extracurricular activity outside of author CP's cited affiliation.

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Interleukin-13 in Asthma and Other Eosinophilic Disorders

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Asthma is characterized by episodic, reversible airflow obstruction associated with variable levels of inflammation. Over the past several decades, there has been an increasing appreciation that the clinical presentation of asthma comprises a diverse set of underlying pathologies. Rather than being viewed as a single disease entity, asthma is now thought of as a clinical syndrome with the involvement of multiple pathological mechanisms. While it is appreciated that eosinophilia is present in only a subset of patients, it remains a key feature of asthma and other eosinophilic disorders such as atopic dermatitis, eosinophilic esophagitis, and chronic rhinosinusitis with nasal polyps. Eosinophils are bone marrow-derived leukocytes present in low numbers in health; however, during disease the type 2 cytokines [interleukins (IL)-4, -5, and -13] can induce rapid eosinophilopoiesis, prolonged eosinophil survival, and trafficking to the site of injury. In diseases such as allergic asthma there is an aberrant inflammatory response leading to eosinophilia, tissue damage, and airway pathology. IL-13 is a pleiotropic type 2 cytokine that has been shown to be integral in the pathogenesis of asthma and other eosinophilic disorders. IL-13 levels are elevated in animal models of eosinophilic inflammation and in the blood and tissue of patients diagnosed with eosinophilic disorders. IL-13 signaling elicits many pathogenic mechanisms including the promotion of eosinophil survival, activation, and trafficking. Data from preclinical models and clinical trials of IL-13 inhibitors in patients have revealed mechanistic insights into the role of this cytokine in driving eosinophilia. Promising results from clinical trials further support a key mechanistic role of IL-13 in asthma and other eosinophilic disorders. Here, we provide a perspective on the role of IL-13 in asthma and other eosinophilic disorders and describe ongoing clinical trials targeting this pathway in patients with significant unmet medical needs.

Keywords: interleukin-13, eosinophils, chemokines, asthma, eosinophilic disorders

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INTRODUCTION

Eosinophils are bone marrow-derived leukocytes that are present in low numbers in the blood during health (typically < 5% of all white blood cells) and rapidly migrate to select tissues where they reside. However, increased blood and tissue eosinophil counts have been associated with multiple pathologies. During parasitic infection and allergic diseases rampant eosinophilopoiesis occurs leading to increased numbers in the peripheral blood. Eosinophils then become activated and migrate to the site of injury where they can release mediators, including cytokines, chemokines, and cytotoxic

granule proteins. This ultimately leads to parasite expulsion, or in the case of allergic diseases, tissue injury (1).

Investigation of mouse models and human disease has found that increased eosinophil numbers are associated with type 2 inflammation and an increase of interleukin (IL)-4, -5, and -13. Indeed, eosinophilic disorders are predominantly characterized by type 2 inflammation. IL-13 is a pleiotropic type 2 cytokine that has been shown to be important in the pathogenesis of asthma and other eosinophilic disorders. The effects of IL-13 in these conditions include induction of goblet cell metaplasia and increased mucus secretion, increased airway hyperreactivity, and, indirectly, trafficking of eosinophils to the site of tissue injury *via* chemotaxis (2).

The prevalence of eosinophilic syndromes is continuing to increase with more severe forms of disease refractory to standard of care thus necessitating a better understanding of underlying biology to enable the development of new treatments. Therapeutics targeting type 2 inflammation, including IL-4, IL-5, and IL-13, are currently in development to treat eosinophilic diseases. However, due to the overlapping biology of these cytokines it has been a challenge to delineate the exact roles each play in type 2/eosinophilic disease. Here, we provide a review of the literature describing the role of IL-13 and the ongoing clinical development of therapeutics targeting IL-13 in asthma and other eosinophilic disorders such as atopic dermatitis (AD), eosinophilic esophagitis (EoE), and chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP).

INFLAMMATION IN EOSINOPHILIC DISEASES

Eosinophils develop from pluripotent progenitors in bone marrow and migrate into peripheral blood once mature. Mature eosinophils have distinct bilobed nuclei and secretory granules allowing them to be easily identified by routine tissue histology using hematoxylin and eosin staining. Eosinophils are terminal cytotoxic effector cells and make unique contributions to both innate and adaptive immunity (3). They have a half-life of ~18 h in blood and under homeostatic conditions quickly migrate to spleen, lymph nodes, thymus, gastrointestinal tract, uterus, and mammary glands, recruited by chemotactic factors (4). The evolutionary function of type 2 inflammation is primarily to respond to and control infection by extracellular parasitic organisms. Infection with parasitic worms elicits a Th2-mediated response that is required for the successful expulsion of the parasitic burden and protection of the host. Classical Th2 effector mechanisms are employed to expel the infectious organisms including mastocytosis, eosinophilia, increased mucus production, smooth muscle hypercontractility, and IgE synthesis. At the site of infection, eosinophils degranulate releasing cytotoxic granules to assist with killing of the parasite. They also secrete many mediators including IL-4 and IL-13 to perpetuate further type 2 inflammation (5, 6).

However, the presence of eosinophils in classic type 2 diseases such as asthma, AD, EoE, and CRSwNP can be pathogenic. The relationship between the presence of eosinophils in tissue and pathology has long been established, as seen in postmortem examinations of patients who suffered from fatal asthma exacerbations (7). In the instance of asthma, there is an aberrant response to non-parasite triggers such as allergens, viruses, or mucosal injury leading to epithelial cells producing cytokines, including IL-25, IL-33, thymic stromal lymphopoietin (TSLP), and IL-1α. These so-called type 2 alarmins can then promote differentiation of T helper 2 (Th2) cells, as well as activation of mast cells, macrophages, and type 2 innate lymphoid cells (ILC2s). IL-4, IL-5, and IL-13 secreted from these cells can subsequently elicit further immune activation including eosinophilic responses. IL-5 is the major cytokine responsible for eosinophilopoiesis, along with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3, which also support eosinophil survival (8, 9). The role of IL-5 in eosinophilic diseases is reviewed elsewhere in this issue.

Human IL-13 was first discovered in 1993 and has since been shown to be produced by multiple cell types. Increased IL-13 expression can elicit many of the pathological findings associated with type 2 diseases (10). The functions of IL-13 *in vivo* were elucidated by the generation of a mouse strain selectively overexpressing IL-13 in the lung *via* a transgene regulated by the club cell-specific CC10 promoter (11). This airway-specific IL-13 transgenic mouse presented with eosinophilic lung inflammation, airway epithelial cell hypertrophy, goblet cell metaplasia, mucus hypersecretion, subepithelial fibrosis, and airway hyperresponsiveness (AHR). In an ovalbumin (OVA) challenge model, IL-13 was found to be essential for the maintenance of AHR and mucus hypersecretion as administration of an IL-13 neutralizing antibody resulted in attenuation of these responses (12). ILC2s were found to expand *in vivo* in response to the innate type 2 cytokines IL-25 and IL-33 and represented the predominant early source of IL-13 during *Nippostrongylus brasiliensis* infection to allow for efficient helminth expulsion (13).

Interestingly, IL-4 and IL-13 both signal through the IL-4 receptor α (IL-4Rα). IL-4Rα is a component of the type I (IL-4Rα and γc) and type II (IL-4Rα and IL-13Rα1) IL-4R complexes. IL-4 can signal through both type I and II receptor complexes, whereas IL-13 signals only through the type II receptor complex. IL-4 and IL-13 activate the Janus kinase–signal transducer and activator of transcription (JAK-STAT) pathway. For example, IL-13 engages with its cell surface receptor IL-13Rα1 that then associates with IL-4Rα resulting in phosphorylation of JAK1 and TYK2. These activated kinases then phosphorylate the cytoplasmic domain of the receptor, creating binding sites for STAT6. STAT6 molecules are in turn phosphorylated, whereupon they dimerize and translocate to the nucleus. There they regulate gene transcription, ultimately leading to the production of type 2 cytokines such as IL-13, eotaxins, and other mediators involved in eosinophilic inflammation (**Figure 1**) (14). IL-4Rα and IL-13Rα1 are expressed on both hematopoietic and non-hematopoietic cells such as macrophages, B cells, fibroblasts, and airway epithelial cells. It is thought that this receptor configuration is responsible for the fact that IL-4 and IL-13 have overlapping functions as well as the ability to act independently of each other. For example, IL-4 alone has been implicated in initiating and potentiating polarization of naive T cells to Th2 cells and has a more dominant role than IL-13 in antibody class switching to IgE (15). IL-13 on the other hand plays a key role in fibrosis and mucus secretion (2). These distinct

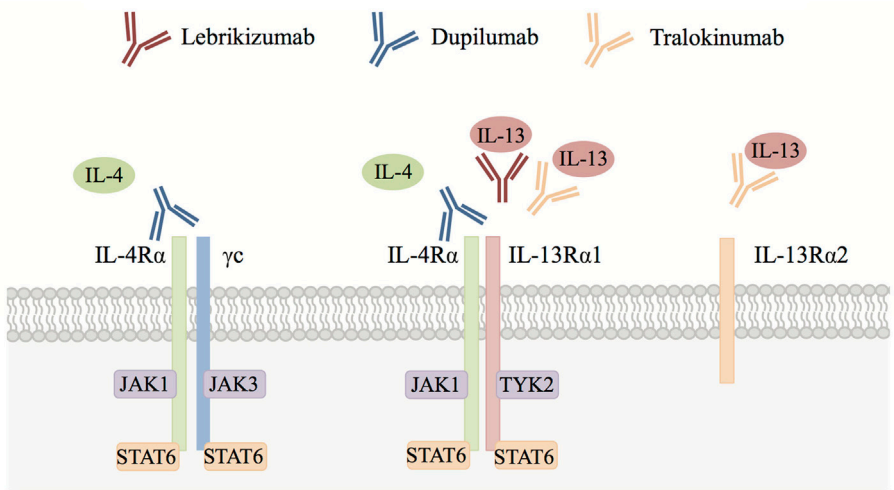

FIGURE 1 | Interleukin (IL)-4/IL-13 cytokine signaling. IL-4 signals through both the IL-4 receptor α (IL-4Rα)/γc (type I) and IL-4Rα/IL-13Rα1 (type II) receptor complexes, whereas IL-13 signals only through the IL-4Rα/IL-13Rα1 receptor complex. IL-13 can also bind to the IL-13Rα2 chain, which is thought to act primarily as a decoy receptor. Both IL-4 and IL-13 activate signal transducer and activator of transcription 6 (STAT6) via Janus kinase (JAK) family kinases leading to type 2 responses and eosinophilic inflammation in tissues orchestrated by chemokines, growth factors, and factors that position eosinophils in the tissue (see text for details). Blocking antibodies including lebrikizumab, dupilumab, and tralokinumab have been developed to inhibit IL-4 and/or IL-13 signaling in eosinophilic diseases.

functions may be due to both differential expression of type I and type II receptor complexes and differential spatiotemporal secretion of IL-4 and IL-13. However, both IL-4 and IL-13 can contribute to inflammation, AHR, and induction of chemokines that drive chemotaxis of blood eosinophils to injured tissue (16). Of note, IL-13 can also bind to the IL-13Rα2 chain, which does not contain a transmembrane-signaling domain and thus is thought to primarily act as a decoy receptor (17, 18).

In eosinophilic disorders such as asthma, there is increased eosinophilopoiesis and subsequent migration of eosinophils to the lung due to: (i) elevated levels of IL-3, IL-5, and GM-CSF to stimulate eosinophil development in bone marrow and survival in the blood and (ii) increased levels of type 2 cytokines (IL-4 and IL-13) to upregulate chemokine production, including CCL11 (eotaxin 1), CCL24 (eotaxin 2), CCL26 (eotaxin 3), CCL13 (MCP4), and CCL5 (RANTES), which enhance chemotaxis for eosinophil trafficking from the circulation to the airway (19). These chemokines bind to the chemokine receptor, CCR3, activating adhesion molecules such as integrins on the surface of blood eosinophils. In turn, this allows eosinophils to interact with endothelial cells *via* intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and periostin leading to infiltration from blood to the airway tissue (20) (**Figure 2**). Chemokine knockout mice such as *CCL11*$^{-/-}$ and *CCL24*$^{-/-}$ show decreased trafficking of eosinophils to the airway during allergen challenge (21, 22). In an *Aspergillus fumigatus*-induced asthma model, CCR3 knockout mice had decreased eosinophilic airway inflammation along with reduced levels of type 2 cytokines, including IL-13 (23).

Two eosinophil-deficient mouse strains have been developed (ΔdblGATA and PHIL), however, studies inducing allergic airway inflammation in these models have reported conflicting results. OVA-challenged ΔdblGATA (24) mice had similar airway hyperreactivity and airway inflammation but reduced collagen deposition and airway smooth muscle mass compared to WT mice (25). However, OVA-challenged PHIL mice were protected from airway hyperreactivity and goblet cell metaplasia and mucus secretion (26). In another study, ΔdblGATA mice were protected from *A. fumigatus*-induced allergic airway inflammation and had decreased type 2 cytokines and airway mucus production (23). However, there are numerous caveats comparing these studies including the different strains of mice, variations in the models used, and disparities in experimental readouts. Further investigation is required to definitively characterize the relationship between IL-13 and eosinophils in mouse models of allergic disease.

Multiple biologics and small molecule therapeutic candidates targeting eosinophilic inflammation have been or are currently being evaluated in preclinical or clinical settings. A number of biologics blocking soluble inflammatory mediators and their receptors associated with eosinophilic inflammation, including IgE, IL-4, IL-4Rα, IL-5, IL-5R, IL-13, TSLP, IL-25, and IL-33, are being investigated. Anti-Siglec-8 antibodies have been proposed to inhibit eosinophil activation and induce eosinophil apoptosis (27). The advantages and potential limitations of different targeted therapies for eosinophilic disorders have been recently reviewed elsewhere (28).

ASTHMA

Asthma is one of the most common chronic disorders in the world. Despite a vast body of research and the large clinical burden associated with asthma, its complexity and heterogeneity make it difficult to establish a standardized definition of what constitutes "disease." Asthma is typically characterized by airway inflammation and a history of respiratory symptoms (including

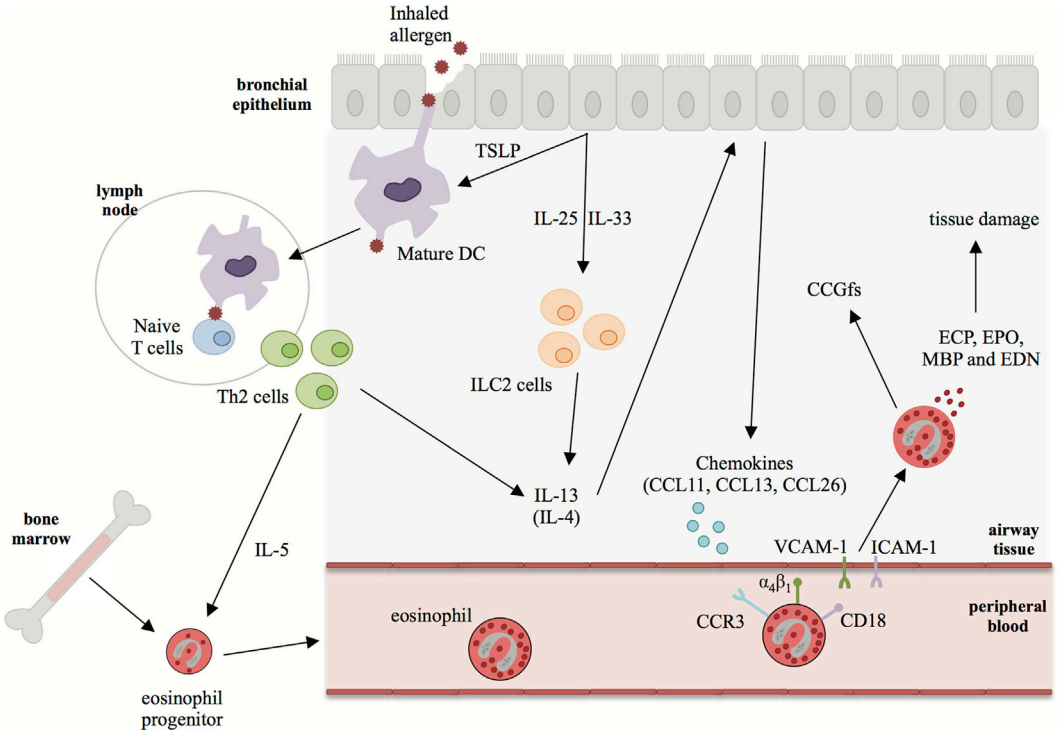

FIGURE 2 | The role of interleukin (IL)-13 in driving eosinophilia in asthma. In asthma, bronchial epithelial cell injury leads to production of type 2 alarmins such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP). These alarmins can promote the differentiation of Th2 cells and activation of ILC2s leading to production of IL-4, IL-5, and IL-13. IL-5 is the major cytokine responsible for eosinophilopoiesis and eosinophil survival in the periphery. IL-13 (and, to a lesser extent, IL-4) induces the secretion of chemokines, such as CCL11, CCL13, and CCL26 from epithelial cells. Chemokines bind to CCR3 on eosinophils trafficking them to the site of injury where they extravasate into the lung tissue. In the lung eosinophils degranulate causing tissue damage *via* the secretion of eosinophil granule proteins and cytokines, chemokines, and growth factors (CCGfs).

wheeze, shortness of breath, chest tightness, and cough) together with a variable airflow limitation (29).

Investigation into the complexity of asthma has led to the identification of multiple different clinical and molecular phenotypes (30). The most commonly described clinical phenotypes include those defined by severity, rates of exacerbations, response to treatment, age of onset, and obesity. The current molecular phenotypes include type 2/eosinophilic, neutrophilic, and paucigranulocytic inflammation (30, 31). These clinical and molecular phenotypes are not mutually exclusive, may change over time in individual patients, and may interact, contributing to differences in responsiveness to asthma therapies.

Role of Eosinophils in Asthma Pathophysiology

Arguably, molecular phenotyping of asthma patients has been most valuable in developing novel targeted therapies, particularly in understanding the biology of type 2/eosinophilic asthma. Eosinophils have been observed in increased numbers in peripheral blood, bronchoalveolar lavage (BAL) fluid, and bronchial tissue in asthma patients. It has also been reported that elevated eosinophil counts are significantly correlated with disease severity, indicating that these cells may play an important role in asthma pathogenesis (32). Measurement of eosinophils in induced sputum has been shown to be a biomarker of airway inflammation and a useful tool for adjusting the intensity of corticosteroid treatment to achieve optimal asthma control (33–35). However, measurement of sputum eosinophils is not widely used in the clinical setting, as it is time-consuming, requires specialized technical expertise, and the collection process may cause some discomfort to patients. Elevated blood eosinophil counts are correlated with lung function and asthma symptom scores, and therefore, can be useful in both the diagnosis and the management of patients with asthma (32, 36–43). A statistically significant correlation between blood eosinophils and sputum eosinophils in asthma patients has been reported (44). Another study later showed that blood eosinophil counts could accurately predict airway eosinophilia in asthma patients with persistent uncontrolled disease despite treatment (45). Therefore, blood eosinophils may be a good surrogate biomarker to identify patients with airway eosinophilia. However, Wenzel et al. described two subtypes of asthma, eosinophilic and non-eosinophilic, with different pathological, physiological, and clinical characteristics, although it should be noted that these characteristics exist along a continuum rather than being completely independent. In their study, the presence of eosinophils in bronchial biopsies was associated with significantly increased tissue lymphocytes, mast cells, and macrophages, basement membrane thickening, and patient intubations compared to the non-eosinophilic asthmatics (46). Due to disease heterogeneity in poorly controlled asthma patients, there was a need to develop

biomarkers that could enable the identification of a particular subset of patients. This has been valuable in recent clinical trials, for example, blood eosinophil counts correlated with response to anti-IL-13 therapies in Phase 2 clinical trials, wherein patients with higher levels of blood eosinophils had a greater benefit than patients with lower counts (47–50). In addition to blood eosinophil counts there are other biomarkers of type 2 diseases, such as serum periostin and fractional exhaled nitric oxide (FeNO), which are being investigated in asthma. Measurement of these biomarkers in a population of asthmatics revealed that they are continuously distributed and correlated with each other (44). This continuous distribution of biomarkers and airway pathology is a critical nuance to appreciate when interpreting clinical data, as cutoffs defining "biomarker-high" vs. "biomarker-low" populations are arbitrary and typically fall near the median of continuously distributed values rather than defining clear distinctions between subgroups (51).

IL-13 and Eosinophils in Asthma

Interleukin-13 has been implicated in promoting eosinophil survival, activation, and recruitment. *In vitro* cultures of eosinophils with recombinant IL-13 showed prolonged survival in a dose-dependent manner, which was attributed to inhibition of apoptosis. This was mediated by an autocrine mechanism through stimulation or release of IL-3 and GM-CSF by eosinophils. A major function of IL-13 (and IL-4) in the asthmatic airway is to induce chemotaxis of eosinophils to the site of injury. A number of *in vitro* studies have investigated the role of IL-13-induced chemotaxis and activation of eosinophils. Significant dose-dependent chemotactic activity was observed in an experiment in which eosinophils were cultured in the upper compartment of chemotactic chambers with recombinant IL-13 in the lower compartment (52). *In vitro* culture of eosinophils stimulated with IL-13 resulted in a concentration-dependent upregulation of the activation marker CD69. Furthermore, the addition of an anti-IL-13 antibody to these cultures led to inhibition of this activation (53). IL-13 induces VCAM-1 expression in endothelial cells, leading to increased adhesiveness of eosinophils to endothelium via VCAM-1/integrin α4 interactions. This might be a potential mechanism by which IL-13 promotes arrest and extravasation of eosinophils to the asthmatic airway (54). In clinical studies, increased IL-13 mRNA expression in sputum specimens and bronchial mucosa was significantly positively correlated with the percentage of eosinophils in the airway lumen (55, 56). In a study of human bronchial epithelial cells from asthma patients, an IL-13-inducible gene signature (*POSTN*, *CLCA1*, and *SERPINB2*) was identified that served as a surrogate marker of type 2 airway inflammation. This signature was observed in about half of asthmatics and was associated with distinct features of asthma including airway eosinophilia (56). In addition, eosinophils in the bronchial submucosa were found to express IL-13 (57). IL-13 is produced and consumed locally at sites of inflammation, therefore, peripheral levels are very low, and developing reliable assays to measure circulating IL-13 has been an on-going challenge. Recently, we developed an assay to detect human serum IL-13 with femtograms per milliliter sensitivity and excellent specificity (58). Using this assay, we found significantly higher levels of serum IL-13 in severe asthma patients relative to healthy volunteers and these levels strongly correlated with the type 2 gene signature in bronchial epithelium. Interestingly, in moderate to severe asthma patients, serum IL-13 was strongly positively correlated with blood eosinophil counts. It has also been demonstrated that human eosinophils derived from both periphery and tissue are capable of synthesizing, and upon stimulation releasing over 35 cytokines, chemokines, and growth factors. IL-13 is an abundant cytokine in eosinophils and upon release may directly orchestrate inflammatory responses with other immunomodulators (59). It is therefore possible that the elevated serum IL-13 levels may be in part a consequence of release from both airway and blood eosinophils (58).

IL-13 and Eosinophil Activation in Asthma

Type 2 cytokines, including IL-13, regulate the secretion of various chemokines that can bind to eosinophils *via* the CCR3 receptor leading to eosinophil activation and migration to the lung *via* chemotaxis. Eosinophils recruited to the asthmatic airway are highly activated and localize with inflammatory mediators and other immune cells that accumulate at the site of injury. While eosinophils can secrete cytokines and other mediators without degranulating, the ultimate result of eosinophil activation is degranulation. Human eosinophil granules contain four cationic proteins, major basic protein (MBP) primarily present in the crystalline core, eosinophil peroxidase (EPO/EPX), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) enriched in the granule matrix. The secretion of eosinophil granule proteins has been shown to facilitate the killing of parasites; *in vitro* ECP and MBP were found to be toxic to the larvae of parasites such as *Schistosoma mansoni* and *Trichinella spiralis* (60). However, they also have cytotoxic effects on tissues and their levels have been suggested to be associated with asthma severity, bronchial epithelial cell damage, and remodeling (61).

The most commonly observed forms of eosinophil degranulation in the inflammatory airway are piecemeal degranulation and cytolytic degranulation. Piecemeal degranulation is a form of exocytosis, in which specific granule contents are transported to the cell surface in small cytoplasmic secretory vesicles, while cells remain viable (62). Cytolytic degranulation on the other hand involves eosinophil chromatolysis and cell membrane rupture leading to the release of intact secretory granules. Cell-free eosinophil granules can store and further release their contents (61). One study has described eosinophil granules in airway macrophages that had presumably phagocytosed apoptotic eosinophils. Increasing numbers of macrophages containing ECP and EPO were observed with increasing severity of asthma (63). Besides the most common piecemeal and cytolytic degranulation, another form of eosinophil degranulation is to generate extracellular traps (ETosis) containing granule proteins in response to exposure to bacteria, C5a, or CCL11 (64, 65). The process of eosinophil degranulation in the peripheral blood is less clearly defined, and there have been conflicting reports as to whether peripheral blood eosinophil degranulation contributes to disease. It has been shown that blood eosinophils in allergic diseases such as asthma and AD display no morphological signs of either piecemeal or cytolytic degranulation while eosinophils from matched

diseased tissue biopsies exhibited degranulation through both piecemeal and cytolytic processes. These observations suggest that eosinophils exist in a resting status in the circulation and are activated at the site of tissue pathology (66). However, a small study with mild allergic asthmatics and healthy volunteers demonstrated that blood eosinophils in allergic patients underwent piecemeal degranulation during pollen season (67). A study in children comparing blood eosinophils from healthy controls, symptom-free asthmatics, and asthmatics with acute exacerbations showed that the proportion of activated blood eosinophils with significant morphologic changes were highest in the children with acute asthma exacerbations compared to symptom-free asthmatics and healthy controls (68). Circulating levels of eosinophil granule proteins have also been demonstrated to correlate with some aspects of disease activity. Therefore, in addition to simply measuring blood eosinophil counts, assessing eosinophil activation status may have clinical utility. Indeed, it has been reported that serum ECP and EPX levels predicted asthma risk more accurately than standard blood eosinophil counts in patients with allergic rhinitis (69).

Of note, treatments targeting the IL-13 pathway have consistently reported increases in blood eosinophil counts. However, the activation status of the eosinophils has not been characterized. To address this we analyzed serum levels of two eosinophil granule proteins, ECP and EDN, using pooled data from two independent Phase 2 studies investigating the efficacy of lebrikizumab (an anti-IL-13 monoclonal antibody) in patients with uncontrolled asthma despite maintenance therapy with inhaled corticosteroids (ICS) and a second controller. All patients provided written informed consent for their samples to be used for research purposes. Patients received either placebo or lebrikizumab (37.5, 125, or 250 mg) (47). Blood eosinophils, FeNO, and serum periostin were measured during this study, and pharmacodynamic (PD) effects were observed on each of these biomarkers. Blood eosinophil counts increased in response to treatment, and there was a slight trend toward a dose response. FeNO and serum periostin levels decreased after treatment but this was not dose dependent. Given the similar PD effects across treatment arms they were combined to analyze the eosinophil activation status after lebrikizumab treatment. Serum levels of ECP and EDN were measured at baseline and after 16 and 24 weeks of placebo or lebrikizumab treatment from a subset of patients who had comparable baseline characteristics to the overall study population (**Table 1**). These previously unpublished data demonstrated that at baseline there was a strong positive intercorrelation between blood eosinophils and serum ECP and EDN, suggesting that serum ECP and EDN may be secreted from blood eosinophils (**Figure 3**). Patients treated with lebrikizumab had increased blood eosinophil numbers after 16 and 24 weeks of treatment. However, serum ECP and EDN levels remained unchanged suggesting that while lebrikizumab treatment led to increased blood eosinophil levels it did not result in blood eosinophil activation (**Figure 4**). Of note, serum ECP and EDN levels in the placebo group significantly declined at 16 and 24 weeks. This decrease was unexpected given the unchanged blood eosinophil numbers over time in the placebo arm and therefore needs to be further explored.

While these data are potentially interesting and begin to shed light on the relationship between IL-13 and eosinophils in asthma there are some caveats. This was a *post hoc* analysis carried out in a subset of patients from two independent Phase 2 studies and therefore will require further validation to better understand the

TABLE 1 | Summary of key patient characteristics at baseline.

	Placebo (*n* = 64)	Lebrikizumab (*n* = 191)
Age, mean (SD), years	48.9 (13.7)	48.4 (12.8)
Female, *n* (%)	41 (64.1)	112 (58.6)
Baseline ICS dose ≥1,000 μg/day, *n* (%)	14 (21.9)	65 (34.0)
Pre-bronchodilator FEV_1 (% of predicted), mean (SD)	61.3 (10.7)	62.5 (10.2)
Time in placebo-controlled period, median (range), weeks	28.6 (19.0–48.1)	32.1 (19.3–49.0)
Blood eosinophils, mean (SD), 10^9/L	0.32 (0.29)	0.30 (0.27)
ECP, mean (SD), ng/mL	27 (29)	25 (24)
EDN, mean (SD), ng/mL	57.2 (35.4)	55.7 (30.6)

There were no significant differences in these characteristics between placebo and lebrikizumab treatment arms.
ICS, inhaled corticosteroids; FEV_1, forced expiratory volume in 1 second; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin.

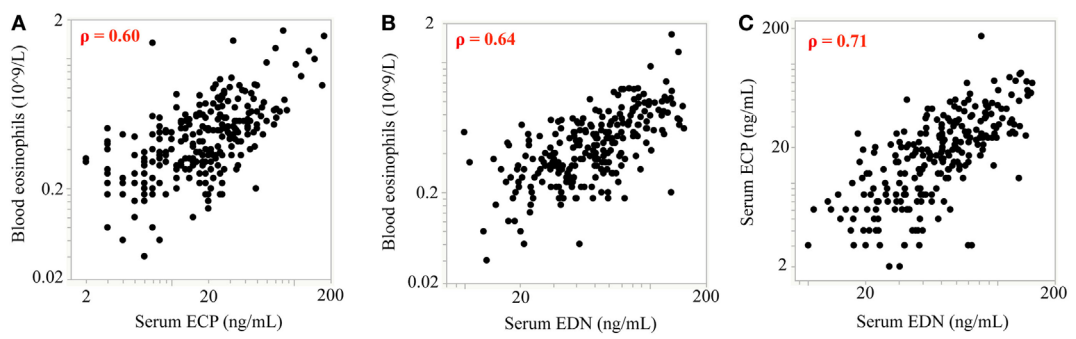

FIGURE 3 | Baseline measurements of blood eosinophil counts are positively correlated with eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) levels. At baseline, there was a strong positive intercorrelation between blood eosinophils and **(A)** serum ECP and **(B)** serum EDN. **(C)** ECP and EDN levels were also strongly positively correlated. Spearman's correlation coefficient was employed for statistical analysis. For all correlations $p < 0.0001$; rho (ρ) values are shown on each plot.

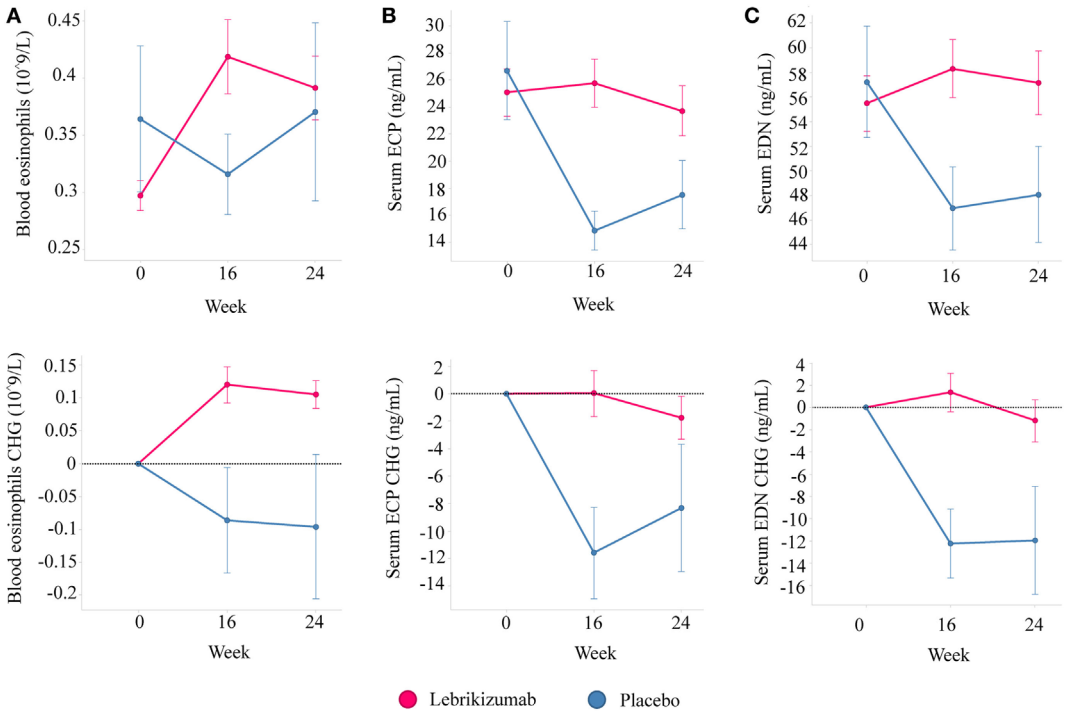

FIGURE 4 | Lebrikizumab treatment increases peripheral blood eosinophil counts but circulating levels of eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) remain unchanged. **(A)** Absolute levels and changes from baseline show increased blood eosinophils in patients treated with lebrikizumab at 16 and 24 weeks [mean change of 0.12 × 10^9/L (95% CI 0.08–0.16) and 0.10 × 10^9/L (95% CI 0.06–0.15) respectively, $p < 0.001$] but no significant change in the placebo arm was observed. **(B)** Absolute levels and changes from baseline show no change in serum ECP in patients treated with lebrikizumab but a decrease in patients in the placebo arm at 16 and 24 weeks [mean change of −11.59 ng/mL (95% CI −18.80 to −4.38), $p = 0.018$ and −8.31 ng/mL (95% CI −16.71 to 0.08), $p = 0.052$ respectively]. **(C)** Absolute levels and changes from baseline show no change in serum EDN in patients treated with lebrikizumab but a decrease in patients in the placebo arm at 16 and 24 weeks [mean change of −12.25 ng/mL (95% CI −19.33 to −5.17), $p = 0.0008$ and −11.94 ng/mL (95% CI −20.25 to −3.64), $p = 0.0051$, respectively]. Graphs show mean ± SE and paired Student's *t*-test were carried out (CHG, change).

role of IL-13 in the number and activation status of eosinophils in asthma. Further investigation is also required to definitively address what are the consequences, if any, of increased blood eosinophils in asthma patients in response to anti-IL-13 therapies.

Targeting IL-13 in Asthma

Moderate to severe asthma patients with poorly controlled disease represent a substantial unmet medical need. Compelling evidence for the role of IL-4 and IL-13 in driving type 2/eosinophilic asthma has led to the development of several therapeutic candidates to target these pathways (**Figure 1**; **Table 2**). Lebrikizumab is a humanized monoclonal antibody that binds to soluble IL-13 with high affinity and blocks signaling through the IL-4Rα/IL-13Rα1 heterodimer. Phase 2 clinical trials assessing lebrikizumab in moderate to severe uncontrolled asthma showed that treatment significantly improved lung function (49) and reduced the rate of exacerbations (47), compared to placebo. However, in two Phase 3 studies conducted in parallel there were inconsistent results; LAVOLTA I reported a significant reduction in exacerbations in lebrikizumab-treated patients compared to placebo but this did not replicate in LAVOLTA II. However, lung function as determined by forced expiratory volume in 1 second (FEV_1) improvement was observed in both studies (70). Tralokinumab, a human IL-13-neutralizing monoclonal antibody blocking binding of IL-13 to both IL-13Rα1 and IL-13Rα2, has been assessed in clinical trials of moderate to severe uncontrolled asthma patients. A Phase 2 study investigating different dosing regimens of tralokinumab reported a trend for improved lung function after 16 weeks of treatment, but no change in asthma control questionnaire (ACQ)-6 score (71). A subsequent Phase 2b trial investigating two and four weekly dose regimens found that there was no significant reduction in asthma exacerbation rates but patients dosed every 2 weeks showed an improvement in lung function (48). Phase 3 studies to evaluate the efficacy and safety of tralokinumab in adults and adolescents with inadequately controlled asthma are currently underway (NCT02161757, NCT02449473, and NCT02281357). Dupilumab, a humanized monoclonal antibody to IL-4Rα that inhibits both IL-4 and IL-13 signaling, is being assessed in patients with uncontrolled asthma. An initial study evaluating the efficacy of dupilumab was carried out in persistent, moderate-to-severe asthma patients with elevated eosinophil levels (blood eosinophil count of at least 300 cells/mL or sputum eosinophil levels of at least 3%). In this study, patients on background ICS and long-acting beta-agonist (LABA) therapy were randomized to receive dupilumab or placebo and background treatment was withdrawn. In this context,

TABLE 2 | IL-13-targeted therapies in eosinophilic diseases.

Drug (company)	Mechanism of blocking IL-13	Disease—target patients	Clinical trial outcome
Lebrikizumab (Roche)	Binds soluble IL-13 blocking IL-13Rα1 signaling	Asthma—moderate to severe	• Phase III—inconsistent results across 2 studies. Study 1—reduced exacerbations and improved FEV_1. Study 2—no statistical significant effect on exacerbations but improved FEV_1 (60)
		AD—moderate to severe	• Ongoing Phase II trial
Tralokinumab (Medimmune/ AstraZeneca)	Binds soluble IL-13 blocking both IL-13Rα1 and IL-13Rα2 signaling	Asthma—moderate to severe	• Phase IIb—no effect on exacerbations but improved FEV_1 (48) Ongoing Phase III trials
		AD—moderate to severe	• Phase IIb—improvement in EASI, SCORAD and DLQI
Dupilumab (Regeneron/ Sanofi)	Binds IL-4Rα blocking both IL-4 and IL-13 signaling	Asthma—uncontrolled	• Phase IIb—reduced exacerbations and improved FEV_1 (62) Ongoing Phase III trial
		AD—moderate to severe	• Phase III—improved EASI, IGA and symptoms of depression and anxiety (95)
		EoE—active, moderate to severe	• Ongoing phase II trial
		CRSwNP—refractory to intranasal corticosteroids	• Phase II—reduced endoscopic nasal polyp burden (125) Ongoing Phase III trial
QAX576 (Novartis)	Monoclonal antibody binding to IL-13	EoE	• Primary end point not met but decreased esophageal eosinophil counts
RPC4046 (Celgene)	Blocking both IL-13Rα1 and IL-13Rα2	EoE	• Ongoing Phase II trial

IL, interleukin; FEV_1, forced expiratory volume in 1 second; AD, atopic dermatitis; EASI, Eczema Area Severity Index; SCORAD, Scoring Atopic Dermatitis; DLQI, Dermatology Life Quality Index; EoE, eosinophilic esophagitis; CRSwNP, chronic rhinosinusitis with nasal polyps; IGA, Investigator's Global Assessment.

dupilumab treatment led to a reduction of asthma exacerbations and improved lung function compared to placebo (50). A pivotal Phase 2b 24-week study in persistent, moderate-to-severe asthmatics on background ICS in which patients were enrolled irrespective of baseline eosinophil levels reported lung function improvement and a relative annualized exacerbation rate reduction in both eosinophil high and low patients. However, the lung function improvement and absolute exacerbation rate reduction were greater in the eosinophil high group (72). Phase 3 studies to evaluate the efficacy and safety of dupilumab in patients with persistent asthma (NCT02414854 and NCT02528214) are ongoing.

While clinical trials of lebrikizumab, tralokinumab, and dupilumab targeting IL-13 had an acceptable overall safety profile, increases in blood eosinophil numbers were reported for each intervention (48, 70, 72). It is hypothesized that elevated blood eosinophil levels may be a result of reduced trafficking of eosinophils from the circulation to the airway and/or other tissues, where they can exert their pathogenic effects, due to decreased expression of IL-13-induced chemokines. Indeed, unpublished data from our preclinical studies testing the efficacy of anti-IL-13 in a mouse model of asthma found this to be the case. C57/B6 mice were challenged with the house dust mite extract, *Dermatophagoides farinae*, and treated prophylactically with either anti-IL-13 or an isotype control antibody. Administration of anti-IL-13 resulted in decreased BAL eosinophilia compared to control, however, there was concomitant upregulation of blood eosinophils in the anti-IL-13-treated mice but not in the controls. Eosinophil dynamics upon treatment with anti-IL-13 and anti-IL-5 have also been studied using a mathematical model. The model incorporated levels of eotaxin and periostin as chemoattractants for eosinophils to the lung. It predicted that treatment with anti-IL-13 would result in a decrease in lung eosinophils and an increase of blood eosinophils while anti-IL-5 treatment would result in a decrease in both blood and airway eosinophils (73). Of note, treatment with the anti-IL-5 therapy, mepolizumab, consistently leads to decreased blood eosinophil levels. However, differential reductions in airway eosinophils have been observed depending on which compartment of the lung is being sampled. Sputum eosinophil levels decreased significantly in response to mepolizumab treatment but tissue eosinophil numbers did not (74, 75). Further investigation of eosinophil dynamics in humans is required to confirm these animal data and modeled predictions, and several studies are ongoing. To evaluate the effect of blocking IL-13 on human airway eosinophil dynamics, studies with lebrikizumab (NCT02099656) and tralokinumab (NCT02449473) are being conducted in inadequately controlled asthmatics. In addition, the effect on inflammatory cells in the airway after blocking IL-4 and IL-13 signaling by dupilumab is being examined in patients with persistent asthma (NCT02573233). The results of these studies should shed significant light on the relationships between IL-13 and airway eosinophilia and other pathologies in asthma patients *in vivo*.

ATOPIC DERMATITIS

Atopic dermatitis is the most common recurring inflammatory skin disease in children, with an average world prevalence of 7.9% in 6–7-year olds (76). Disease manifestations include dry skin, eczematous lesions, intense pruritus, and high serum IgE levels. In AD, compromised epidermal barrier function leads to enhanced allergen penetration and systemic IgE sensitization. Patients with AD exhibit blood eosinophilia (77), eosinophil infiltrates in skin lesions (78), and deposition of eosinophilic granule proteins (79).

IL-13 and Eosinophils in AD

Signatures of responsiveness to Th2, Th22, Th17, and Th1 cytokines are associated with AD skin at various stages of the disease. In particular, the Th2 cytokines IL-4 and IL-13 have

been shown to play central roles in AD by modulating the epidermal barrier, including suppression of keratinocyte epidermal differentiation complex (EDC) genes (80) and inhibition of antimicrobial peptide production (81, 82). IL-13 mRNA has been shown to positively correlate with AD disease severity in acute and chronic lesional skin (83–85). Patients with AD have higher levels of serum IL-13 compared to healthy controls (58, 86). Children with more severe AD exhibited a higher percentage of IL-13-expressing $CD4^+$ T cells in peripheral blood (87). *In vitro* treatment of normal human epidermal keratinocytes with IL-13 led to increased expression of a key chemokine for eosinophil recruitment, CCL26 (88). In mice, transgenic overexpression of IL-13 in the skin induced key features of AD, including pruritus, elevated IgE, and eosinophilic infiltration. There were also elevated levels of eosinophil chemoattractants such as CCL11 in the skin, driving recruitment of eosinophils from the blood to the tissue. This established a clear role for IL-13 in AD (89). IL-4 and IL-13 share overlapping biological functions and pathophysiological roles in AD in part due to the shared use of the IL-4Rα/IL-13Rα1 receptor complex and subsequent signaling through STAT6. Mice constitutively expressing active STAT6 (Stat6VT) were found to develop spontaneous AD-like disease with decreased EDC gene expression and increased IL-4, IL-13, and eosinophils in the lesional skin. IL-4 deficiency in these mice (IL-$4^{-/-}$ Stat6VT) attenuated development of allergic skin disease and eosinophilic inflammation, while therapeutic blockade with anti-IL-13 in the Stat6VT mice led to the rescue of EDC gene expression (90). Similarly, blockade of IL-13 by topical delivery of IL-13 antisense oligonucleotides reduced AD-related cytokines, IgE, and inflammatory cells in the skin in an epicutaneous OVA sensitization model (91). As described earlier, IL-13 also binds to IL-13Rα2, a decoy receptor that lacks an intracellular signaling motif and which may serve as a negative feedback regulator of IL-13 signaling. Keratinocytes from lesional skin of AD patients showed elevated expression of IL-13Rα2 (92). IL-13 also induced the expression of IL-13Rα2 in human keratinocytes in a STAT6-dependent manner (93). Consistent with its role as a decoy receptor, mice deficient for IL-13Rα2 showed increased transepidermal water loss, skin inflammation, peripheral eosinophilia, and IgE in a model of AD compared to control mice (94).

Targeting IL-13 in AD

Given the strong biologic rationale various companies have moved forward with therapeutic candidates targeting type 2 cytokines in AD (**Table 2**). Dupilumab was investigated in patients with moderate to severe AD inadequately controlled by topical treatment. In two Phase 3 trials, dupilumab improved the signs and symptoms of AD, anxiety and depression, and quality of life compared to placebo (95). These results further validate the hypothesis that the type 2 cytokines IL-4 and IL-13 are key drivers of AD. Of note and similar to therapies targeting the IL-13 pathway in asthma, these trials reported elevated blood eosinophil levels in patients treated with dupilumab compared to placebo. Biologics specifically targeting IL-13 have completed Phase 2 studies for the treatment of AD. In an ongoing study, lebrikizumab was evaluated in patients with persistent, moderate to severe AD inadequately controlled by topical corticosteroids (TCS) (NCT02340234). Efficacy of tralokinumab was assessed in patients with moderate to severe AD on a background of TCS (NCT02347176) and showed statistically significant improvements in symptoms of AD. These results support a key role of IL-13 signaling in AD pathophysiology. However, due to the differences and limitations in trial designs, the relative contributions of IL-4 vs. IL-13 and a role of IL-13Rα2 in human AD could not be fully elucidated and will require further investigation.

EOSINOPHILIC ESOPHAGITIS

Eosinophilic esophagitis is a chronic inflammatory disease of the esophagus. It is one of the most common conditions diagnosed during the assessment of feeding problems in children and dysphagia and food impaction in adults (96). EoE occurs worldwide with increasing prevalence, currently at 0.4% in Western countries (97). The diagnosis of the EoE has been challenging. Two key components are required: (i) clinical symptoms including feeding problems, vomiting, and abdominal pain in children, and dysphagia and food impaction in adolescents and adults and (ii) histological evaluation of 15 or more eosinophils per high-powered field in esophageal mucosal biopsy following treatment with proton pump inhibitors (98).

IL-13 and Eosinophils in EoE

Defective barrier function, evidenced by thickening of the mucosal basal layer, dilated interepithelial spaces and altered epithelial barrier function have been observed in esophageal tissues from patients with EoE (99). The resulting increased epithelial permeability is believed in turn to enhance antigen presentation and eosinophil recruitment. Both environmental and genetic predispositions modulate immune responses that play an important role in the pathogenesis of EoE. Type 2 responses induced primarily by food antigens have been thought to be a major driver of the disease. In particular, IL-13 is overexpressed in biopsies from patients with EoE (100). IL-13 has been shown to affect epithelial barrier function by downregulating EDC genes such as filaggrin (*FLG)* (101). *In vitro* studies found that IL-13 upregulates IL-5, CCL26, and other related cytokines that contribute to eosinophilia (101, 102). Furthermore, a genome-wide association study (GWAS) revealed a potent IL-13 inducer, *TSLP*, and IL-13 downstream response genes, *CCL26* and calpain 14 (*CAPN14*) were associated with EoE (102–105). EoE transcriptome signatures identified by microarray and RNA-seq analyses for dysregulated genes in the esophagi of patients with EoE revealed a significant involvement of IL-13 and exhibited a striking degree of overlap with the gene expression pattern observed in endobronchial biopsies of "Th2-high" asthma patients (106–108). The long non-coding RNA *BANCR* is induced by IL-13 and its expression correlates with levels of eosinophils and transcripts known to be involved in EoE pathogenesis. Another transcriptional target of IL-13, neurotropic tyrosine kinase receptor type 1 (NTRK1), was upregulated in EoE esophageal tissues. This upregulation is believed to cause enhanced responsiveness of epithelial cells to NGF, a ligand of NTRK1, and the subsequent induction of the eosinophil chemokine, CCL26 (109).

Targeting IL-13 in EoE

Several biologics targeting IL-13 have been tested in clinical trials for the treatment of EoE (**Table 2**). QAX576, an anti-IL-13 monoclonal antibody, was tested in a small cohort of patients with proton pump inhibitor-resistant EoE for its efficacy in reducing peak eosinophil counts in the esophageal tissue after 8 weeks of treatment. The primary end point was not met; nevertheless, QAX576 reduced esophageal eosinophil counts by 60% compared to an increase of 23% with placebo and there was a slight trend toward improved symptoms. Transcriptomics were also carried out on biopsy specimens collected on day 85 of the study and showed that EoE-related genes were downregulated, including the eosinophil chemoattractant CCL26, which suggests that IL-13 is a significant driver of the differential gene expression observed in EoE. QAX576 had no effect on blood eosinophil counts (110). RPC4046, an anti-IL-13 monoclonal antibody that blocks both IL-13Rα1 and IL-13Rα2, is currently being studied in a dose ranging Phase 2 study in EoE with mean eosinophil count as a primary outcome (NCT02098473). The efficacy of dupilumab is also being investigated in a Phase 2 trial in patients with active, moderate to severe EoE. However, the primary outcome for this trial is change in the Straumann Dysphagia Instrument (SDI) patient-reported outcome (PRO) score and changes in eosinophil counts will be evaluated as one of the secondary end points (NCT02379052).

CRS WITH NASAL POLYPS

Chronic rhinosinusitis is an inflammatory pathological condition of the nose and paranasal sinuses. Patients with CRS are characterized by nasal obstruction, drainage, compromised olfaction, and prolonged facial pain or pressure (111). CRS is classified into two subtypes: CRS without nasal polyps (CRSsNP) and CRS with nasal polyps (CRSwNP) (112). In the United States and Europe, the majority of CRSwNP patients have significant eosinophilic infiltration in their polyp tissue (113).

IL-13 and Eosinophils in CRSwNP

Eosinophilic CRSwNP (ECRSwNP) has been increasing in prevalence worldwide, estimated to be 2.1%–2.7% in adults (114–116). It represents a recalcitrant form of the disease resistant to medical or surgical intervention (113). ECRSwNP is characterized by type 2 inflammation with elevated levels of IL-5, IL-13, and eosinophils in the polyp tissue (117). Th2 cells, ILC2s, mast cells, and eosinophils are hypothesized to be the major sources of type 2 cytokines in ECRSwNP. Similar to asthma, AD, and EoE, it is believed that IL-4 and IL-13 play important roles in the pathophysiology of ECRSwNP. IL-13 has been shown to affect the integrity of the sinonasal epithelial barrier by inhibiting the expression of tight junction proteins (118) and antimicrobial peptide production (119). The expression of eosinophil chemoattractants, such as CCL11, CCL24, and CCL26, are elevated in ECRSwNP (120–122). *In vitro*, IL-4 or IL-13 in combination with TNF induced elevated expression of CCL11 in fibroblasts and airway epithelial cells derived from these patients, suggesting a positive feedback loop between eosinophil recruitment and type 2 inflammation (123). Transcriptomic analysis of RNA-seq data comparing nasal polyps from eosinophilic and non-ECRSwNP and nasal mucosa from control subjects revealed distinct expression profiles between these subgroups. Notably, IL-13 and CCL26 are specifically overexpressed in ECRSwNP, along with other chemokines that mediate eosinophilic inflammation (124).

Targeting IL-13 in Nasal Polyps

There are currently no biologic therapies approved for the treatment of nasal polyps. Dupilumab has been evaluated in a Phase 2 clinical trial for its efficacy in patients with chronic sinusitis and nasal polyposis refractory to intranasal corticosteroids and showed encouraging results (125). Patients who received dupilumab together with mometasone furoate nasal spray experienced reduced endoscopic nasal polyp burden compared to patients that received mometasone plus placebo (**Table 2**). Longitudinal analysis of plasma CCL26 showed a decrease after dupilumab treatment, while there was a trend toward decreased serum CCL17 levels. A transient increase in blood eosinophils was observed in response to treatment in some patients. Phase 3 clinical studies are currently underway (NCT02912468, NCT02898454).

CONCLUSION

Asthma and other eosinophilic disorders such as AD, EoE, and CRSwNP are highly prevalent and numbers of people affected are continuing to increase. There are subsets of patients with each of these conditions that do not respond adequately to standard of care, experience significant morbidity, consume substantial healthcare expenditures, and thus represent an unmet clinical need.

Molecular phenotyping has been instrumental in understanding the underlying biology of these diseases. As such, we are now beginning to understand the roles of various cytokines and immune effector cells, in particular type 2 cytokines and eosinophils. IL-5 was identified as the key driver of eosinophilopoiesis, leading to the development of multiple therapies targeting IL-5 and eosinophils. The efficacy of these drugs indicates the vital role of eosinophils in diseases such as asthma. In addition, substantial evidence exists from *in vitro* cell culture models, *in vivo* animal models and observational human studies for the role of IL-13 (and IL-4) in driving eosinophilia and type 2 inflammation, which led to interventional studies targeting these pathways in human disease. Biologics targeting the IL-13 pathway have demonstrated efficacy, particularly in subsets of patients with evidence of eosinophilic disease. Ongoing clinical trials will help to further dissect the contributions of IL-13 to tissue eosinophilia. Notably, blood eosinophil counts themselves are used as a biomarker in many of these clinical studies. Eosinophil "high" patients have experienced greater clinical benefit from anti-type 2 therapies compared to eosinophil "low" patients. This further necessitates the need to fully understand the role of eosinophils in type 2 driven diseases, as well as define the mechanisms contributing to disease pathology in patients with low levels of type 2 inflammation and eosinophils.

The advent of better mouse models and ongoing clinical trials targeting multiple pathways, including IL-13, will help garner a

better understanding of eosinophil biology and improve therapeutic strategies for treating eosinophilic disorders in the future.

AUTHOR CONTRIBUTIONS

ED, CH, KW, and JA made substantial contributions to important intellectual content, drafting of the manuscript and revisions. FC and JB also carried out experiments, analyzed and interpreted data. All authors approved the final version of the manuscript.

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MINI REVIEW
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Platelet–Eosinophil Interactions As a Potential Therapeutic Target in Allergic Inflammation and Asthma

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The importance of platelet activation during hemostasis is well understood. An understanding of these mechanisms has led to the use of several classes of anti-platelet drugs to inhibit aggregation for the prevention of thrombi during cardiovascular disease. It is now also recognized that platelets can function very differently during inflammation, as part of their role in the innate immune response against pathogens. This dichotomy in platelet function occurs through distinct physiological processes and alternative signaling pathways compared to that of hemostasis (leading to platelet aggregation) and is manifested as increased rheological interactions with leukocytes, the ability to undergo chemotaxis, communication with antigen-presenting cells, and direct anti-pathogen responses. Mounting evidence suggests platelets are also critical in the pathogenesis of allergic diseases such as asthma, where they have been associated with antigen presentation, bronchoconstriction, bronchial hyperresponsiveness, airway inflammation, and airway remodeling in both clinical and experimental studies. In particular, platelets have been reported bound to eosinophils in the blood of patients with asthma and the incidence of these events increases after both spontaneous asthma attacks in a biphasic manner, or after allergen challenge in the clinic. Platelet depletion in animal models of allergic airway inflammation causes a profound reduction in eosinophil recruitment to the lung, suggesting that the association of platelets with eosinophils is indeed an important event during eosinophil activation. Furthermore, in cases of severe asthma, and in animal models of allergic airways inflammation, platelet–eosinophil complexes move into the lung through a platelet P-selectin-mediated, eosinophil β1-integrin activation-dependent process, while platelets increase adherence of eosinophils to the vascular endothelium *in vitro*, demonstrating a clear interaction between these cell types in allergic inflammatory diseases. This review will explore non-thrombotic platelet activation in the context of allergy and the association of platelets with eosinophils, to reveal how these phenomena may lead to the discovery of novel therapeutic targets.

Keywords: platelets, eosinophils, asthma, P-selectin, allergy, IgE

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INTRODUCTION

Platelets are small, anuclear cell fragments that are essential for hemostasis. Activation of platelets during hemostasis leads to shape change, α-granule, dense δ-granule, and lysosomal λ-granule release, along with surface expression of adhesion molecules and receptors, leading to platelet aggregation and clot formation (1–3). In addition to these hemostatic responses, it is now understood that platelets

contribute distinct functions to non-thrombotic processes such as innate immunity to pathogens, and inflammatory disorders where there is often no associated changes in hemostatic functions of platelets. The difference in platelet function in hemostasis compared with inflammation has led to the hypothesis that there is a dichotomy in platelet activation (4), which has recently been confirmed experimentally (5, 6). It is necessary to comprehend such a distinction when analyzing the relationship platelets have with eosinophils in the context of allergic inflammation and host defense.

Platelets express an array of physiologically relevant and functional receptors that might be considered relevant to the inflammatory response in asthma and allergic inflammation, including chemokine receptors (CCR1, CCR3, CCR4, and CXCR4 receptors) (7), immunoglobulin receptors (FcγRI, FcγRII, FcγRIII; FcεRI, FcεRII, FcαRI/CD89) (8–11), toll-like receptors (TLR2, TLR4, and TLR9) (12), and certain adhesion molecules (PSGL-1, P-Selectin, and ICAM-2) (10, 13). Platelets also store inflammatory mediators in granules that can be released on activation, such as platelet factor-4 (PF4, CXCL4) (14), interleukin-1β (IL-1β) (15, 16), regulated upon activation normally T-cell expressed and secreted (RANTES, CCL5), and thymus activation regulated chemokine (CCL17) (3, 17). Therefore, platelets indeed possess the requisite components to behave as inflammatory cells, as has been demonstrated in allergic diseases including asthma, allergic rhinitis, and eczema. This short review will examine non-thrombotic platelet activation in asthma and allergic inflammation, and the association of platelets with eosinophils in these disease states, which are perhaps an inappropriate manifestation of interactions between platelets and eosinophils that occur as part of host defense against parasitic infections.

PLATELETS IN ASTHMA AND ALLERGIC INFLAMMATION

Evidence has suggested platelet activation occurs in allergic diseases since the 1970s (18). We refer the reader to recent extensive reviews on the implications of platelet activation in asthma and give a summary below (19, 20). Following bronchial provocation of patients with asthma, there is an increased release of platelet-specific chemokines, for example, PF4 and beta-thromboglobulin (β-TG, CXCL7) (21), and mediators derived from platelets, for example, 5-hydroxytriptamine (5-HT) (22), free radical species (23), and RANTES (9). Thus, the activation of platelets *in vivo* from patients with asthma is demonstrated by the fact that *ex vivo* analysis of platelets from subjects with asthma have a diminished store of mediators, which has been linked to an apparent lack of *in vitro* responsiveness due to prior activation *in vivo*: the so-called "platelet exhaustion" (24, 25). Patients with asthma have also been reported to exhibit mild thrombocytopenia after allergen provocation (26–28) and to have shortened platelet lifespans in the circulation compared with healthy individuals (29), demonstrating that continuous platelet activation may occur as part of this disease. The mild thrombocytopenic effects observed within minutes after allergen exposure in patients with asthma suggests that platelets are recruited to the lungs (26, 28). Indeed, platelets are present in bronchoalveolar lavage fluid and in bronchial biopsies of patients with asthma. In particular, platelets are found in extravascular compartments and also in fibrous material within the airway luminal edge, indicating platelets have the ability to migrate into inflamed tissue (30, 31). Platelet chemotaxis toward inflammatory chemokines, formyl-methionyl-leucyl phenylalanine (fMLP), macrophage-derived chemokine (CCL22), and stromal cell-derived factor 1α (SDF-1_{α}, CXCL12) has been demonstrated *in vitro*, through *N*-formyl-peptide (32), CCR4 and CXCR4 receptor activation, respectively (33, 34). These migratory effects may well be attributed to the fact that platelets can release enzymes contributing to movement such as cathepsin D, cathepsin E, heparinase, and β-N-acetylhexosaminidase and possess the necessary machinery to extend pseudopod-like processes and undergo actin cytoskeleton rearrangements, indicating that platelets have the mechanical capability to migrate in response to situations caused by allergic inflammation (34, 35).

Platelets and Cellular Motility

Indeed, platelets have been shown to undergo chemotaxis directly toward allergen *in vitro* and migration through lung tissue *in vivo via* an IgE-/FcεRI-dependent mechanism (11). The induction of cellular chemotaxis by allergen appears to be atypical of how other molecules induce chemotaxis *via* GPCRs, although chemotaxis can be induced by structurally very varied molecules. It has been reported that basophils (36), mast cells (37, 38), and eosinophils (39) can undergo chemotaxis to specific allergens. These studies investigated a range of allergens and laboratory antigens and reported that cellular chemotaxis toward allergen was also IgE and FcεRI dependent (11, 37, 38), while others reported an affect *via* formyl peptide receptors (40). Clearly, the molecular mechanisms are not as well understood as chemotaxis induced by *via* GPCR activation. It is also interesting that FcεRI signaling can modify, or be modified by GPCRs, including certain chemokine receptors (41, 42). The significance of this is unknown with respect to cell motility. While it is speculative to consider whether platelet motility toward allergen might therefore modify eosinophil chemotaxis, platelets have recently been reported to be important for the intravascular crawling of neutrophils (43, 44), and platelet-dependent neutrophil chemotaxis to a range of chemokines *in vitro* (5, 45–48), and platelets might have a similar relationship with eosinophils.

Platelets in Bronchoconstriction

It has been postulated that the platelets found within the airways of patients with asthma could propagate disease progression *via* various mechanisms. In patients with asthma, platelets can become activated following exposure to allergens, endotoxins, pollutants, and inflammatory mediators (19). The consequence of this is unknown. However, in models of allergic airway inflammation in rabbits and guinea pigs, platelet depletion prevented bronchoconstriction induced by certain substances such as bradykinin and capsaicin (49, 50), but had no effect on direct acting spasmogens such as histamine, substance P, and methacholine. Human platelets are able to produce a number of bronchoactive mediators within their granules such as histamine,

serotonin, thromboxane A_2 (TXA_2), adenosine, 12-HETE, and cytotoxic compounds (19).

Thus, platelets that have accumulated within the lung may release stored spasmogens and affect the action of other spasmogens leading to bronchoconstriction. Furthermore, agents released by platelets might stimulate eosinophils *in situ* to cause eosinophil-dependent bronchoconstriction (19, 51, 52).

Airway Remodeling

In bronchial asthma, chronic inflammation can alter the airway architecture that contributes to adverse effects on respiratory function. Platelet activation has been shown to persist long after the initial allergen challenge and outlasts the presence of platelet–leukocyte conjugates in the blood, displaying a potential role for platelets in chronic airway remodeling (28). Platelets produce mitogens such as TXA_2, transforming growth factor-β, platelet-derived growth factor, epidermal growth factor, and vascular endothelial growth factor, which can have proliferative actions on cells located in the airways (19). Platelet depletion in a mouse model of allergic airways inflammation decreased epithelial thickening, smooth muscle thickening, and sub-epithelial reticular fiber deposition (53). Thus, platelet activation appears to play an important role in airway remodeling by release of extracellular matrix modifying enzymes and hypertrophic factors, causing smooth muscle hyperplasia and collagen deposition. While some of these events appeared to be independent of leukocyte activity (53), the association of platelets with eosinophil activation discussed in the following sections implies that eosinophil-associated remodeling events might be dependent on platelet activation.

PLATELET–EOSINOPHIL INTERACTIONS FORM A PART OF HOST DEFENSE AGAINST PARASITE INFECTIONS

Platelet participation is necessary during our immune defense against pathogens of bacterial, viral, and fungal origin (54), and platelet activation also occurs in immunity against parasites, for example, helminths (Schistosomiasis), protozoa (toxoplasmosis), and malaria (54–57). In particular, a role of eosinophils in helminth infections remains an important area of study (58, 59). There are therefore correlations in platelet and eosinophil activities, and that of their activation by IgE in IgE-dependent killing of schistosomes that are applicable to our understanding of the association between these two cell types in allergy (23, 58). While platelets express other immunoglobulin receptors (FcγRI, FcγRII, FcγRIII; FcαRI/CD89), the roles of these receptors in platelet responses to infection, and possible platelet effects on eosinophils have not been as extensively reported. Furthermore, it is possible that platelet–eosinophil interactions during host defense occur *via* non-immunoglobulin-associated activation pathways. We therefore discuss below a data set that is restricted to IgE/IgE receptor interactions as one example by which platelets and eosinophils can interact with each other, and which may have implications in allergic diseases. Human platelets express both high-affinity (8) and low-affinity (60) IgE receptors (FcεR1 and FcεRII or CD23, respectively). The level of expression of FcεR1 and CD23 are variable, and they are not found on all platelets from a given donor, platelet stimulation by IgE or allergen interactions can cause non-thrombotic platelet activation leading to release of cytotoxic mediators, such as reactive oxygen metabolites, free radicals, and catonic proteins (8, 61). While the physiological consequence of platelet-derived cytotoxic substances, compared to other cellular sources of the same or similar material is not known, the expression of IgE receptors on platelet surfaces was demonstrated to be important for host defense against parasitic infections as originally demonstrated in the seminal work of Capron and Joseph in the 1980s, who showed that IgE receptor stimulation of platelets was essential in killing certain types of parasites, by stimulating this generation and release of cytotoxic free radicals (8, 61). Various lines of enquiry have revealed that platelets can release chemokines in response to IgE stimulation (for example, RANTES and PF-4) that are potent toward eosinophils (62–65); and these also stimulate eosinophil free radical oxygen products (66). Platelets have been reported to release GM-CSF and thus inhibit eosinophil apoptosis, prolonging survival (67). Early studies related to the mechanism by which eosinophils engaged with *Schistosoma mansoni* revealed that selectin and Lewis X-related structures might act as coreceptors for eosinophil-mediated killing of worms (68). P-selectin mediates adhesion of platelets to eosinophils (69), and PSGL-1–P-selectin interactions between platelets and eosinophils can lead to CD18-dependent eosinophil stable adhesion (70). It is therefore of interest that there are perhaps parallels with how platelets enhance neutrophil responses to pathogens and NET formation *via* P-selectin interactions (19). Thus, it would appear that platelets have the capacity to enhance eosinophil functions against parasitic infections (see **Figure 1**).

The relationship between platelets and eosinophils is symbiotic. Eosinophils also release products that can potentially stimulate platelets, for example, platelet-activating factor (PAF) (71). Although the physiological consequences are unclear, an early *in vitro* investigation by the group of Gerald Gleich reported that eosinophil granule proteins, such as major basic protein (MBP) and eosinophil peroxidase (EPO) are very potent at inducing platelet α granule (β-TG), dense granule (5-HT), and lysosomal granule (β-*N*-acetylglucosaminidase) release, whereas eosinophil-derived neurotoxin and eosinophil cationic protein (ECP) had no effect (72). It was also noted that MBP and EPO stimulation of platelets was different in nature to thrombin stimulation (72). It is therefore of interest that a recent study reported that platelet aggregation was inhibited by eosinophil supernatant, and ECP in particular (73). The implications of platelet activation being induced by eosinophil-derived mediators on the one hand and inhibition of platelet aggregation to agonists by eosinophil-derived mediators on the other is difficult to interpret. It is possible that eosinophils activate or prime platelets for functions unrelated to aggregation, thereby revealing evidence for the "dichotomy in platelet function" (4). Alternatively, it is possible that different mediators derived from eosinophils promote selective functions of platelets, since there is an association of eosinophils with thrombi in patients with acute myocardial infarction (74, 75). However, the role of eosinophils in thrombosis, and a

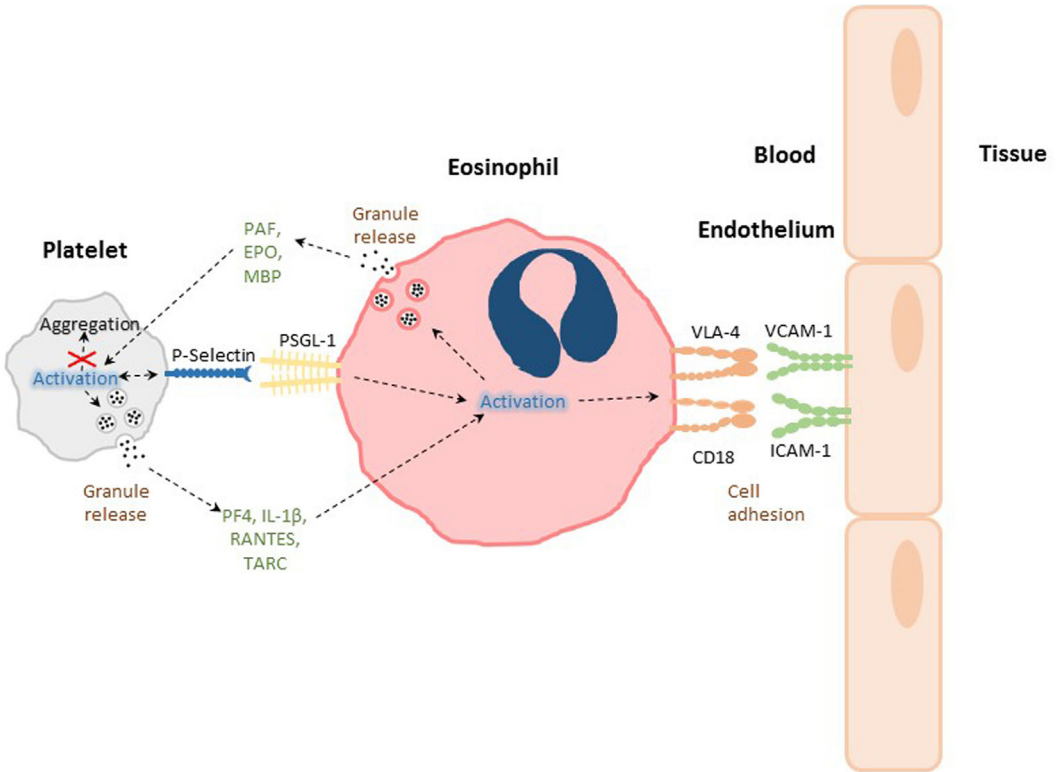

FIGURE 1 | The interactions between platelets and eosinophils. Platelets and eosinophils can directly interact with one another *via* both contact-dependent (e.g., P-selectin/PSGL-1) and soluble mediator-dependent [eosinophil: platelet-activating factor (PAF), eosinophil peroxidase (EPO), major basic protein (MBP); platelet: PF-4, RANTES, IL-1β, thymus activation regulated chemokine (TARC)] mechanisms. The activation of platelets during allergic airways inflammation leads to platelet P-selectin-dependent eosinophil tissue recruitment, and activation eosinophils to express integrins very late antigen-4 (VLA-4) and CD18, or to release granule products. Granule constituents of eosinophils can likewise induce platelet activation and functions pertinent to inflammation and host defense, rather than aggregation, associated with intravascular thrombi formation.

causal link to platelet activation (aggregation) has not been made, although patients with allergies have been reported to have less calcification of the major arteries, suggesting allergy might be protective (76).

PLATELET–EOSINOPHIL INTERACTIONS IN ASTHMA AND ALLERGY

Eosinophilic inflammation is associated with atopic asthma, rhinitis, and aspirin-induced exacerbated respiratory disease (AERD) (77–79), therefore the interactions between eosinophils and platelets that can occur during host defense may be important for the pathogenesis of these respiratory conditions. Lellouch-Tubiana and colleagues first demonstrated in an allergic guinea pig model of asthma that eosinophil infiltration into the lung is reduced when platelet numbers are depleted in the circulation using an anti-platelet antiserum (APAS) (80). These findings were later supported by data which showed that platelet depletion *via* APAS, caused a reduction in eosinophil infiltration, and decreased hyperresponsiveness into the lungs of allergic rabbits and mice (49, 81). Subsequently, correlations between eosinophil and platelet activity have been reported in patients with asthma. Nasal wash levels of ECP and P-selectin as measures of activation of eosinophils and platelets, respectively, revealed a positive association between eosinophils and platelets, which was negatively associated with asthma-related quality of life measurements (51). Furthermore, an association of platelets with eosinophils was reported in 1992, and more recently, staining of mixed leukocyte cytospins from whole blood revealed 5–25% eosinophils attached to platelets from patients with mild or moderate asthma (82, 83), and in AERD (84) suggesting a possible role of platelets in human lung eosinophilia. While *ex vivo* measurements of circulating platelet–leukocyte (eosinophil) complexes cannot on their own be suggestive of a mechanism by which platelets influence eosinophil tissue recruitment, due to complexities of blood processing, the association between eosinophils and platelets does have functional consequences for recruitment, because eosinophils isolated from patients with asthma adhere to endothelial cells under flow conditions to a greater degree compared to eosinophils from healthy subject, and platelets promote this adhesion (85), and this important phenomenon is discussed below.

A role of platelet P-selectin-mediated events has been widely investigated in the evaluation of pulmonary eosinophil recruitment and activation, since platelets adhere to

eosinophils *via* P-selectin/PSGL-1-dependent interactions, and P-selectin is important for pulmonary leukocyte recruitment (13, 69, 70, 86–90). Specifically, the adhesion of platelets and eosinophils has been investigated *in vitro*, by comparing different stimuli that activate either eosinophils, platelets or both: fMLP, thrombin, and LPS (91, 92). A blocking antibody to P-selectin and fucoidan (a non-selective selectin antagonist) was reported to suppress the rossetting of platelets around eosinophils, while abciximab (integrin αIIbβ3 antagonist) and blocking anti-L-selectin antibody had no effect (92). Furthermore, the addition of aspirin had a rather minor effect on platelet–eosinophil rosettes, while WEB2170 (PAF receptor antagonist) and MK886 (an inhibitor of FLAP) actually increased the phenomenon (92).

The specific mechanisms behind platelet and eosinophil interactions are therefore due to surface expression of adhesion molecules on activated cells (see **Figure 1**). In a mouse model of allergic airways inflammation, P-selectin expression on platelets was critical in eosinophil recruitment to the lung, following allergen challenge. Platelet-depleted mice that had been sensitized and exposed to experimental allergen had reduced pulmonary eosinophil recruitment after transfusion of unstimulated platelets, when compared with transfusion with stimulated platelets expressing P-selectin (13). Johansson and colleagues have reported that eosinophils taken from the blood of patients with non-severe asthma have increased levels of surface associated platelets expressing P-selectin after whole-lung antigen challenge, and these were associated with increased α4β1-integrin very late antigen-4, but not αMβ2 integrin MAC-1 expression on a proportion of eosinophils (52). β1-integrin and P-selectin appeared to colocalize on activated eosinophils, when investigated by immunofluorescence microscopy (83). The addition of soluble P-selectin to whole blood caused enhanced activation of α4β1-integrin on eosinophils and also enhanced eosinophil adhesion to vascular cell adhesion molecule-1 *in vitro* (83). Further investigations found that after whole-lung antigen challenge of patients with asthma, circulating eosinophils associated with P-selectin disappeared from the circulation, suggesting a migration of platelet–eosinophil complexes into the lungs (52). This hypothesis is supported by findings that demonstrate under flow conditions in the blood of patients with asthma, blocking antibodies directed against P-selectin, causes a decrease in eosinophil binding and clustering to activated endothelium (85). Indeed, *in situ* staining reveals platelets attached to intravascular eosinophils after allergen challenge in a murine model of allergic airways inflammation (13). Therefore, early contact-dependent interactions between platelets and eosinophils are likely to be important in subsequent eosinophil recruitment, since it is now recognized that platelet contact *via* platelet P-selectin with neutrophils is necessary for efficient neutrophil adhesion, and this has been visualized *via* intra-vital and multiphoton microscopy (6, 43, 44, 93). Such events could be initiated through the increased preponderance of platelet–eosinophil complexes. Nevertheless, along with the direct physical interactions between platelets and eosinophils, platelets can also influence eosinophil function *via* inflammatory mediator release. The platelet-specific chemokine, PF-4, is capable of accelerated eosinophil–endothelial adhesion due to upregulation of adhesion molecules (14, 94).

PHARMACOLOGICAL STRATEGIES FOR MODULATING PLATELET–EOSINOPHIL INTERACTIONS

The development of therapeutic strategies to inhibit platelet–eosinophil interactions would be considered part of a larger research effort to modulate platelet function during inflammation (rather than solely platelets and eosinophils). Such a strategy is clearly nascent. Anti-platelet drugs (e.g., aspirin, $P2Y_{12}$ antagonists clopidogrel, and prasugrel) used in the prevention of thrombi in patients with cardiovascular disease have not been extensively tested in non-thrombotic diseases, and especially asthma, although prasugrel was reported to have a limited (if any) beneficial effect in patients with asthma (95). Other $P2Y_{12}$ antagonists had no effect of pulmonary leukocyte (including eosinophil) recruitment in a murine model of allergic airways inflammation (5). This would suggest that a dichotomy in platelet function exists between inflammatory responses pertinent to host defense, and platelet aggregation in response to vascular injury (4). Therefore, platelet activation and signaling pathways are likely to be separated and require a different anti-platelet pharmacy to be effective compared with established anti-platelet drugs used to inhibit the formation of thrombi. In this regard, the mechanisms of platelet activation that are necessary for pulmonary leukocyte (and eosinophil) recruitment in models of allergic and non-allergic inflammation have been shown to be platelet $P2Y_1$ (5), and $P2Y_{14}$ (96) dependent *via* signaling pathways involving Rho GTPases (RhoA, Rac-1) that are largely redundant during platelet activation in the context of hemostasis (5, 6).

Other strategies that inhibit processes associated with platelets (e.g., platelet P-selectin-dependent leukocyte recruitment) have gained traction. Bimosiamose, a small molecule antagonist to P-selectin, attenuated late asthmatic reactions following allergen challenge in mild asthmatics in a randomized, double-blind, placebo-controlled clinical cross over trial (97). Selectins are difficult structures to create effective small molecule antagonists against. Consequently, several drugs that inhibit the synthesis of PSGL-1 and therefore have the potential to suppress P-selectin-dependent eosinophil recruitment are being examined (98). These compounds have been reported to inhibit the synthesis of PSGL-1 under inflammatory conditions, rather than affecting expression at resting state, and might therefore provide an important safety benefit of not affecting the necessary immunosurveillance of the host (98). Heparin is known to inhibit P-selectin-dependent events, and a non-anticoagulant form of heparin (*N*-acetyl-de-*O*-sulfated-heparin), has recently been reported to disrupt platelet-dependent eosinophil recruitment in animal models (93).

Thus, there is a therapeutic potential in disrupting platelet–eosinophil interactions, or steps of platelet activation that have consequence on eosinophil functions, to alter the pathology of diseases associated with eosinophilia. Furthermore, an expanding volume of research is uncovering molecules whose biological pathways might in the future lead to drug development, for

example, platelet-derived: 5-HT (22), IL-33 (99), Dickkopf-1 (100), and CD154 (101).

CONCLUSION AND IMPLICATIONS

Evidence now demonstrates the importance of platelets and their interactions with eosinophils in allergic disease states, with a dichotomy in their activation that is distinct to the function of platelets during hemostasis. Further research into the relationship between platelets and eosinophils may yield novel targets for drug intervention in respiratory diseases characterized by eosinophilia, for example, atopic asthma, rhinitis, and AERD, by controlling the relationship between these essential blood components.

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REVIEW
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Bidirectional Mast Cell–Eosinophil Interactions in Inflammatory Disorders and Cancer

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Human mast cells (MCs) and eosinophils were first described and named by Paul Ehrlich. These cells have distinct myeloid progenitors and differ morphologically, ultrastructurally, immunologically, biochemically, and pharmacologically. However, MCs and eosinophils play a pivotal role in several allergic disorders. In addition, these cells are involved in autoimmune disorders, cardiovascular diseases, and cancer. MCs are distributed throughout all normal human tissues, whereas eosinophils are present only in gastrointestinal tract, secondary lymphoid tissues, and adipose tissue, thymus, mammary gland, and uterus. However, in allergic disorders, MCs and eosinophils can form the "allergic effector unit." Moreover, in several tumors, MCs and eosinophils can be found in close proximity. Therefore, it is likely that MCs have the capacity to modulate eosinophil functions and *vice versa*. For example, interleukin 5, stem cell factor, histamine, platelet-activating factor (PAF), prostaglandin D_2 (PGD_2), cysteinyl leukotrienes, and vascular endothelial growth factors (VEGFs), produced by activated MCs, can modulate eosinophil functions through the engagement of specific receptors. In contrast, eosinophil cationic proteins such as eosinophil cationic protein and major basic protein (MBP), nerve growth factor, and VEGFs released by activated eosinophils can modulate MC functions. These bidirectional interactions between MCs and eosinophils might be relevant not only in allergic diseases but also in several inflammatory and neoplastic disorders.

Keywords: allergy, asthma, cancer, eosinophils, inflammation, mast cells, mastocytosis

Abbreviations: SCF, stem cell factor; MCs, mast cells; H_4R, histamine 4 receptor; PAR-2, protease-activated receptor 2; ECP, eosinophil cationic protein; MBP; major basic protein; MRGPRX2, Mas-related G protein-coupled receptor member X2; OPN, osteopontin; IL-5, interleukin 5; GM-CSF, granulocyte-macrophage colony-stimulating factor; LTC_4, leukotriene C_4; PGD_2, prostaglandin D_2; PAF, platelet-activating factor; NGF, nerve growth factor; VEGF-A, vascular endothelial growth factor A; IL-5R, interleukin 5 receptor; $CysLTR_{1/2}$, cysteinyl leukotriene receptor 1/2; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; PAFR, platelet-activating factor receptor; TrkA, tyrosine kinase receptor A; VEGF-R1/2, vascular endothelial growth factor receptor 1/2.

INTRODUCTION

Mast cells (MCs) and eosinophils are important cells of the immune system with critical roles in allergic (1–3) and autoimmune disorders (4–7), cardiovascular diseases (8–15), and cancer (16–19). Human MCs and eosinophils were first described and named in 1878 and 1879, respectively, by Paul Ehrlich who discovered their property to be stained by specific dyes (20–22). Mature MCs and eosinophils differ morphologically, ultrastructurally, immunologically, biochemically, and pharmacologically (23, 24). Moreover, they synthesize a plethora of distinct mediators and display a constellation of different surface receptors (24, 25).

The recent assessment of the transcriptional profiles of MCs and eosinophils revealed the MC heterogeneity across different tissues and their different gene expression program compared to eosinophils (26). The latter findings are consistent with the identification of a distinct myeloid progenitor expressing the gene encoding the transcription factor GATA-1, which generates separately eosinophils and MCs (27). Human MCs derive from $CD34^+$, $CD117^+$ pluripotent hematopoietic stem cells in the bone marrow (28). MC progenitors enter the circulation and complete their maturation in different tissues such as skin, bronchi, tonsils, nasal and intestinal mucosa, conjunctiva, lymph nodes, and breast parenchyma (29). The main differentiation, maturation, survival, priming, and chemotactic factor for human MCs is stem cell factor (SCF), which acts by binding the tyrosine kinase receptor Kit (CD117) (30). $CD34^+$ IL-$5R\alpha^+$ eosinophil lineage-committed progenitors give rise to mature cells in the bone marrow under the control of critical transcription factors such as GATA-1, PU-1, and C/EBP (31). Eosinophil maturation in the bone marrow is driven by interleukin 5 (IL-5), IL-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF) that share the common receptor β subunit (βc) (3, 32). Recent evidence indicates that IL-33 precedes IL-5 in regulating eosinophil commitment and is required for eosinophil homeostasis (33). Under the effect of chemotactic stimuli, together with IL-5, eosinophils migrate to the gastrointestinal tract, secondary lymphoid tissues, and adipose tissue, thymus, mammary gland, and uterus, where they reside under homeostatic conditions (see Marichal et al. in this issue) (34–36). In response to inflammatory stimuli (e.g., eotaxins), peripheral blood eosinophils migrate into inflamed tissues, where their survival is presumably prolonged (37, 38).

BIDIRECTIONAL MC–EOSINOPHIL INTERACTIONS

Although human MCs are distributed throughout, nearly all normal tissues (39, 40) their density is increased at sites of allergic reactions (1), autoimmune disorders (4, 5), and at the edge of several solid (41–55) and hematologic tumors (56–64). In several allergic disorders (e.g., bronchial asthma, allergic rhinitis, chronic urticaria, and eosinophilic esophagitis), MCs and eosinophils can be found in close proximity forming the "allergic effector unit" (AEU) (65). In addition, there is *in vitro* evidence that the physical interaction between MCs and eosinophils induces a hyperactivation state and release of soluble mediators (65–67). Therefore, MCs likely have the capacity to modulate eosinophil functions and *vice versa*. We discuss examples of such two-way interactions below.

MC Mediators

Histamine, released immunologically and non-immunologically from MCs, induces eosinophil chemotaxis through the engagement of the histamine 4 receptor (H_4R) (68, 69). Similar to eosinophils, histamine-induced chemotaxis can be also observed in MCs (69).

Adenosine, an endogenous nucleoside released by activated MCs (70), acts in an autocrine and paracrine fashion *via* binding to four G protein-coupled receptors: the A_1, A_{2a}, A_{2b}, and A_3 receptors (71) and is involved in airway hyperresponsiveness in asthma (72). Adenosine and its stable analogs potentiate mediator release from human lung MCs (HLMCs) (73, 74) through the activation of adenosine receptors (75) and modulate eosinophil functions (76, 77). MC tryptase can stimulate eosinophil activation and degranulation by cleavage of protease-activated receptor 2 (78).

Eosinophil Mediators

On the other side, eosinophil granule proteins such as MBP and eosinophil cationic protein (ECP) act as complete secretagogues on MCs isolated from human heart (HHMC) (8, 9). ECP, and to a lesser extent MBP, induces the release of histamine and tryptase and the *de novo* synthesis of PGD_2 from HHMC. This observation highlights a mechanism by which infiltrating eosinophils can cause myocardial damage in patients with eosinophilia (3, 79–84). ECP and MBP do not induce histamine release from isolated HLMCs (8, 9). Interestingly, Piliponsky et al. reported that HLMCs became responsive to MBP only in coculture with human lung fibroblasts (85). Recently, the Mas-related gene X2 (MRGPRX2) has been identified as a receptor for several basic peptides on human and rodent MCs (26, 86), and indeed ECP and MBP activate human MCs through the interaction of the MRGPRX2 receptor expressed on their surface (87). Eosinophil MBP-1 activates MCs through the interaction with integrin-β1 (88).

MC and Eosinophil Mediators

Stem cell factor (SCF) is a potent activator of human MCs (89, 90) and induces the release of eosinophil peroxidase (EPO) and cysteinyl leukotriene C_4 (LTC_4) from eosinophils (91). SCF, produced by both human MCs (90) and eosinophils (92), acts on Kit receptor (CD117) on MCs (30) and eosinophils (93).

Osteopontin (OPN) is a multifunctional glycoprotein implicated in allergic disorders and cancer. OPN can be released by IL-5-activated human eosinophils and induces their migration (94). OPN is also produced by MCs and modulates their IgE-mediated degranulation and migration (95).

Interleukin-5, produced by human MCs, activates the IL-5R, highly expressed on the surface of human eosinophils, basophils, and MCs (96). In addition to MCs, Th2 cells, group 2 innate lymphoid cells (ILC2), invariant NK T cells, and eosinophils themselves are major cellular sources of IL-5 (97). GM-CSF released by activated human MCs (98), and eosinophils binds

its receptor expressed by both cell types (99). The cysteinyl leukotrienes (CysLTs LTC_4 and LTD_4), produced by activated MCs (18, 100), stimulate the proliferation of eosinophil progenitors in the presence of IL-5 and GM-CSF (101). In addition, CysLTs acting through CysLTR1/2 induce the release of IL-4 from human eosinophils (102). PGD_2 is the major cyclooxygenase metabolite released by activated MCs (8) and a minor product of eosinophils (103). PGD_2 is involved in asthma and allergic rhinitis (104, 105), mastocytosis, rheumatoid arthritis, and cardiac dysfunction (6, 106). PGD_2 induces eosinophil and MC chemotaxis in a paracrine and autocrine fashion *via* binding to CRTH2 receptor on these cells (107, 108). Platelet-activating factor (PAF), synthesized by human MCs and eosinophils (109, 110), is involved in asthma (111) and exerts multiple effects on eosinophils (112, 113).

Nerve growth factor (NGF), produced by both MCs (114, 115) and eosinophils (116, 117), is increased in patients with asthma (118). NGF enhances MC survival (119) through the activation of TrkA receptor (115). NGF is preformed in and activates human eosinophils (116).

Human MCs produce several proangiogenic (VEGF-A, VEGF-B, and FGF-2) (120–125) and lymphangiogenic factors (VEGF-C and VEGF-D) (100, 124). Human eosinophils induce angiogenesis (126) through the production of VEGF-A (127, 128), MBP (129), and OPN (94). Interestingly, VEGF-A, produced by both MCs and eosinophils, is also chemotactic for MCs through the engagement of VEGFR-1/2 present on their surface (124).

The bidirectional interactions between MCs and eosinophils mediated by soluble mediators and the autocrine modulation of these cells are schematically illustrated in **Figures 1A,B**.

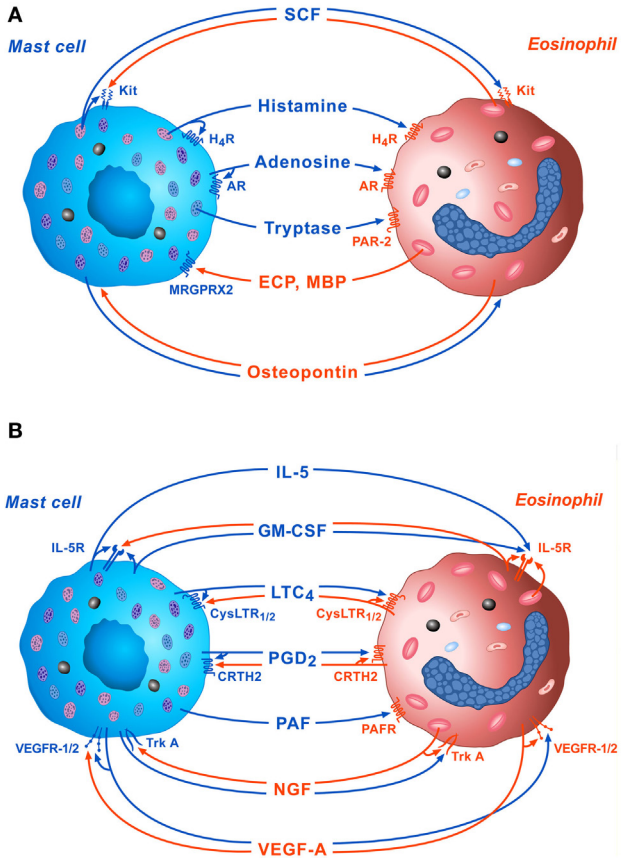

FIGURE 1 | Schematic representation of some of the bidirectional interactions between MCs and eosinophils. **(A)** Several preformed mediators such as stem cell factor (SCF), histamine, adenosine, and tryptase, released by activated MCs can exert paracrine and/or autocrine functions through the engagement of Kit, H_4R, adenosine receptors, and protease-activated receptor 2 (PAR-2), respectively. On the other side, cationic proteins [eosinophil cationic protein (ECP) and MBP], released by activated eosinophils modulate mast cell functions through the activation of MRGPRX2 on their surface. Osteopontin released by both activated eosinophils and MCs exert paracrine and autocrine effects. **(B)** Several *de novo* synthesized mediators such as IL-5, granulocyte-macrophage colony-stimulating factor (GM-CSF), LTC_4, PGD_2, platelet-activating factor (PFA), nerve growth factor (NGF), and VEGF-A, released by activated MCs, can modulate eosinophil functions *via* the activation of IL-5R, $CysLTR_{1/2}$, CRTH2, platelet-activating factor receptor (PAFR), TrkA, and VEGF-R1/2, respectively, on their surface. IL-5, GM-CSF, LTC_4, PGD_2, NGF, and VEGF-A can also exert paracrine and/or autocrine effects.

DISORDERS IN WHICH MCs AND EOSINOPHILS ARE PRESENT AND LIKELY DRIVE DISEASE PATHOGENESIS

Asthma

Asthma is a chronic inflammatory disorder of the airways in which cells of the innate and adaptive immune system act together with epithelial cells to cause bronchial hyperreactivity, mucus overproduction, airway wall remodeling, angiogenesis, and airway narrowing (123, 130, 131). MCs and their mediators display important roles in the pathogenesis of asthma (2, 39). Indeed, MC-derived histamine, proteases, chemotactic factors, cytokines, and metabolites of arachidonic acid act on vasculature, smooth muscle, connective tissue, goblet cells, and inflammatory cells in the airway inducing acute bronchoconstriction (1). MCs synthesize and release a vast array of pro-inflammatory chemokines and cytokines and recruit other immune cells, such as eosinophils, activated macrophages, and lymphocytes. Therefore, MCs are involved both in the early and the late phases of allergic responses in sensitized individuals (132). Compelling evidence suggests that in asthma MCs are constantly activated resulting in enhanced mediator release and the establishment of chronic airway inflammation. Moreover, MCs reside close to key structures of the bronchial wall, such as airway smooth muscle (ASM) epithelium and submucosal glands, contributing to ASM hypertrophy and other remodeling features (133).

Numerous stages of the MC life cycle have the potential for therapeutic intervention in allergic disorders (134). Targeting the progenitor recruitment offers an upstream checkpoint that could be used to limit tissue MC activity. However, since the mechanisms regulating MC progenitor recruitment to the human lung are not fully understood, no potential therapeutic targets at this level of MC biology have been defined so far. Once within tissue, MC survival, growth, differentiation, and maturation are driven by the local cytokine melieu, with a pivotal role played by

SCF and its receptor Kit, which retains protein tyrosine kinase (TK) activity. MC eradication *via* TK inhibitors may also be a means to treat MC-driven diseases such as asthma. Indeed, the TK inhibitor imatinib decreased airway hyperresponsiveness, MC counts, and tryptase release in patients with severe asthma (135). In addition, masitinib, an inhibitor of Kit and the platelet-derived growth factor receptor, showed some benefit in a small phase II trial over 16 weeks in severe glucocorticoid-dependent asthma (136).

High-affinity receptor for the Fc region of IgE (FcεRI) is expressed on MCs and basophils as a tetrameric complex of three chains with the stoichiometry $\alpha\beta\gamma_2$. FcεRI is also expressed in either a trimeric form, $\alpha\gamma_2$, or the tetramer, on a range of other cell types [e.g., various antigen-presenting cells (APCs), dendritic cells, Langerhans cells, macrophages, eosinophils, and platelets] contributing to IgE-mediated allergic pathophysiology (137). The "low affinity" FcεRII, first discovered on B cells, is also expressed on several other cell types, including various APCs, and also airway and gut epithelial cells (137). FcεRI-dependent MC activation occurs following exposure to allergens, bacterial and viral superantigens, and IgE antibodies. This pathway has been targeted successfully with omalizumab, which prevents IgE binding to the FcεRI and has been approved for use in asthma and chronic urticaria (138, 139). Indeed, anti-IgE therapy with omalizumab, added to medium- or high-dose inhaled glucocorticoids, has proven effective in the treatment of patients with moderate-to-severe and severe allergic (IgE-mediated) asthma by reducing exacerbations and associated use of systemic glucocorticoids in addition to improving other clinical outcomes (140, 141). Since omalizumab reduces the expression of FcεRI on circulating basophils and MCs, it seems to lower the activity potentials of basophils and MCs, thereby reducing the potential reactivity of these cells. Concordantly, serum tryptase was reported to decrease under omalizumab therapy in two mastocytosis patients, but it remained unchanged in two other patients (142). A recent study performed on 18 non-atopic asthmatic patients showed improved lung function and reduced total bronchial mucosal IgE^+ cells in bronchial biopsies, but not changed total MCs, plasma cells, B lymphocytes, eosinophils, and plasmablast (143). A pooled analysis of five randomized, double-blind, placebo-controlled trials demonstrated that the reduction of serum-free IgE by omalizumab was associated with a reduction in peripheral eosinophil counts in patients with moderate-to-severe asthma receiving moderate-to-high doses of glucocorticoids [see Stokes in this issue and Ref. (144)]. Smaller studies already reported an inhibitory effect of omalizumab on eosinophils, in the peripheral blood and in the sputum or in bronchial biopsies, but no significant results on tissue MC numbers (145–149). A decrease in blood eosinophilia during omalizumab therapy was proposed as predictor of less asthma exacerbations (150) as well as higher IL-13 levels in sputum predicted the response to omalizumab (151). However, despite these clinical evidences, the mechanisms whereby reductions in circulating IgE lead to a reduction in eosinophils remain unclear. It is possible that omalizumab leads to the inhibition of the release of pro-inflammatory mediators, cytokines, and chemokines from MCs/basophils or inhibition of the allergen-induced differentiation of T cells to Th2 cells by reducing the expression of FcεRI on APCs. Indeed, omalizumab was shown to reduce $IL\text{-}4^+$ cells in the bronchial submucosa (145). A reduction in circulating IL-13 has also been reported in patients with moderate-to-severe allergic asthma treated with omalizumab (147). A decrease in eotaxin expression in exhaled breath condensate, exhaled NO, eosinophil blood count, serum ECP after 16 weeks of omalizumab treatment was observed (152). Increased eosinophil apoptosis and reduced numbers of $GM\text{-}CSF^+$ lymphocytes have been observed in peripheral blood of omalizumab-treated patients with coexisting allergic asthma and rhinitis, which may also contribute to the inhibitory action of omalizumab on eosinophils (146). A direct effect of omalizumab on eosinophils may be possible *via* the FcεRI that have been detected on eosinophils, even though their functional significance has yet to be established (153).

Given the pivotal role played by eosinophils in the pathogenesis of severe eosinophilic asthma (3, 154), targeting IL-5 or IL-5Rα appears an interesting therapeutic approach (3, 131). Several randomized, double-blind, placebo-controlled studies demonstrated that mepolizumab (155, 156), reslizumab (157, 158), and benralizumab (159, 160) improved lung function and decreased asthma exacerbations in adult patients with severe eosinophilic asthma.

Eosinophilic Esophagitis

Eosinophils, normally present in the gastrointestinal tract, are absent in the esophagus of healthy subjects. Eosinophilic esophagitis (EoE) is a chronic, immune-mediated esophageal disease, characterized by dysphagia, abdominal pain, and presence of ≥15 eosinophils/field at 400× magnification in the proximal and distal esophagus (161). In EoE, eosinophils are present in all layers of the esophagus, but predominate in the lamina propria and submucosal regions, and are considered the main effector cells in this disorder (161). Activated MCs and their products (e.g., TGF-β) have been described in the esophageal biopsies of active EoE patients (162, 163). The relative contribution of MCs and eosinophils to disease pathogenesis is still under investigation. There is no evidence supporting MC-targeting therapies in EoE (164–166). However, an open label, single arm, unblinded small study showed a statistically significant reduction in MCs and eosinophils in endoscopic biopsies of EoE patients following omalizumab treatment, which correlated with clinical outcome (167). IL-5 targeting therapies resulted in a reduction of esophageal inflammation, but only in minimal symptom relief (168). Interestingly, mepolizumab did not deplete eosinophils nor MCs in the duodenal mucosa of patients (169). In contrast, a pediatric retrospective study showed a reduction in esophageal eosinophil numbers upon mepolizumab treatment, which was more pronounced in a subgroup of responders that also displayed a marked reduction of $tryptase^+$ MCs after treatment. These esophageal MCs were found adjacent to eosinophils, and the frequency of these MC/eosinophil couplets in the esophagus of the responders was reduced after mepolizumab treatment. Moreover, activated MBP^+ eosinophils and unidentified cells adjacent to $tryptase^+$ MCs in the esophagus produced IL-9, a pleiotropic cytokine with a pivotal role in activation and

maturation of MCs. Interestingly, the authors reported that the esophageal MC numbers correlated with the severity of EoE symptoms, but the reduction of eosinophil numbers did not correlate with symptoms severity. In the subgroup of patients with a greater than 70% decrease in MC density, numbers of MCs correlated with the severity of symptoms. By contrast, there was no correlation between eosinophil numbers and symptom severity. This study suggests an additional role for eosinophils in EoE, as providers of IL-9 that promotes esophageal mastocytosis and indicates that interactions between MCs and eosinophils can regulate the severity of EoE symptoms (170). Reslizumab reduced intraepithelial esophageal eosinophils without improvements in symptoms (171). Thus, although the involvement of eosinophils and presumably MCs in EoE is likely, their relative contribution to the pathogenesis and symptoms of EoE is not fully understood.

Eosinophilic Granulomatosis with Polyangiitis (EGPA)

Eosinophilic granulomatosis with polyangiitis, previously known as Churg–Strauss syndrome, is characterized by increased blood level of IL-5 and eosinophilia in peripheral blood and affected tissues (172). In EGPA, eosinophilic inflammation affects the upper (chronic rhinosinusitis) and lower airways (asthma) (173). Endocardial inflammation, coronary vasculitis, and pericarditis can be observed in patients with EGPA (79, 80). A preliminary study in a small group of EGPA patients demonstrated the efficacy of mepolizumab in reducing blood eosinophils, but not in improving the pulmonary functions (174). A recent multicenter, double-blind, parallel-group, phase 3 trial demonstrated that in patients with EGPA mepolizumab (300 mg s.c. every 4 weeks) was associated with more accrued time in remission than was placebo, which allowed for reductions in the glucocorticoid dose over a period of 52 weeks (175). We have found that omalizumab resulted in clinical improvement of asthma, reduction of peripheral blood eosinophils, and prednisone administration in EGPA patients (173, 176). However, the role of MC in the pathogenesis of EGPA is not fully understood.

Eosinophilic Endomyocarditis and Atherosclerosis

The association between endomyocardial disease and eosinophilia was first described by Löffler in 1936 (177). Cardiac involvement is the most common cause of morbidity and mortality in patients with hypereosinophilia (3, 81–84). Eosinophils and their granule proteins have been found in cardiac biopsies from patients with eosinophilic endomyocardial disease (178, 179). Recently, an association of EoE and cardiomyopathy has been reported (180).

Eosinophil cationic protein and, to a lesser extent, MBP stimulate the release of preformed (histamine and tryptase) and the *de novo* synthesis of PGD_2 and LTC_4 from human HHMC (8, 181). Activated HHMCs release histamine and CysLTs, which exert profound cardiovascular and metabolic effects (182, 183). In addition, MBP and eosinophil peroxidase induce platelet aggregation (184). These observations suggest that infiltrating eosinophils and their mediators contribute to cardiac dysfunction in patients with eosinophilia.

Activated MCs are increased at site of atheromatous rupture in myocardial infarction (10). MCs in human coronary plaques release angiogenic factors, such as FGF-β (11), which enhance atherosclerotic plaque progression. Cardiac MC-derived renin promotes local angiotensin formation leading to cardiac dysfunction (12). Activated MCs may also promote abdominal aortic aneurysms (13, 14) presumably through the release of chymase (185, 186) and CysLTs (15).

Skin Disorders

Bullous pemphigoid (BP) is the most frequent autoimmune blistering dermatosis, characterized by autoantibodies directed against the dermal–epidermal junction proteins BP180/BP230 typically causing pruritic bullous eruptions. The immune response leading to blister formation in BP involves different inflammatory cells and molecules, including CD4 T cells, B cells, complement factors, neutrophils, as well as MCs and eosinophils (187). Serum levels of ECP were elevated in patients with active BP compared with healthy controls. Moreover, MC tryptase serum levels were associated with circulating anti-BP180 autoantibodies and decreased at the time of clinical remission (188). In a murine model of BP, blistering was dependent on C5a–C5aR interaction on MCs, which led to the activation of the p38 MAPK pathway in MC and their degranulation (189). Moreover, blood, skin, and blister-derived eosinophils were activated in patients with BP compared to controls. Activated eosinophils produced CCL26, IL-6, IL-8, and IL-1α in BP skin and blister fluid and displayed apoptosis features (190). Interestingly, IL-5-activated eosinophils were shown *ex vivo* to directly contribute to BP blister formation in the presence of BP autoantibodies. Indeed, IL-5-activated eosinophils induced dermal–epidermal separation, which was dependent on eosinophil adhesion, FcγR activation, ROS production, degranulation, and eosinophil extracellular trap formation (191).

Psoriasis is a frequent, chronic recurrent inflammatory skin disease which results from dysregulation between environmental factors, epithelial cells and immune cells (100). MCs infiltrate skin lesions of psoriatic patients and were identified as high producers of IL-17A and IL-22, both cytokines involved in psoriasis pathogenesis (192, 193). MCs and keratinocytes also induced angiogenesis by producing IL-8 and VEGF-A (194). In contrast, eosinophils were not increased in the skin or peripheral blood of psoriatic patients.

Atopic dermatitis (AD) is a common chronic inflammatory skin disease driven by specific genetic and immunological mechanisms (100). MC-derived histamine, tryptase, chymase, and other inflammatory mediators contribute to itching and inflammation in patients with AD (195). However, MCs were not required for the development of disease in a murine model of AD (196). AD is characterized by an increased number of circulating eosinophils and dermal and epidermal infiltrates of eosinophils. Tissue and blood eosinophilia and increased circulating levels of ECP, MBP, and eosinophil-derived neurotoxin have been correlated with disease activity. Serum levels of

IL-5 were increased in AD patients and correlated with disease activity. However, although eosinophils might have important roles in AD pathogenesis, their exact mechanisms are not fully understood (197).

Tumor-Associated MCs (TAMCs) and Tumor-Associated Eosinophils in Cancer

Tumor-associated eosinophilia was first described in 1893 (198). Eosinophilia is frequently observed in patients with solid tumors (199–201) and Hodgkin's lymphoma (202). Eosinophils are recruited to tumors by chemoattractant CCL11 (eotaxin-1), which binds to CCR3 (203) and damage-associated molecular patterns, notably the alarmin high-mobility group box 1, released by necrotic tumor cells (204, 205).

Clinical studies addressing the role of eosinophils in tumors provided conflicting results. Tumor-associated eosinophilia was related to good prognosis in colorectal, head and neck, bladder and prostate cancers (206–208). By contrast, in Hodgkin's lymphoma, oral squamous cell carcinoma, and cervical carcinoma, eosinophils have been linked to poor prognosis (206, 207, 209, 210).

Experimental studies also provided inconclusive results (211). Indeed, human eosinophils exert tumoricidal activity toward cancer cells through the release of TNF-α and granzyme A, contained in their secondary granules (17, 212). On the other hand, tumor-recruited eosinophils influence tumor angiogenesis, through distinct mechanisms. Human eosinophils and their supernatants induce endothelial cell proliferation *in vitro* and angiogenesis *in vivo* (213). Eosinophils contain VEGF in their secretory granules that can be secreted upon activation by IL-5 (127). In addition, eosinophils can contribute to tumor angiogenesis through the release of other proangiogenic molecules such as OPN (94) and MBP (129). Recently, activated eosinophils were shown to be essential for tumor rejection (16). Indeed, tumor-homing eosinophils secreted chemoattractants such as CCL5, CXCL9, and CXCL10, which recruited $CD8^+$ T cells to the tumor (16).

Tumor-associated MCs are present in several human solid (41–55) and hematologic tumors (56–64). Peritumoral and/or intratumoral MC density is increased in different types of human cancer (18). Although the role of MCs and their mediators in experimental and human tumors is still controversial (19, 214), the bidirectional interaction between MCs and eosinophils can influence tumor angiogenesis and lymphangiogenesis.

Tumor immunologists have just scratched the surface of the complexity of the multidirectional interactions between eosinophils, MCs, and their neighboring tumor cells in tumor microenvironment.

MCs and Eosinophils in Myeloproliferative Disorders

Mast cells and eosinophils can also found to be increased in primary myeloproliferative disorders of the bone marrow. The mechanism of the increased numbers of MCs and eosinophils in myeloproliferative disorders involves a primary defect in a tyrosine kinase gene resulting in uncontrolled proliferation and dysregulated apoptosis. Two such disorders are particularly associated with increased numbers of both cell types: systemic mastocytosis (SM) and chronic eosinophilic leukemia (CEL).

Mastocytosis is an abnormal clonal MC expansion and accumulation in several tissues including the bone marrow and the skin (215, 216). Cutaneous mastocytosis is associated with gain-of-function *Kit* mutations in approximately 8% of cases (217). Almost all patients with SM present a somatic mutation in codon 816 (D816V) of the gene encoding the receptor Kit, which leads to the substitution of a valine for an aspartate in the protein. Because of the D816V mutation, Kit is constitutively active, resulting in autophosphorylation and enhancement of MC differentiation and survival. A variable percentage (15–28%) of patients with SM also presents peripheral blood eosinophilia, which predicted poorer prognosis in some studies (218–220). In patients with cutaneous or SM, a correlation between the levels of soluble IL-5Rα (sIL-5Rα) and eosinophils in peripheral blood was also found (219).

In 2003, the FIP1L1–PDGFRA fusion was identified in patients with idiopathic hypereosinophilic syndrome and its presence redefined such patients having a neoplasm instead of idiopathic hypereosinophilic syndrome. Before the discovery of this cytogenetic rearrangement, the patients carried a poor prognosis due to early cardiac death in the absence of effective treatment. The identification of this fusion rearrangement as therapeutic target of imatinib dramatically changed the perspectives of these patients, due to a prompt hematologic and clinical remission. Patients with FIP1L1–$PDGFRA^+$ CEL exhibit features of myeloproliferative syndromes such as splenomegaly, hypercellular bone marrow, and clinicopathological aspects that overlap with systemic MC diseases, such as increased number of abnormal MCs, elevated circulating tryptase levels, and bone marrow fibrosis (221). These similarities raised the doubt that FIP1L1–$PDGFRA^+$ CEL could be considered a subtype of SM, rather than a primary eosinophil disease (222). Indeed, even though less dense clusters of MCs compared to the typical multifocal aggregates of D816V Kit^+ SM, in some cases MCs exhibited spindle-shaped morphology and aberrant surface expression of CD25, both minor criteria for SM according to the WHO criteria (223). In the revised 2008 WHO semi-molecular classification of myeloid neoplasms, FIP1L1–$PDGFRA^+$ disease is not considered a subtype of SM. To date, FIP1L1–PDGFRA and D816V *Kit* mutations appear to be mutually exclusive. In the D816V Kit^+ patients, gastrointestinal symptoms, urticaria pigmentosa, thrombocytosis, median serum tryptase value, and the presence of MC dense infiltrates in the bone marrow were increased compared to patients with FIP1L1–PDGFRA mutation. By contrast, cardiac and pulmonary symptoms, median eosinophil count, eosinophil to tryptase ratio, and serum B_{12} levels were higher in the FIP1L1–$PDGFRA^+$ patients. Whether a patient with peripheral eosinophilia and increased bone marrow MC infiltration carries a D816V Kit or FIP1L1–PDGFRA mutation is important for guiding therapeutic decisions. Indeed, FIP1L1–PDGFRA mutation is highly sensitive to imatinib treatment, which induces clinical remission as early as 4 weeks. By contrast, the vast majority of SM carrying D816V Kit mutation are imatinib resistant and candidate to second-line tyrosine kinase inhibitors or cytoreductive therapy (224).

CONCLUSION

Mast cells and eosinophils were identified and named by Paul Ehrlich based on their capacity to be stained by specific dyes. These cells and their mediators have been classically associated with the pathogenesis of allergic disorders. However, there is now evidence that MCs and eosinophils are involved in autoimmune disorders, vasculitis, cardiovascular diseases, as well as solid and hematologic tumors. MCs and eosinophils play complex, sometimes complementary, but also distinct roles in these conditions. The latter findings are not surprising given the observations that these cells have distinct myeloid progenitors, are activated by different agonists, and differ morphologically, ultrastructurally, immunologically, biochemically, and pharmacologically.

In allergic disorders (e.g., asthma, allergic rhinitis, chronic urticaria) and certain solid (e.g., gastric and prostate cancers) and hematologic tumors (e.g., Hodgkin's lymphoma), MCs and eosinophils can be found in close proximity. In particular, in allergic diseases, these cells can form the AEU (65). It is now clear that MCs modulate several eosinophil functions through the release of a plethora of preformed (e.g., SCF, histamine, and adenosine) and *de novo* synthesized mediators (e.g., IL-5, LTC_4, SCF, PGD_2, and PAF). On the other side, eosinophils modulate MC functions through the production of several mediators (e.g., IL-5, PAF, ECP, MBP, and NGF). These bidirectional interactions between MCs and eosinophils might be important not only in allergic diseases but also in several inflammatory and neoplastic disorders.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and have approved the final version of the manuscript.

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REVIEW
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Asthma Endotypes and an Overview of Targeted Therapy for Asthma

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Guidelines for the management of severe asthma do not emphasize the measurement of the inflammatory component of airway disease to indicate appropriate treatments or to monitor response to treatment. Inflammation is a central component of asthma and contributes to symptoms, physiological, and structural abnormalities. It can be assessed by a number of endotyping strategies based on "omics" technology such as proteomics, transcriptomics, and metabolomics. It can also be assessed using simple cellular responses by quantitative cytometry in sputum. Bronchitis may be eosinophilic, neutrophilic, mixed-granulocytic, or paucigranulocytic (eosinophils and neutrophils not elevated). Eosinophilic bronchitis is usually a Type 2 (T2)-driven process and therefore a sputum eosinophilia of greater than 3% usually indicates a response to treatment with corticosteroids or novel therapies directed against T2 cytokines such as IL-4, IL-5, and IL-13. Neutrophilic bronchitis represents a non-T2-driven disease, which is generally a predictor of response to antibiotics and may be a predictor to therapies targeted at pathways that lead to neutrophil recruitment such as TNF, IL-1, IL-6, IL-8, IL-23, and IL-17. Paucigranulocytic disease may not warrant anti-inflammatory therapy. These patients, whose symptoms may be driven largely by airway hyper-responsiveness may benefit from smooth muscle-directed therapies such as bronchial thermoplasty or mast-cell directed therapies. This review will briefly summarize the current knowledge regarding "omics-based signatures" and cellular endotyping of severe asthma and give an overview of segmentation of asthma therapeutics guided by the endotype.

Keywords: endotype, severe asthma, omics, sputum cytometry, type 2-low asthma, type 2-high asthma

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INTRODUCTION

The definition of asthma has not changed in over 50 years (1). The variability in airflow that characterizes the disease may be driven by airway hyper-responsiveness or by airway inflammation that is one of the determinants of airway hyper-responsiveness (2). Despite this heterogeneity, guideline-based therapy with inhaled beta agonists and corticosteroids do not consider measurements of these individual components in routine clinical care. While the majority of asthmatics are responsive to guideline-based therapy and have reduced symptoms, improved quality-of-life, increased lung function as well as decreased exacerbation frequency (3), in approximately 5–10% of asthmatics, anticipated outcomes are not achieved (4). These patients with severe disease are responsible for the majority of indirect and direct asthma-related costs and economic burden. The advent of biologic therapies calls for a new paradigm of personalized medicine based on inflammatory endotype to better-inform who is most likely to benefit from specific targeted therapies.

Traditional asthma phenotyping (description of observable characteristics that do not provide an insight into the underlying pathobiology) classifies patients according to clinical characteristics such as exacerbating factors (allergens, exercise, and infections), age of onset, concomitant comorbidities (sinusitis and obesity), and response to treatment. More recently, unbiased clustering algorithms that have the capacity to incorporate a range of clinical variables (e.g., FEV_1, BMI, ACQ, atopic status, and blood eosinophils) have been applied to large patient datasets to objectively identify clinical phenotypes of asthma. Such datasets include the Severe Asthma Research Program (SARP) (5), Airways Disease Endotyping for Personalized Therapeutics (ADEPT) (6), and Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes (U-BIOPRED) (6, 7) cohorts, which have been partitioned into up-to five clinical phenotypes. However, composites of observable characteristics do not provide us insight into mechanisms of persistent symptoms, physiological abnormalities, or inflammation, and therefore are of limited value to choose the appropriate biological agent. As opposed to phenotypes, characterizing severe asthmatics according to their endotype, a term applied to a "subtype of a condition that is defined by a distinct functional or pathophysiological mechanism," may be more useful to directing therapy (8). This notion is strongly supported by the efficacy of biologic therapies that target-specific inflammatory mediators (e.g., IL-5) in well-defined patients characterized based on inflammatory biomarkers (9, 10). Currently, however, asthma management guidelines fail to adequately emphasize the measurement of the inflammatory component of airway disease (bronchitis).

Inflammatory endotype characterization should be considered a central component of the workup and management of severe asthma. Widespread acceptance of this notion, however, has been slow, perhaps because there is no consensus as to how to best identify asthma endotypes and what therapy to use for a given endotype. While novel omics-based signatures of severe asthma have emerged, they have not been evaluated clinically. We suggest that asthma endotype characterization can be reliably done on the nature of underlying airway inflammation assessed by sputum cytometry (1). This review aimed to summarize the current knowledge regarding cellular endotyping and novel "omics-based signatures" of severe asthma and give an overview of segmentation of asthma therapeutics guided by the inflammatory endotype. Molecular pathways and mechanisms associated with asthma endotypes were recently reviewed and therefore have not been discussed in detail here.

INFLAMMATORY ENDOTYPES

Wenzel et al. (11) defined two distinct inflammatory endotypes of severe, corticosteroid-dependent asthma based on the presence or absence of eosinophils in endobronchial biopsy and lavage. Since this landmark study, T-helper type 2 (Th2)-high and Th2-low have remained the most well recognized and described endotypes of severe asthma. The Th2-high endotype is characterized by the presence of eosinophilic airway inflammation, whereas the Th2-low endotype is usually characterized by neutrophilic or paucigranulocytic airway inflammation. While several non-invasive biomarkers exist for the detection of the Th2-high endotype [blood eosinophils, serum IgE, serum periostin, and exhaled nitric oxide (eNO)], sputum cytometry is currently the most clinically validated quantitative and responsive method to assess airway inflammation. Perceived difficulties in implementing this approach in routine clinical practice have limited its widespread use. In fact, as described by Lim and Nair (12), many easily accessible biomarkers with demonstrated clinical utility remain confined to the research arena or are only exploited in a small number of specialized academic centers. This may be due to the lack of measurement standardization, validated diagnostic or predictive thresholds, and evidence-based reference guidelines to inform how biomarkers should be used and interpreted in clinical practice. Cost and infrastructure constraints also limit generalizability of validated biomarkers. To overcome these issues, readily available or point-of-care diagnostic methods are welcomed. Quantitative cytometry of induced or spontaneous sputum is currently the most sensitive and specific non-invasive method to directly characterize the type and severity of airway inflammation in asthma (13). Importantly, standardized methodology for sputum induction, processing, and quantification (14, 15) is safe (16) and well tolerated by the majority of patients (17–19). The nature of bronchitis assessed by sputum cytometry can be stratified into four groups based on the percentage of sputum granulocytes: (1) eosinophilic, (2) neutrophilic, (3) mixed-granulocytic (eosinophils and neutrophils elevated), and (4) paucigranulocytic (eosinophils nor neutrophils elevated) (17, 20). In non-smoking healthy adults, Belda et al. established the 90th percentile for sputum eosinophil and neutrophil counts, reporting 1.1 and 64.4%, respectively, with a total cell count of 9.7 million cells/g (21). However, standardized stratification cutoffs have not been established in asthma and as a result have varied between studies. Proposed thresholds for sputum eosinophilia have ranged between >1.1 and 4% of the total cell count (17, 20, 22) with studies strongly suggesting that a cutoff of >3% is clinically relevant and can be used to guide treatment and reduce exacerbations (23). To indicate neutrophilia, thresholds >61% of the total cell count have been proposed (20). Overlooked by many centers, it is important to acknowledge that the presence of eosinophil free granules also indicates uncontrolled eosinophilic bronchitis (24). The prevalence of these groups have been reported in stable, severe, and exacerbated disease with the proportion of eosinophilic asthma ranging from 35 to 50% (17, 20). The groups differ with respect to their clinical characteristics and response to therapy. Patients with mixed-granulocytic asthma have more severe airflow obstruction, a higher frequency of exacerbations and daily wheeze, and increased health care utilization than patients with either eosinophilic or neutrophilic bronchitis alone (25). It is also important to note that asthmatics who exhibit concordant blood and sputum eosinophilia experience more airway symptoms than those with isolated blood or sputum eosinophilia alone (19).

As mentioned above, there is no consensus on the specific definition of inflammatory cellular endotypes. Based on our clinical experience of over 20 years in over 5,000 patients, a patient can be determined to have eosinophilic asthma if there is evidence of elevated sputum (>3% with or without degranulation) and/or

blood eosinophils (≥400 cells/μL) on at least two occasions, and if treatment strategies aimed at suppressing eosinophils are effective in controlling symptoms and exacerbations (26). Likewise, a patient can be determined to have neutrophilic asthma if there is evidence of elevated neutrophils (≥64%) but not eosinophils (<3%) with an increased total cell count (≥9.7 million cells/g) on at least two occasions and if treatment strategies aimed at suppressing eosinophils are not effective in controlling symptoms and exacerbations. Patients can exhibit features of both the eosinophilic and neutrophilic endotype. A patient can be determined to have mixed-granulocytic asthma if there is evidence of both neutrophils and eosinophils, independently or concurrently, on at least two occasions. Finally, a patient can be determined to have paucigranulocytic asthma if there is no evidence of elevated eosinophils (<3%) or neutrophils (<64%) and if treatment strategies aimed at suppressing eosinophils and neutrophils are not effective in controlling symptoms and exacerbations. The workup and therapy regimen for these inflammatory endotypes have been described at the end of this review.

NOVEL OMICS ENDOTYPING STRATEGIES

The maturation of omics-based technologies has facilitated the investigation of transcriptomics (27–33), proteomics (25, 34), and metabolomics (35, 36) to better understand the molecular characteristics of asthma, which have all been recently reviewed. Large multicenter initiatives are now ongoing, including the U-BIOPRED project (37), which aim to identify distinct severe asthma endotypes by integrating inflammatory biomarkers derived from "omics" and clinical data. Thus far, omics measurements have been utilized to (1) identify asthma endotypes (38), (2) identify genes related to inflammatory characteristics (39), and (3) to describe the molecular characteristics of clinical asthma phenotypes (6). Studies are required to investigate the clinical benefit of these more sophisticated and computationally intensive endotyping strategies, both to initiate the appropriate treatment and to longitudinally monitor responses to various anti-inflammatory (including biologics) therapies in asthma.

Transcriptomics

When compared with non-asthmatics, whole-genome expression in peripheral blood of severe asthmatics is different such that a severe asthma disease signature comprised of nearly 1,700 genes was identified by Bigler et al. (38). Within severe asthma, distinct gene signatures associated with eosinophilia, mast cells, and group 3 innate lymphoid cells have been identified in patients with adult-onset asthma when compared with patients with childhood-onset asthma (32). Beyond asthma versus non-asthma stratification, numerous studies (summarized in **Table 1**) have aimed to define transcriptomic endotypes of asthma by analyzing differential gene expression in bronchial epithelium (27) and induced sputum samples (28, 30, 31, 33). Woodruff and colleagues were the first to identify two evenly sized "Th2-high" and "Th2-low" subgroups of mild, steroid naive asthma based on unsupervised hierarchical clustering of the expression levels of three Th2 cytokine induced genes (POSTN, CLCA1, and SERPINB2) in bronchial epithelial brushings. These subgroups were different with respect to their expression levels of IL-5 and IL-13 within the airway, airway hyper-responsiveness, IgE, blood and airway eosinophilia, and reticular basement membrane thickness. Not surprisingly, Th2-high gene expression was predictive of corticosteroid responsiveness as clinically relevant improvements in FEV_1 following 8 weeks of fluticasone use were only observed in the Th2-high group (27). Wilson et al. (39) identified seven genes (COX-2, ADAM-7, SLCO1A2, TMEFE2, and TRPM-1, and two unnamed genes) in bronchial brushing samples with expression levels that were moderately correlated with submucosal eosinophils, suggesting that they may also predict corticosteroid responsiveness. Given the limited clinical applicability of invasive bronchoscopic samples, Woodruff and colleagues went on to evaluate the variable expression of 14 genes relevant to Th2 inflammation in induced sputum samples (30). Generating a quantitative composite metric of IL-4, IL-5, and IL-13 gene expression, termed the "Th2 gene mean," the study population was again dichotomized into Th2-high (70%) and Th2-low (30%) subgroups (30). When compared with the Th2-low cluster, the Th2-high cluster had higher eNO levels as well as sputum and blood eosinophil counts (30). It is notable that sputum (AUC = 0.89) and peripheral blood (AUC = 0.89) eosinophil counts alone, but not eNO (AUC = 0.76), performed well as biomarkers of Th2-high asthma as assessed by the sputum cell "Th2 gene mean" (30). Acknowledging the limitations of analyzing pre-selected genes, Baines et al. (28) subjected whole-genome gene expression profiles from induced sputum of adults with stable asthma to unsupervised hierarchical cluster analysis. Three distinct transcriptional asthma phenotypes (TAPs) were identified that had similarities to previously defined sputum inflammatory phenotypes of eosinophilic (TAP1), neutrophilic (TAP2), and paucigranulocytic (TAP3) asthma (28). In fact, 92% of the differentially expressed genes between the TAPs overlapped when the population was grouped according to sputum quantitative cell count (eosinophilic, neutrophilic, and paucigranulocytic). The same investigators subsequently identified a sputum gene expression signature comprised of six genes (CLC, CPA3, DNASE1L3, IL1B, ALPL, and CXCR2) that was able to discriminate asthmatics according to their inflammatory endotype and predict ICS response (29). Of most interest, the six-gene expression signature outperformed the ability of sputum eosinophil count to predict corticosteroid response (FEV_1 responder vs. non-responder; AUC = 91.5) (29). Somewhat contradictory to the findings of Baines et al., when unbiased hierarchical clustering was performed on 508 genes that were differentially expressed between severe asthmatics with and without eosinophilic airway inflammation confirmed by sputum cytometry, one Th2-high and two non-Th2 transcriptome-associated clusters (TACs) were defined (33). When examining the distribution of the three TACs according to sputum inflammatory endotype, two TACs were associated with eosinophilic (TAC1 or TAC3) and neutrophilic (TAC2 or TAC3) inflammation, suggesting that two distinct transcriptional signatures are associated with both sputum eosinophilia and neutrophilia (33). Similarly, Yan et al. (31) did not detect significant between cluster differences

TABLE 1 | Summary of transcriptomics studies.

Reference	Subjects	Transcriptomic analysis	Computational analysis		Predictive investigation?
			Approach	Result	
Blood					
Bigler et al. (38)	Severe asthma non-smokers (n = 309); severe asthma current/ex-smokers (n = 110); mild–moderate asthma (n = 87); healthy controls (n = 100)	Microarray	Whole-genome gene expression data were filtered and 1,693 entities differentially expressed between severe asthmatics and non-asthmatics ("severe asthma disease signature") were subjected to unsupervised hierarchical clustering and topological analysis	Two clusters: "Severe asthma-enriched cluster" and "mixed cluster"	No
Bronchial brushings					
Woodruff et al. (27)	Mild-to-moderate asthma (n = 42); healthy controls (n = 28)	Microarray and qPCR	Unsupervised hierarchical clustering based on the gene expression profile of three IL-13-inducible genes (POSTN, CLCA1, and SERPINB2)	Two clusters: "Th2-high" and "Th2-low" asthma	Yes, ICS response
Wilson et al. (39)	Severe asthma non-smokers (n = 46); severe asthma current/ex-smokers (n = 16); mild–moderate asthma (n = 34); healthy controls (n = 41)	Microarray	No cluster analysis. Association of gene expression profiles with eosinophil and neutrophil counts evaluated using a general linear model	NA	No
Sputum					
Baines et al. (28, 29)	Adults with stable asthma (n = 59); healthy controls (n = 13)	qPCR	Whole-genome gene expression data (22,218 entities) were filtered and 7,436 entities present in all asthmatics were subjected to unsupervised hierarchical clustering	Three "transcriptional asthma phenotypes"	Yes, ICS response
Peters et al. (30)	Asthma (n = 37); healthy controls (n = 15)	qPCR	Supervised analysis of gene expression profiles of 14 genes relevant to Th2 inflammation	Single quantitative metric: "Th2 gene mean"	Yes, Th2-high and Th2-low endotype
Yan et al. (31)	Asthma (n = 100); healthy controls (n = 12)	Microarray	5,500 gene expression profiles from 186 Kyoto Encyclopedia of Genes and Genomes pathways were used to assess the pathway-based distance between samples followed by unsupervised k-means clustering	Three "transcriptomic endotypes of asthma"	No
Kuo et al. (33)	Moderate-to-severe asthma (n = 104); healthy controls (n = 16)	Microarray	508 differentially expressed genes between eosinophil (≥1.5%) and non-eosinophil (<1.5%) associated sputum inflammation were subjected to unbiased hierarchical clustering	Three "transcriptome-associated clusters"	No
Sputum, nasal brushings, bronchial brushings, and biopsies					
Hekking et al. (32)	Adults with adult-onset (n = 253) and childhood-onset severe asthma (n = 158)	Microarray	105 predefined genes associated with the presence of asthma, leukocytes, and induced lung injury were subjected to gene set variation analysis to form gene signatures associated with adult-onset severe asthma	Significantly different asthma, leukocyte, and induced lung injury gene signatures in adult-onset severe asthma patients (bronchial brushings n = 6; nasal brushings n = 5; sputum n = 3)	No

in sputum cell differentials suggesting an imperfect association with Th2 pathophysiology. Taken together, conflicting evidence surrounds the association between transcriptomic endotypes and sputum quantitative cell count.

Proteomics

While gene expression studies have dominated the omics landscape, it is their translated products, the proteins, which mediate airway inflammation in asthma by regulating cell recruitment. Numerous studies, limited by sample size, have investigated the "proteomic profile" of asthma in bronchoalveolar lavage fluid (BALF) (40, 41), bronchial biopsy (42), and sputum supernatants (43, 44). One relatively large study by SARP investigators (34) focused on 18 cytokines detectable in BALF and discriminated mild-to-moderate and severe asthmatics into four groups based on cytokine expression. The groups were independent of corticosteroid use and phenotypically distinct with respect to BALF cellularity and lung function. In another study, Hastie et al. (25) investigated the hypothesis that sputum inflammatory granulocytes define phenotypic subgroups of asthma with different patterns of inflammatory proteins. Protein microarray data of induced sputum were stratified by sputum cell differential (eosinophilic: ≥2% eos and <40% neuts; neutrophilic: <2% eos and ≥40% neuts; mixed-granulocytic: ≥2% eos and ≥40% neuts; paucigranulocytic: <2% eos and <40% neuts) and revealed differentially increased inflammatory proteins between the groups (25). Of note, 19 inflammatory mediators were significantly elevated in those asthmatics with neutrophilic bronchitis, a subset of which were correlated with neutrophil counts.

Metabolomics

Metabolomics, the exploration of biochemical molecules derived from metabolic processes, was recently reviewed for its applications in asthma (36). Studies to date strongly suggest that metabolic profiles measured in exhaled breath, urine, plasma, and serum may be applied as a point-of-care tool to discriminate asthma endotypes (36). Of most interest, "breathomics" (45) profiles volatile organic compounds in exhaled breath using an electronic nose and has demonstrated ability to discriminate asthmatics from healthy controls (46–48). Electronic nose "breathprints" are correlated with the percentage of sputum eosinophils (48, 49). One highly relevant study concluded that exhaled breath analysis outperformed the ability of both eNO and the percentage of sputum eosinophils to predict corticosteroid response in asthmatics from whom corticosteroids had been withdrawn (48).

Important Considerations and Future Studies

There are numerous limitations to clustering techniques applied in the studies discussed above; therefore, these results should be interpreted with caution. One such concern is cluster stability. Specifically, clusters identified at a specific point in time may not be reproducible at subsequent time points. Longitudinal stability assessment of the clusters that have been described is required to understand how they behave over time and in response to treatment and/or exacerbations. Similarly, different clusters may be identified across different asthma populations and therefore validation of cluster classification in independent cohorts of asthmatic patients is necessary to understand generalizability. These important considerations were evaluated for the first time by Loza et al. (6) who defined four severe asthma phenotypes in two independent severe asthma cohorts (ADEPT and U-BIOPRED), performing both external validation and longitudinal stability assessments. Cluster number and composition may also be influenced by the clustering methodology applied. Finally, it is also important to consider statistical robustness as the majority of studies to date define clusters comprised of a small number of asthma patients and hence have limited statistical power.

None of the omics-signatures discussed above have been translated to clinical practice as prognostic or predictive tests. In fact, the clinical utility of these signatures is currently unknown as the majority of studies to date have been observational and hypothesis generating. The ultimate potential of a biomarker-based clinical test is dependent on its analytical and clinical validity in addition to its clinical utility. A 30-point checklist of criteria, developed by McShane et al., should be considered to gauge the potential clinical utility of the omics-based signatures discussed in this review (50). Important considerations include those related to the specimen and assays used, and the appropriateness of the statistical methods used to develop and validate the signature (50). Studies are now required to address the paucity of evidence concerning (1) the longitudinal variability of omics data and endotypes and (2) the responsiveness of omics to therapies. Randomized clinical trials (RCTs) will be necessary to definitively confirm the clinical utility of novel omics-based signatures and design consideration for biomarker RCTs have been proposed (51). Specifically, intervention studies will be necessary to shed light on the capacity of these signatures to direct personalized therapy and to adjust doses of drugs during exacerbations in severe asthma. The qualitative nature of omics is an obvious limitation, therefore the development and application of qualitative metrics are certainly desirable. Similar to that described by Hinks et al. (52), multi-dimensional clinicopathobiologic clustering should also be considered to maximally leverage the measurements available to tertiary care clinicians. It is clear however that omics-based endotypes have similar molecular, physiological, and clinical characteristics to the inflammatory endotypes of eosinophilic, neutrophilic, mixed-granulocytic, and paucigranulocytic asthma.

INFLAMMATORY ENDOTYPE-GUIDED THERAPEUTIC STRATEGY

In this section, we focus our discussion on the segmentation of therapy in severe asthma [as defined by the European Respiratory Society/American Thoracic Society (4)] guided by inflammatory endotype. We support the notion that the specific nature of bronchitis reveals the underlying mechanism driving the bronchitis and therefore predicts therapy response. As summarized in **Figure 1**, our therapeutic strategy is dependent on the type of bronchitis (assessed using quantitative cytometry in induced sputum) (53) and severity of airway hyper-responsiveness (assessed using methacholine inhalation challenge). We emphasize the

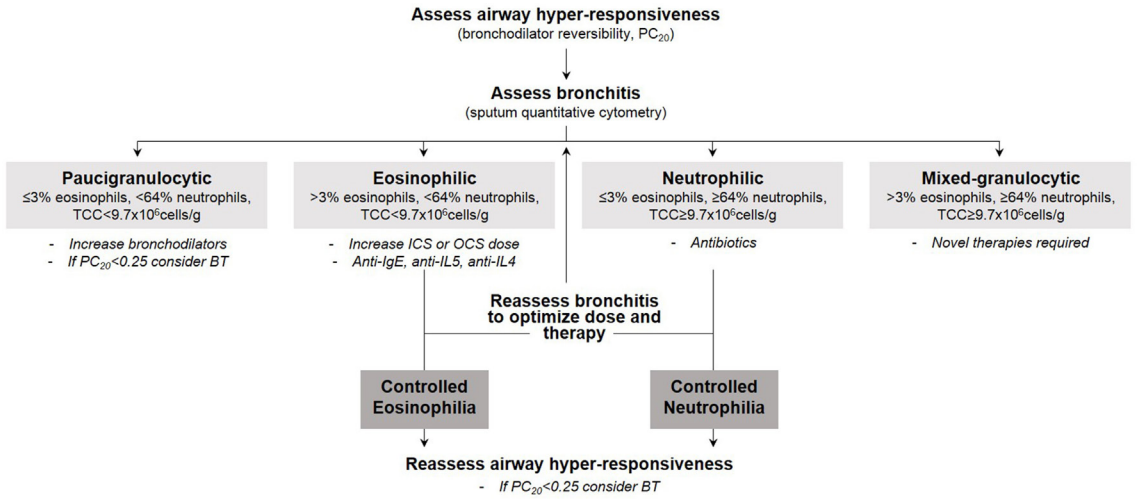

FIGURE 1 | Our therapeutic strategy in severe asthma guided by inflammatory endotype and severity of airway hyper-responsiveness.

importance of identifying the particular component of airways disease that drives symptoms in each patient prior to therapy selection. Furthermore, the components of airways disease should be assessed and subsequently reassessed to optimize therapy such that symptom and inflammation control is achieved. Careful endotyping is probably not necessary to manage most patients with asthma. This strategy has not been shown to reduce exacerbations in patients with mild asthma (reference our own Jayaram et al., study). It is currently recommended only for patients with more severe asthma.

Management of the T2-High Endotype

Although most commonly referred to as Th2-high, type 2 (T2)-high has emerged as a more appropriate and inclusive term given the involvement of numerous cell types (including type 2 innate lymphoid cells and natural killer cells) that are outside the originally described Th2 cell population (54). *Eosinophilic bronchitis* indicates a T2-driven mechanism and is usually steroid responsive. When bronchitis is eosinophilic in nature with a differential cell count of more than 3% (or eosinophil-free granules are observed), inhaled corticosteroids should be initiated and the dose titrated to keep the sputum eosinophil count below 3%. In situations where high-dose inhaled corticosteroids do not control sputum eosinophilia, oral corticosteroids are required. In corticosteroid-treated patients, absent eosinophils may suggest that the current steroid dose is unwarranted and therefore should be reduced to avoid over-treatment. In RCTs in adults, moderate-to-severe asthmatics managed by normalizing induced sputum eosinophils had significantly reduced exacerbations and hospital admissions when compared with those managed by national asthma guidelines (23, 55). When this strategy was applied clinically in 52 OCS-dependent asthmatics, maintained symptom control, reduced exacerbations, and preserved lung function was observed over 5 years (56). Novel biologic therapies should be considered for their steroid sparing effect, also for the minority of patients who are corticosteroid-insensitive (unresolved sputum eosinophilia despite high doses of oral corticosteroids) (57).

To date, all biologic therapies that have been approved and the majority of those in development aim to target T2 inflammation [recently reviewed (58)] and are therefore directed toward the T2-high eosinophilic asthma endotype. Detailed illustrations of asthma pathobiology and the mechanism of action of targeted therapies are provided in recent reviews (59, 60). Anti-IgE [omalizumab (61)] therapy was the first approved monoclonal antibody and is effective in patients with allergic asthma, confirmed by a positive skin prick test and serum IgE levels ≥30 IU/mL. Two anti-IL-5 therapies are approved [mepolizumab (62) and reslizumab (63)] and one is in phase 3 development [benralizumab (64, 65)] for use in severe eosinophilic asthma. With the potential to block both IL-4 and IL-13, one anti-IL-4 receptor alpha therapy is currently in phase 3 development (dupilumab) following successful phase 2b trials (66, 67). The efficacy of strategies targeting IL-13 alone [lebrikizumab (68) and tralokinumab (69)] is inconclusive as only modest clinical benefit has been shown. Drugs that target both IL-4 and IL-13 signaling (e.g., dupilumab) have reported more clinically relevant effects in phase 2 clinical programs. The reason for this difference is not immediately obvious. Perhaps one of the reasons may be related to the lack of accurate endotyping to identify patients in whom IL-13-mediated biology was not the dominant pathobiology of asthma. Selecting patients with significant airway hyper-responsiveness and mucus secretion may have demonstrated greater clinical effect. Other novel therapies that are currently being investigated include anti-thymic stromal lymphopoietin (70), IL-33 blocking agents (71), and prostaglandin antagonists (CRTH2) (72). A general scheme to choose the appropriate monoclonal antibody based on simple clinical features (e.g., clinical history of allergy, severity of asthma based on the dose of corticosteroids, and readily available biomarkers such as blood and sputum eosinophils and fraction of eNO) is shown in **Figure 2**. This is based on our clinical experience, the lack of evidence supporting omalizumab in prednisone-dependent asthmatics (73), the lack of efficacy of mepolizumab 100 mg subcutaneous in patients with persistent sputum eosinophilia despite high-dose inhaled and oral corticosteroid use (74), and

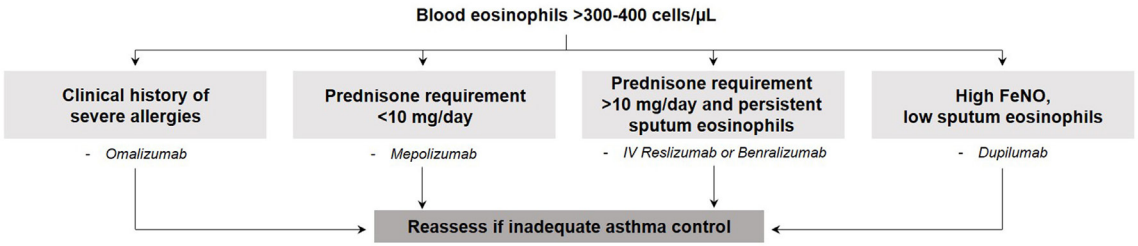

FIGURE 2 | General scheme to choose the appropriate monoclonal antibody based on simple clinical features.

the efficacy of benralizumab (75) and reslizumab (76) in severe prednisone-dependent patients. However, this approach needs to be prospectively validated in clinical trials.

Management of the T2-Low Endotype

Neutrophilic bronchitis with a raised total cell count is suggestive of a non-T2-driven disease and is not usually steroid-responsive, but instead a predictor of response to antibiotics. Macrolides, including clarithromycin (77) and azithromycin (78), have demonstrated effectiveness in non-eosinophilic asthmatics. To identify the causative pathogen that may help to direct antibiotic therapy, molecular microbiology, and extended cultures, including 16 s deep sequencing, should be considered in those patients with recurrent infective exacerbations. Logically, neutrophilic bronchitis may be a predictor of therapies targeted at pathways that lead to neutrophil recruitment such as TNF, IL-1, IL-6, IL-8, IL-23, and IL-17. Several small molecule anti-neutrophilic biologics have been developed, although there are currently no active phase 3 trials. Such molecules include CXCR2 antagonists (79, 80), 5-lipoxygenase-activating protein inhibitors (81), anti-IL-17 (82), and anti-TNFα (83). It is evident that few therapeutic options exist for these patients; therefore, intense study of the underlying mechanisms contributing to the T2-low endotype is urgent. Similarly, despite the severity of their disease, currently there are no treatment options for patients with *mixed-granulocytic asthma*. In fact, no interventions have been evaluated for this specific inflammatory endotype although preliminary evidence suggests therapies targeting the IL-6 pathway may be beneficial (84).

Asthmatics with *paucigranulocytic bronchitis* may not warrant anti-inflammatory therapy as symptoms in these patients may be driven solely by smooth muscle dysfunction (airway hyper-responsiveness). Therefore, these patients may benefit from smooth muscle-directed therapies such as additional bronchodilators and long-acting anti-muscarinic antagonists, mast-cell directed therapies, or in the most severe cases, bronchial thermoplasty. Our clinical experience suggests that bronchial thermoplasty is indicated when severe airway hyper-responsiveness ($PC_{20} < 0.25$) and frequent exacerbations persist despite absent or controlled airway inflammation (85); however, clinical trials are required to confirm this hypothesis. Bronchial thermoplasty after therapy has been optimized to control eosinophilic and/or neutrophilic inflammation has been previously described by Cox et al. (85) for those patients with persistent symptoms. Although the mechanism of action is uncertain, bronchial thermoplasty aims to attenuate airway smooth muscle mass through the delivery of localized thermal energy (86).

CONCLUDING REMARKS

One of the major challenges of respiratory medicine is the management of patients with severe asthma. Identifying a specific endotype may have profound implications on advanced targeted therapy selection and intern clinical outcomes. It should be acknowledged that the mere presence of a particular gene, protein or a cell do not necessarily make them a therapeutic target or disease biomarker. Teasing out "association" from "causality" is of paramount importance. Koch's postulates, described in 1890, remain a useful benchmark to establish whether there is causal relationship between a particular molecular or cellular observation and a disease presentation. Persistence of a particular cell type or cytokine and their temporal association with exacerbation and resolution provide strong evidence to support a causative role. A consensus as to how to best identify asthma endotypes and what therapy to use for a given endotype is now required. In the meantime, quantitative cell counts in sputum provide the most reliable method to identify T2 (most eosinophilic) and non-T2 (most neutrophilic) inflammatory processes.

AUTHOR CONTRIBUTIONS

PN conceived the idea. SS prepared the first draft. PN and SS edited and reviewed the manuscript and have approved the final version of the manuscript.

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MINI REVIEW
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(A Critical Appraisal of) Classification of Hypereosinophilic Disorders

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Hypereosinophilia (HE) is a heterogeneous condition that can be reported in various (namely inflammatory, allergic, infectious, or neoplastic) diseases with distinct pathophysiological pathways. In 1975, Chusid et al. published the first diagnostic criteria of hypereosinophilic syndromes (HES). Over the years, as both basic and clinical knowledge improved, several updates have been suggested, with a focus on better distinguishing isolated or asymptomatic eosinophilia from diseases with specific eosinophil-related organ damage. Moreover, underlying molecular and cellular mechanisms of eosinophilia gradually became the cornerstone of successive attempts to classify HE-related diseases. In 2011, the International Cooperative Working Group on Eosinophil Disorders criteria emerged from a multidisciplinary Working Conference on Eosinophil Disorders and Syndromes, and provided substantial contribution to the clarification of general concepts and definitions in the field of HE. Yet, owing to the low prevalence of HE/HES, to the numerous diseases encompassed in the spectrum of HE-related disorders (with sometimes overlapping phenotypes), many questions are left unanswered (e.g., the need to better standardize the use of modern molecular tools, or the clinical relevance of distinguishing different subtypes of idiopathic HES). Here, we review the current state of knowledge in the fields of classification and diagnosis criteria of HE-related diseases, with emphasis on the analysis of both strengths and weaknesses of present concepts and their usefulness in daily practice.

Keywords: hypereosinophilic syndrome, hypereosinophilia, classification, eosinophilic granulomatosis with polyangiitis, eosinophilic disorders

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INTRODUCTION

The concept of "hypereosinophilic syndromes" (HES) was introduced by Hardy and Anderson in 1968 (1), and Chusid et al. later suggested in 1975 diagnostic criteria for HES (2). These readable and easy-to-use criteria comprised chronic (i.e., longer than 6 months) hypereosinophilia (HE) (i.e., above 1.5 × 10^9/L) with no identifiable cause, associated with clinical manifestations. Nowadays, given the various identified molecular mechanisms underpinning HE (e.g., T-cell-dependent IL-5 production, clonal abnormalities of the myeloid lineage) and the subsequent heterogeneity of diseases encompassed in the spectrum of HES, this first set of diagnostic criteria has become outdated.

Hereafter, we will review the main classifications in HES, and discuss their strengths and potential pitfalls.

CURRENT CLASSIFICATIONS AND DEFINITIONS OF EOSINOPHILIC DISORDERS

Initially, the concept of HES was commonly applied to patients with multi-organ damage (often involving the heart) and unexplained chronic HE above 1.5×10^9/L. Yet, different clinico-biological phenotypes were already observed in the first published series of patients with HES, suggesting that various underlying pathophysiological processes could be involved (2, 3). Hence, a subgroup of patients presented with features suggestive of myeloproliferative neoplasm (i.e., anemia, splenomegaly, myelofibrosis, etc.), which led to the concept of myeloproliferative ("leukemic" for Chusid) HES. Decades later, clonal abnormalities involving fusion transcripts (among which PDGFRA and PDGFRB genes) were identified in the same subgroup of patients, thereby validating *ex post* the initial hypothesis (4). Next, the lymphoid variant of HES was defined in another subgroup of patients with dermatologic manifestations that responded to corticosteroids and were originally classified as hypersensitivity (or non-malignant or allergic) HES but in which abnormal clonal T-cells (e.g., $CD3^-CD4^+$ T-cells) that produced eosinophilopoietins were later identified (5).

In the early 2000s, numerous expert classifications embedded the above-defined concepts of molecularly defined myeloproliferative-HES, lymphoid HES, and idiopathic HES (when no molecular or immunological abnormality can be found) (6, 7). Yet, less than two decades later, these classifications already seem outdated due to recent major breakthroughs in molecular biology. Currently, the two main—and partially redundant—classifications are the one proposed by the WHO (which covers only primary/neoplastic HES) (8) and the one proposed by the International Cooperative Working Group on Eosinophil Disorders (ICOG-EO) in 2011 (9). The ICOG-EO (an international and multidisciplinary panel of experts) agreed on unifying terminologies and criteria, and suggested a classification that delineates various forms of HE and HES (including primary and secondary variants) based on specific hematologic and immunologic conditions.

Definitions of Eosinophilia and HE

International Cooperative Working Group on Eosinophil Disorders first provided basic definitions of what should be considered as HE (**Table 1**). The cut-off of 1.5×10^9/L was chosen to differentiate HE ($>1.5 \times 10^9$/L) from "eosinophilia" (between 0.5 and 1.5×10^9/L). The duration of 1 month of blood HE (instead of the 6 month delay comprised in Chusid criteria) was retained as sufficient and indeed makes sense from a clinical viewpoint, considering that life-threatening organ involvement is frequent in HES. Importantly, the latter criteria also include tissue eosinophilia in the field of HE-related disorders, thereby highlighting the fact that discrepancies between blood and tissue eosinophilia (e.g., marked tissue eosinophilia without blood eosinophilia or HE) can be reported in some eosinophilic disorders (e.g., eosinophilic esophagitis or acute eosinophilic pneumonia). Hence, the pathologist's definition of tissue HE includes more than 20% of eosinophils in bone marrow sections, and/or (for other tissues) extensive tissue infiltration by eosinophils, and/or marked deposition of eosinophils granule proteins (**Table 1**).

Definition of HES

The ICOG-EO defined HES as blood HE or tissue HE associated with HE-related organ damage (precluding the absence of an alternative diagnosis for the organ dysfunction) (**Table 1**). As compared with the Chusid criteria, this definition comprises a causal link between the observed tissue HE and organ damage, the probability of which is considered sufficient in presence of the following specific histological findings: (1) fibrosis or (2) thrombosis or (3) cutaneous eosinophilia (with erythema or angioedema or pruritus or eczema or ulceration) or (4) peripheral or central neurologic defect.

TABLE 1 | Summary of the ICOG-EO's definitions of eosinophilic disorders.

Term	Definition and criteria	Subtype
Blood eosinophilia	Eosinophils > 0.5×10^9/L in blood	
Hypereosinophilia	Eosinophils > 1.5×10^9/L in blood on 2 examinations (interval > 1 month) and/or tissue HE defined by the following: 1. Percentage of eosinophils in BM section exceeds 20% of all nucleated cells and/or 2. Pathologist is of the opinion that tissue infiltration by eosinophils is extensive and/or 3. Marked deposition of eosinophil granule proteins is found (in the absence or presence of major tissue infiltration by eosinophils).	HE_{FA} HE_{US} HE_N HE_R
Hypereosinophilic syndrome	1. Criteria for peripheral blood HE fulfilled and 2. Organ damage and/or dysfunction attributable to tissue HE, and 3. Exclusion of other disorders or conditions as major reason for organ damage.	HES_I HES_N HES_R
Eosinophil-associated single-organ diseases	1. Criteria of HE fulfilled and 2. Single-organ disease	

HE, hypereosinophilia; HE_{FA}, familial (hereditary) hypereosinophilia; HES, hypereosinophilic syndrome; HE_{US}, hypereosinophilia of undetermined significance; HEN, Primary (clonal/neoplastic); HE_R, secondary (reactive) hypereosinophilia; HES_I, idiopathic hypereosinophilic syndrome. Adapted from Valent et al. (9).

Definition of Neoplastic HE/HES (HE_N/SHE_N)

Hypereosinophilia or HES are considered as neoplastic (or clonal or primary, HE_N/SHE_N) when an underlying myeloid/lymphoid/stem cell neoplasm with HE and rearrangement of *PDGFRA, PDGFRB, FGFR* or with *PCM1-JAK2* translocation is identified.

HE_N/HES_N also encompasses the broad spectrum of other WHO-defined myeloid neoplasms with associated eosinophilia, such as *BCR-ABL1*$^+$ chronic myeloid leukemia, *JAK2*-mutated myeloproliferative neoplasms, *KIT D816V*$^+$ systemic mastocytosis, acute myeloid leukemia (AML) associated with CBFβ fusion gene, myelodysplastic syndromes associated with HE, and other WHO-defined myeloid neoplasms with HE (10).

Finally, HE_N/SHE_N also includes a last subgroup of patients classified as chronic eosinophilic leukemia not otherwise specified, which should remain an exclusion diagnosis defined by (1) the exclusion of all genetically predisposed conditions described previously, (2) the absence of molecular or cytological features of AML, and (3) the presence of a non-specific clonal cytogenetic or molecular abnormality (i.e., TET2, ASXL1, IDH2, SF3B1) or blast cells >2% in the blood and >5% in the bone marrow.

Definition of Reactive HE/HES

Reactive HE and HES (HE_R and HES_R) aggregate all conditions (e.g., parasitic infections, adverse drug reactions, inflammatory, or neoplastic diseases) in which eosinophils are considered as non-clonal and are thought to be driven by Th-2 (mainly IL-5) cytokines. Importantly, although it may seem counterintuitive at first sight, ICOG-EO classification emphasizes the fact that HE_R and HES_R (with reactive eosinophils) can be observed in neoplastic diseases in which the clonal cells (T-cells, Reed-Steinberg cells, carcinomatous cells, mast-cells, etc.) are the main sources of IL-5 and other eosinophilopoietins. Hence, the lymphocytic variant of HES (an indolent T-cell lymphoproliferative disease) is, therefore, classified as a subtype of HES_R.

Definition of Idiopathic HES

When a patient fulfills the criteria of HES but does not comply with the definitions of both HES_N and HES_R, the diagnosis of idiopathic HES (HES_I) can be retained. Interestingly, in main expert centers in HES, more than half of HES patients are classified as HES_I, while 10–20% of patients each belong to HES_N and HES_R categories (11).

Definition of HE of Undetermined Significance

Patients with isolated blood HE but without organ dysfunction and who will remain completely asymptomatic over time are not that uncommon. Hence, after that an initial comprehensive evaluation excludes HE_N and HE_R, the ICOG-EO classification suggests that such patients should be classified as HE of undetermined significance (HE_{US}). This newly defined subgroup has major clinical implications since recent data strongly suggests that such patients carry a good prognosis and should only be closely followed without treatment (12).

OLD AND NEW CRITERIA FOR THE CLASSIFICATION OF NEOPLASTIC HE/HES

In the 2005 and 2010 classifications of HE/HES, experts brought to the forefront "good old fashioned" features suggestive of a myeloid neoplasm (i.e., hepatosplenomegaly, increased serum vitamin B12 or tryptase, anemia, thrombocytopenia, myelofibrosis) as criteria for "myeloproliferative-HES," even in the absence of a molecularly proven HES_N (6, 7). With the exception of the blast cell count, the ICOG-EO and WHO classifications have nearly completely excluded these patients—which are now classified as HES_I—from the field of HES_N. This distinction of patients with presumed myeloid neoplasm is clinically relevant as response to different treatment options differs (less response to steroids, more likely to respond to imatinib). Hitherto, such patients carry a guarded prognosis [the latter being closer to HES_N than that of other HES_I patients (13, 14)] and, as patients with HES_N, may require treatment with tyrosine kinase (TK) inhibitors, cytotoxic drugs, or even bone marrow transplantation. Hence, the current distinction between patients with molecularly defined HES_N from those with similar clinical features but without any identifiable mutation is questionable, and it seems desirable that further updates of HE/HES classifications should individualize these myeloproliferative-HES patients as a specific subgroup even in the absence of an identified mutation.

Next, due to the development of new sequencing methods and tools in malignant hematology [especially the next-generation sequencing (NGS)], the field of neoplastic HES has considerably evolved. Since the *FIP1L1–PDGFRA* fusion transcript gene was discovered in 2003, the list of genetically defined HE has regularly been implemented over the years and now comprises 72 distinct entities consisting mostly of TK fusion genes (10).

The identification of numerous mutations in myeloproliferative disorders and myelodysplastic syndrome raise the question whether these new mutations should be included in further classifications of HES. Two recent studies report NGS results in two cohorts of 98 and 51 patients with HE_{US} and/or HES (15, 16). Interestingly, such mutations (including *ASXL1*, *TET2*, *SETBP1*, *CSFR3*, and SF3B1) were identified in 11 and 28% of patients, respectively, suggesting that a significant proportion of patients otherwise classified as HE_{US} and/or HES_{US} might rather belong to the HE_N and HES_N subgroups. Yet, none of these studies provided convincing elements demonstrating the transforming capacity of these mutations, suggesting that they may not be the true driving mutation for HES/HE_{US}. In addition, the impact of NGS-identified mutations on survival remains an open question, a poorer prognosis in patients with NGS-mutations being suggested in a single study (16). Hence, NGS seems to be a highly powerful tool to identify molecular defects in HE/HES. Yet, large prospective registries are needed in order to evaluate its potential usefulness in daily practice regarding patients' treatment and prognosis, before this tool be included as a new criterion for HE_N or HES_N.

REACTIVE HES: A DISORDER WITH BYSTANDER HE OR A TRUE REACTIVE HES?

Many pathologic conditions can induce reactive blood and/or tissue eosinophilia (HE_R), due to the overproduction of eosinophilopoietic cytokines, mostly IL-5. Yet, in many of these situations, skin and organ damage seem to be due to an autoimmune process (e.g., bullous pemphigoid), a malignant disease (e.g., cutaneous lymphoma, histiocytosis, mastocytosis) or to the massive tissue infiltration by $IgG4^+$ plasma cells (e.g., IgG4-related disorders) rather than to eosinophilic tissue infiltration. From a pathological viewpoint, substantial effort (including assessment of extracellular deposition of eosinophil granule proteins by immunohistostaining) has been made by the ICOG-EO classification to define eosinophilic tissue infiltration. Yet, these laboratory techniques are often neither standardized nor performed routinely, and not used as a diagnostic tool in daily practice.

On the other side, in some patients with solid cancer, lymphoma or helminthiasis, a pronounced blood and tissue eosinophilia may occur in organs other than those affected by the underlying disease. In such situations, a true eosinophilic endomyocardial fibrosis due to eosinophil toxicity—as well as many other organ involvements—have been reported. Hence, physicians must be aware that the same disease may induce a bystander blood and/or tissue HE without meaningful consequences related to eosinophils, or a true reactive HES_R.

According to the clinical context, the initial workup of an unexplained HE/HES must include broad investigations in order to rule out with certainty an underlying disease likely to favor HE_R/HES_R. The choice of keeping HES_R as part of the ICOG-EO's nosology has clinical implications: (1) HES_I is an exclusion diagnosis which supposes that all etiologies of HES_R must be excluded and (2) the treatment of the underlying cause may reverse HE_R, but in case of organ damage and/or dysfunction attributable to tissue HE (i.e., HES_R), corticosteroids may be considered from the outset in addition to the treatment of the underlying disease.

UNMET NEEDS IN THE DIAGNOSIS OF THE LYMPHOID VARIANT OF HES

HES_L, a subtype of HES_R, is a chronic clonal indolent T-cell lymphoproliferative disorder in which mature peripheral T-cells secrete high amounts of IL-5, leading to the polyclonal expansion of eosinophils. Hence, to some extent, HES_L can be considered as the archetype of Th-2 driven eosinophilic disorders (17). Patients can be aymptomatic or poorly symptomatic for years, with HE being the sole manifestation of the indolent T-cell proliferative disorder (18). Diagnosing HES_L is important for three reasons: (1) its treatment can differ from that of HES_I, notably because of frequent corticosteroid dependency requiring additional corticosteroid-sparing-treatments, (2) clonal T-cells that are found in blood and tissues of HES_L patients can be mistaken for aggressive T-cell lymphoma, and patients wrongly treated as such with chemotherapy, and (3) authentic T-cell lymphomas (e.g., angioimmunoblastic T-cell lymphomas) may occur during disease course of these patients, who should be closely monitored (18–20).

Diagnosing HES_L usually requires the detection of an abnormal T-cell subset in the peripheral blood. Experts agree that three main subsets of HES_L must be systematically searched by flow cytometry: $CD3^-CD4^+TCRab^-$, $CD3^+CD4^+CD7^-$, and $CD3^+CD4^-CD8^-TCRab^+$ (21). Although lacking specificity, further confirmation of HES_L is supported by a clonal TCR rearrangement.

Yet, a clear definition of what should (or should not) be diagnosed as HES_L is lacking in all current classifications. Pertinently, should a cutoff of absolute or relative counts of such abnormal T-cells be defined? Is the demonstration of their ability to produce high levels of IL-5 necessary? Is a clonal TCR rearrangement necessary or sufficient to define HES_L? Could other abnormal subsets of blood cells (e.g., type 2 innate lymphoid cells) define HES_L? Last, several biological biomarkers such as IL-5, CCL-17/TARC, IgE, which demonstrated in many studies potential utility in their ability to distinguish between various subtypes of HES (18, 22) could also be added as additional diagnostic criteria in future classifications.

IDIOPATHIC HES: A MULTIFACETED DISEASE WITH MANY OVERLAPPING CONDITIONS

Should Multi-Organ and Single-Organ Diseases Be Distinguished?

According to current definitions, all patients with blood HE and organ damage with significant eosinophilic infiltration could be classified as HES, whatever the number of organs involved.

Yet, the ICOG-EO classification also makes a clear distinction between HES and several other organ-restricted conditions with HE (e.g., eosinophilic cystitis, eosinophilic esophagitis, eosinophilic gastroenteritis, eosinophilic pneumonia, dermatologic conditions associated with HE, etc.) that by definition only affect a single organ during the entire course of the disease (the main suggested reason being that the causative role of eosinophils in organ damage is unclear) (9). Nevertheless, some patients with HES_L, *FIP1L1–PDGFRA* HES_N or HES_I may only have single-organ involvement at disease onset, with further organ involvement only occurring during follow-up (22). Furthermore, the single-organ damage also depends on the way the clinician looks at the patient: in chronic eosinophilic pneumonia, many patients have sinonasal polyposis. Should they be classified as single-organ disease or HES? Hence, such separation at diagnosis between eosinophilic single-organ diseases and HES appears questionable. In our mind, most patients with eosinophilic single-organ disorders demonstrated by histopathology should be investigated and subsequently followed as HES, even though some of these patients will indeed subsequently never develop multi-organ HES.

Should Outcome Profiles of HE_I/HES_I Be Distinguished?

In daily practice, HE_I/HES_I treating physician are confronted with various patterns of disease courses. First, some patients

will present a single flare of variable duration and will recover (spontaneously or after corticosteroids tapering and discontinuation) without subsequent relapse (**Figure 1A**). Of note, according to previous classifications of HES disorders (6, 7), these latter patients with less than 6 months of disease duration would not have been classified as HES *per se*. Next, other patients periodically relapse during follow-up, with a variable delay in between flares (that can be weeks, months or years in some cases, and whose severity may also fluctuate overtime). Hence, a pragmatic approach to treating such patients could be short courses of corticosteroids but without long-term therapy (**Figure 1B**). Notably, both outcome profiles have been reported in various single-organ eosinophil disorders [e.g., acute eosinophilic pneumonia, eosinophilic gastroenteritis (23), episodic angioedema with HE (Gleich's syndrome) (24)] as well as in HES_I. Last, a third set of patients will present, usually in the context of corticosteroid dependency, chronic persistent disease requiring second-line treatments (**Figure 1C**).

Underlying mechanisms involved in eosinophilia are likely to differ between the three disease patterns described here above. Hence, a single flare of HES without subsequent relapse (pattern A) strongly suggests temporary exposure to an extrinsic trigger (e.g., drug-induced eosinophilia for the disease). Conversely, recurrent or chronic persistent HE_I/HES_I (patterns B and C) suggest an intrinsic dysfunction of eosinophil regulation and/or a persistent unidentified underlying cause. By analogy with multiple sclerosis, it is advisable that these different patterns of disease courses be incorporated into further updates of disease classifications in an effort to homogenize inclusion criteria in clinical trials and to individualize patient care.

Antineutrophil Cytoplasm Antibody (ANCA)-Negative Eosinophil Granulomatosis with Polyangiitis (EGPA) and HES_I: The Diagnostic (and Therapeutic) Dilemma

Eosinophil granulomatosis with polyangiitis is defined in the 2012 International Chapel Hill Consensus Conference on the Nomenclature of Vasculitides as an eosinophil-rich and necrotizing granulomatous inflammation often involving the respiratory tract, with necrotizing vasculitis predominantly affecting small to medium vessels, and associated with asthma and eosinophilia (25). EGPA differs from other ANCA-associated vasculitides (AAV) by the constant presence of asthma, blood and tissue eosinophilia, and the low prevalence of ANCA positivity, which are detected in only 20–40% of patients (26). Next, the phenotype of ANCA-positive and ANCA-negative EGPA patients differ, with ANCA-negative patients having less vasculitic manifestations (purpura, peripheral neuropathy, glomerulonephritis, scleritis) but more frequent cardiomyopathy (often mimicking that observed in HES) (27). Considering that ≈40% of patients with asthma, HE above 1.5 G/L and at least another systemic manifestation had neither symptoms of vasculitis nor ANCA, a recent European Respiratory Society-endorsed Taskforce on EGPA suggested that this subgroup of patients be considered to have hypereosinophilic asthma with systemic (non-vasculitic) manifestations (HASM) rather than genuine EGPA (28). The results of a negative trial testing azathioprine versus placebo in low-risk EGPA (29) and the recent MIRRA study demonstrating the benefit of mepolizumab in EGPA (30) both confirm the overlap between ANCA-negative EGPA and HES. Given the fact that clinical and biological profiles of ANCA-negative EGPA and HES overlap markedly (31), it seems appropriate to consider a diagnosis of HES in patients with HASM should be mentioned in further updates of both AAV and HES classifications (32).

FIGURE 1 | Various patterns of disease courses observed in hypereosinophilic syndromes (HES). Pattern **(A)**: single flare without subsequent relapse. Pattern **(B)**: several relapses with intervals of complete remission. Pattern **(C)**: chronic persistent disease.

CONCLUSION

The concept of HES has evolved considerably since the first classification by Chusid in 1975, and the recent ICOG-EO classification has successfully embedded most of the field's recent breakthroughs. These include, albeit not exclusively, the identification of numerous TK fusion genes, the concept of HES_R (among which HES_L), and the need for a clear histopathological definition of eosinophilic tissue infiltration. Moreover, this classification puts an end to many longstanding issues in the HE/HES domain and is a useful tool for the physician in daily care, allowing for better classification of patients between single-organ disease, HES_N, HES_R, HES_I, and HE_{US} (a condition that does not require therapy). Nevertheless, due to the lack of large prospective cohorts of HE/HES patients, one major limitation of the ICOG-EO classification is that it is mainly expert based and, thus, remains low-evidenced. Pertinently, many points (e.g., the need for a clear definition of HES_L; how to treat patients with a myeloproliferative phenotype but for whom a clonal mutation is not (yet) evidenced; improving the diagnostic workup of patients suspected of ANCA-negative EGPA versus HES_I) are open for improvement and should be the starting point of future HE/HES-targeted research. Hence, the implementation of international multicentric registries is awaited in order to improve current classifications of HE/HE and subsequently patient care.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Eosinophilic Gastrointestinal Disorders Pathology

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Eosinophilic gastrointestinal disorders (EGID) are characterized pathologically by excess eosinophils in mucosal biopsies of one or multiple sites in the gastrointestinal (GI) tract, simultaneously or sequentially. Eosinophilic esophagitis (EoE) is the best characterized EGID, and in most patients it is an abnormal immune-mediated response to food antigens. Current recommendations for diagnosis include signs and symptoms of esophageal dysfunction that do not respond to proton-pump inhibitor therapy, and esophageal biopsies that exhibit at least 15 intraepithelial eosinophils in at least one high power field (HPF). Therapy consists of swallowed glucocorticoids or dietary elimination. Eosinophilic gastritis (EG) is the second most common form of EGID, but like all forms of EGID except EoE consensus recommendations for either clinical or pathological diagnosis do not exist. EG may be associated clinically with peripheral blood eosinophilia, hypoalbuminemia, and anemia, and pathologically with marked expansion of lamina propria by dense eosinophilic infiltrates. Eosinophilic enteritis (EE) may be subdivided into eosinophilic duodenitis, eosinophilic jejunitis, and eosinophilic ileitis. Most investigators believe that EE rarely, if ever, exists as a solitary form of EGID and is encountered only in patients who have at least one other affected portion of the GI tract. Eosinophilic colitis (EC) is perhaps the most enigmatic EGID. Distinction of EC from inflammatory bowel disease may be problematic especially in children. Multiple possible etiologies for EGID include hypereosinophilic syndrome, drug reactions, etc. Currently, the only etiology that can be identified histologically is parasitic infestation, if a portion of an invasive parasite is found in mucosal biopsies. This review will provide guidelines for the pathologic diagnosis of the various forms of EGID.

Keywords: esophagitis, colitis, inflammatory bowel disease, allergy, genome

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INTRODUCTION

In the mid-twentieth century, excess eosinophils in the gastrointestinal (GI) tract were correlated with a multitude of symptoms, based on the examination of resected bowel segments. Increased density of eosinophils in mucosa was found in resected bowels from patients who manifested anemia, hypoproteinemia, and diarrhea, and increased density of eosinophils in the muscularis propria was seen in resected specimens from patients whose major clinical manifestation was bowel obstruction (1). The development of safe and flexible endoscopes resulted in fewer surgical procedures, and therefore resected bowel segments, and greater reliance on mucosal biopsies for diagnosis of

and monitoring response to therapy for GI diseases. Currently, eosinophilic gastrointestinal disorders (EGID) are defined pathologically, virtually, and exclusively by endoscopically obtained mucosal biopsies (2, 3) necessitating greater understanding of the role of eosinophils in GI mucosal health and disease (4, 5). Pathologic confirmation of eosinophilic inflammation confined to the muscularis propria can be accomplished currently by laparoscopic mural bowel biopsies that are guided by imaging studies showing bowel wall thickening. There may also be subserosal dense eosinophil infiltrates that are associated with ascites (1, 6), and in those cases large numbers of eosinophils are found in the ascitic fluid.

Eosinophilic gastrointestinal disorders are subclassified according to the affected site(s) as eosinophilic esophagitis (EoE), eosinophilic gastritis (EG), eosinophilic enteritis (EE), and eosinophilic colitis (EC). Eosinophils are normally found in the mucosa of all parts of the GI tract except the esophagus, but there are few studies that quantify eosinophils in normal GI mucosa complicating the ability to recognize pathologic numbers of eosinophils (7). Consensus recommendations for diagnosis of EGID currently exist only for EoE.

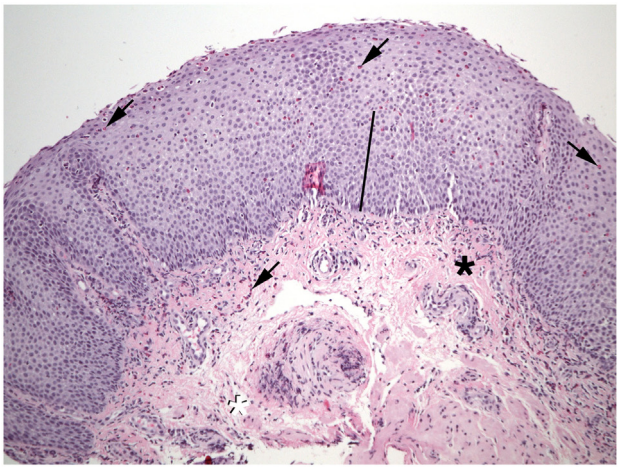

FIGURE 1 | Numerous eosinophils (arrows) are found in the epithelium of this esophageal biopsy. The basal zone is markedly expanded (bar). Lamina propria fibers appear thickened near the epithelium (black asterisk), but not at the deep margin (white asterisk). Eosinophils are also present in the lamina propria (shaded arrows).

EOSINOPHILIC ESOPHAGITIS

In the late-twentieth century, pediatric and adult patients who had abundant eosinophils in esophageal mucosal biopsies and who responded clinically and histologically to dietary restrictions were described (8–10). Characteristic esophageal endoscopic findings were reported in such patients (11). Case reports and small series of affected patients appeared increasingly in the literature. Subsequent retrospective studies of archived pathology slides identified esophageal biopsies, displaying numerous intraepithelial eosinophils in files from the 1980s (12–14). Some of those patients were later diagnosed with EoE, and patients who had as few as 5 eosinophils/HPF were statistically more likely to have signs and symptoms of esophageal dysfunction years later compared to patients whose esophageal biopsies had not displayed intraepithelial eosinophils in the remote past (15).

In 2007, the first set of recommendations for EoE diagnosis and therapy was published (16), and several have subsequently appeared (17–19). All guidelines or recommendations cite a peak eosinophil count of ≥15 eosinophils in at least one high power field (HPF) in an esophageal biopsy from at least one site in the esophagus (distal, mid, or proximal) as the pathologic criterion for diagnosis. All recognize that other pathologic changes are found in EoE biopsies. Recently, an EoE histology scoring system (EoEHSS) was developed that scores eight pathologic features including eosinophil density, but also pathologic features whose definition does not include eosinophils (20). Eosinophil inflammation, basal zone hyperplasia, eosinophil abscess, eosinophil surface layering, dilated intercellular spaces, surface epithelial alteration, dyskeratotic epithelial cells, and lamina propria fibrosis are evaluated in the EoEHSS (**Figures 1** and **2**; **Table 1**). The features are scored separately for severity of change (grade) and for the amount of tissue that is affected by each feature (stage). The EoEHSS scores better identified treated compared to untreated

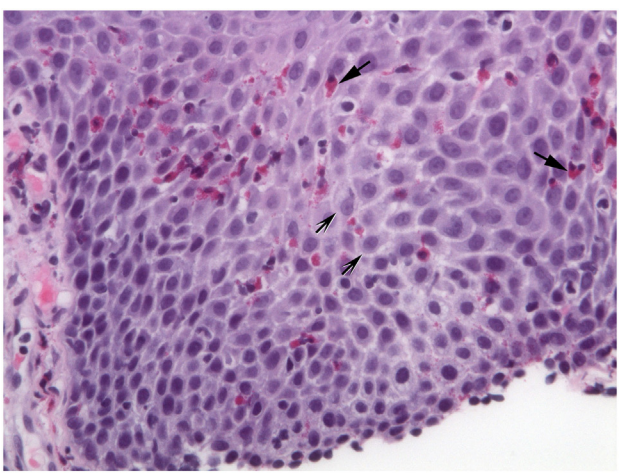

FIGURE 2 | Extracellular eosinophil granules are seen (arrows). Intercellular bridges (shaded arrows) are visible in the dilated intercellular spaces.

subjects' biopsies than did peak eosinophil count, indicating the importance of evaluating more than eosinophil density in esophageal biopsies obtained to diagnose or monitor EoE, and also indicating the importance of determining the amount of tissue damage in addition to the degree of damage. A systematic method to evaluate the myriad endoscopic features of EoE has also been developed (21).

A whole-genome messenger RNA esophageal expression analysis identified a unique EoE transcriptome and eotaxin-3 was the most upregulated gene (22). A diagnostic panel derived from the results of the original microarray study and consisting of 96 genes distinguishes EoE from non-EoE biopsies and can be used on paraffin-embedded tissue samples (23).

TABLE 1 | Eosinophilic esophagitis (EoE) histology scoring system definitions.

Feature	Definition
Eosinophilic inflammation	Based on peak eosinophil count
Basal zone hyperplasia	Basal zone occupies more than 15% of total epithelial thickness
Eosinophil abscess	Eosinophil aggregate that disrupts the underlying epithelial architecture
Eosinophil surface layering	Eosinophils align in one or more rows in the upper third of the epithelium
Dilated intercellular spaces	Intercellular bridges are visible in paracellular spaces
Surface epithelial alteration	Surface epithelial cells stain more darkly than normal and eosinophils that may be present among the altered epithelial cells
Dyskeratotic epithelial cells	Epithelial cells with deeply staining cytoplasm and shrunken hyperchromatic nuclei that generally occur singly and may be found anywhere in the epithelium
Lamina propria fibrosis	Coalesced fibrils form fibers of varying diameter

Prior to treatment, children who have EoE commonly experience vomiting and poor weight gain or weight loss, but adolescents and adults commonly experience dysphagia that may include food impaction (24). Therapy commonly consists of swallowed glucocorticoids (25–30) and elimination diet (31, 32). Response to dietary antigen-removal suggests that Th2 immunity is important in EoE pathogenesis; in fact, IL-13 and IL-5 levels are increased in EoE biopsies (33), and monoclonal antibodies to each of those cytokines reduce esophageal inflammation (34–38). Proton pump inhibitors (PPI) were used to distinguish EoE patients from those who had gastroesophageal reflux disease (GERD): clinical response to a PPI was considered not consistent with EoE and patients were believed to have GERD (16). However, a group of patients who respond initially to PPI, but subsequently may become refractory to PPI emerged and is believed to be a phenotype of EoE (39). Their pre-PPI biopsies are indistinguishable from patients who do not respond to PPI therapy and the genotype identified in their biopsies is very similar to nonresponders (40).

Eosinophilic esophagitis has increased in both prevalence and incidence (41–43) and is currently an important cause for food bolus impaction (44) and esophagitis (41, 45). Care of afflicted patients in the U.S. is estimated to consume approximately one billion dollars annually (46).

EOSINOPHILIC GASTRITIS

In contrast to the esophagus, eosinophils are normally present in gastric mucosa, but in lower concentrations than in the small and large bowel (47–49). The criteria for diagnosing EG histologically include ≥30/HPF in ≥5 HPF and ≥70/HPF in ≥3 HPF (49, 50). Common features of EG biopsies are eosinophil sheets in expanded lamina propria, excess intraepithelial eosinophils, eosinophil cryptitis/abscess, and eosinophils in the muscularis mucosa and submucosa (**Figure 3**). Mast cells and FOXP3-positive lymphocytes are more abundant in EG biopsies compared to controls (51).

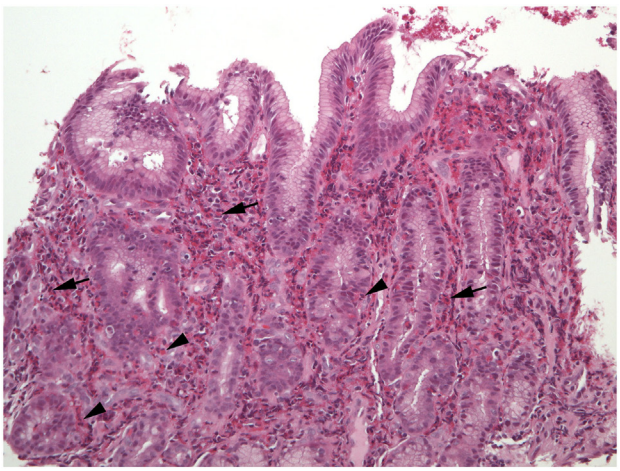

FIGURE 3 | The lamina propria of this section of gastric mucosa is almost entirely occupied by sheets of eosinophils (arrows). Numerous intraepithelial eosinophils are found in gland epithelium (arrowheads).

An EG transcriptome overlaps minimally with the EoE transcriptome (52). However, cadherin 26 (CDH26) is the most upregulated gene in EG and is also among the most upregulated genes in EoE. CDH26 is expressed by esophageal and gastric epithelial cells in EoE and EG respectively, binds to α4 and αE integrins, and regulates leukocyte adhesion and activation. Importantly, CDH26 inhibits CD4+ T-cells *in vitro*, suggesting a role as a downregulator of inflammation in EGID (52).

Clinical features of EG include epigastric pain, peripheral blood eosinophilia, anemia, and hypoalbuminemia (49–51). Endoscopic abnormalities include nodular mucosa, erythema, and ulcers/erosions, but the mucosa may appear normal (49–51). EG may occur in isolation, or may be associated with excess eosinophil infiltrates in the rest of the GI tract, especially the esophagus (50, 51), either simultaneously or sequentially. Antigen restriction successfully reduces symptoms and tissue eosinophilia in some pediatric EG patients (50).

EOSINOPHILIC ENTERITIS

Excess eosinophils in the small intestine could be considered a multiple of the maximum count found in normal biopsies, such as 2 × 26/HPF or 52/HPF in duodenal mucosa, and 2 × 28/HPF or 56/HPF in ileum (7). Excess eosinophils restricted to the small intestine appear to be exceedingly rare, i.e., small bowel mucosal eosinophilia appears to be commonly, perhaps exclusively, associated with excess eosinophil density in the mucosa of other parts of the GI tract. Patients who report dyspepsia and do not have ulcers have increased numbers of duodenal mucosal eosinophils compared to patients who do not report dyspepsia (53). Increased numbers of eosinophils in the lamina propria, increased intraepithelial eosinophils compared to normal counts (48), blunt villi (54), and eosinophils in the muscularis mucosa and submucosa may be found in EE (**Figures 4–6**). Parts of

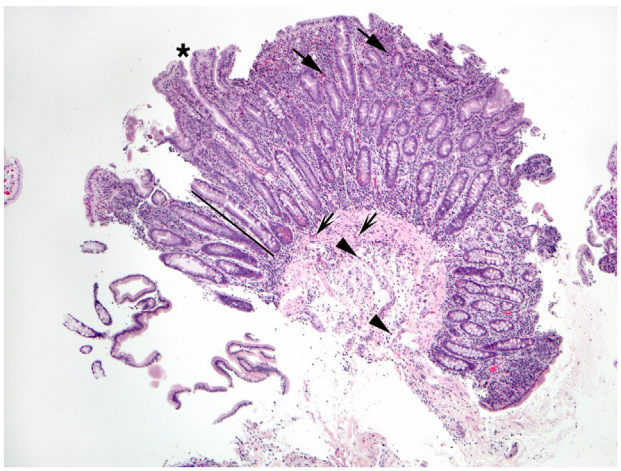

FIGURE 4 | This duodenal biopsy shows few preserved short villi (asterisk), elongated crypts (bar), and numerous eosinophils in the lamina propria (arrows), muscularis mucosa (shaded arrows), and submucosa (arrowheads).

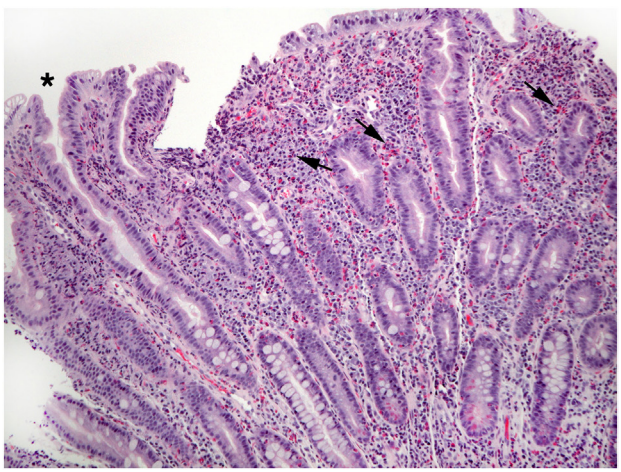

FIGURE 5 | This view of **Figure 4** illustrates blunt villi (asterisk) and confirms numerous lamina propria eosinophils (arrows).

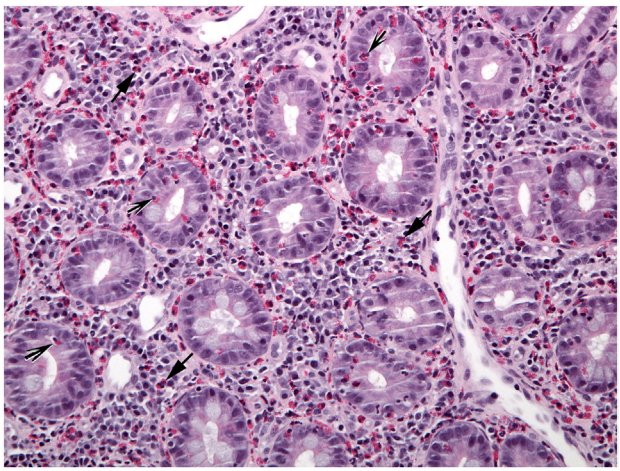

FIGURE 6 | A different biopsy shows numerous eosinophils in duodenal lamina propria (arrows) and crypt epithelium (shaded arrows).

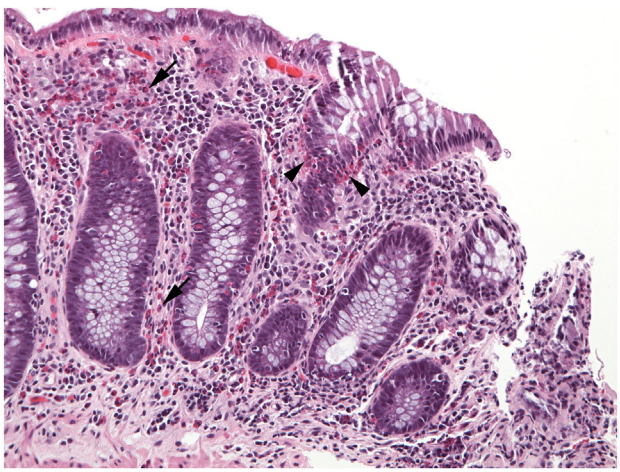

FIGURE 7 | Numerous eosinophils populate the lamina propria (arrows) in this well-oriented section of colonic mucosa and also invade crypt epithelium (arrowheads).

invasive helminths may be found in small bowel mucosal biopsies permitting identification of a specific cause for excess mucosal eosinophils (55).

EOSINOPHILIC COLITIS

Eosinophils are normally present in colon mucosa and are most abundant in the right colon of both children (47, 48, 56) and adults (57). Therefore, using a single threshold value to identify increased eosinophil density is less accurate and potentially misleading compared to applying threshold values appropriate for each colon site (right, transverse, left, rectosigmoid) to properly labeled and separately submitted colon mucosal biopsies. Excess eosinophils could be considered a multiple of the peak count/HPF in normal biopsies, including 2 × 50/HPF or 100/HPF in cecum and ascending colon, 2 × 42/HPF or 84/HPF in transverse and descending colon, and 2 × 32/HPF or 64/HPF in rectosigmoid mucosa (7). Histologic features in colon biopsies showing increased eosinophil density include eosinophil cryptitis/crypt abscesses, crypt architectural abnormalities, increased intraepithelial eosinophils, and eosinophils in muscularis mucosa and submucosa (**Figures 7** and **8**) (57–63).

The prevalence of EC is difficult to ascertain, partly, because guidelines for clinical or pathologic diagnosis do not exist. Nevertheless, a reasonable approach is that EC should be a clinicopathologic diagnosis, akin to EoE, requiring both symptoms referable to colonic dysfunction and colon biopsies showing excess eosinophils. Colon biopsies that displayed eosinophil

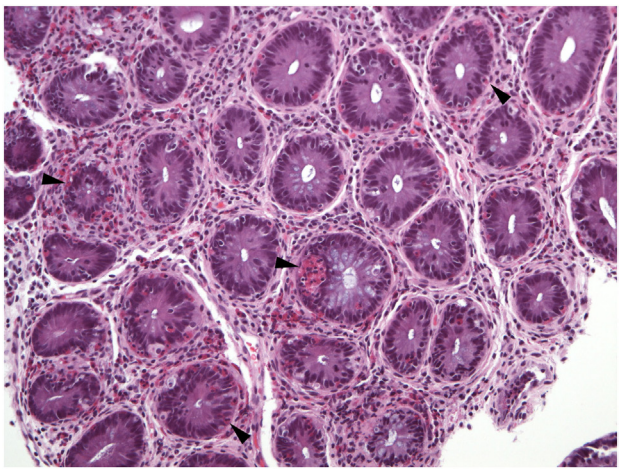

FIGURE 8 | Virtually all crypts in this field of a colon biopsy display increased numbers of intraepithelial eosinophils (arrowheads).

density greater than normal for site plus six SDs from 194 patients were identified in a pathology database of 1.2 million patients (prevalence 1:6,000) (57). Most of those patients had symptoms, mainly diarrhea and abdominal pain, but approximately one-third were asymptomatic. Reported endoscopic abnormalities included erythema, erosions, whitish elevated lesions, pale granular mucosa, and aphthous ulcers.

Several studies have documented that excess eosinophils may be found in mucosal biopsies from patients who have IBD (57, 61, 64, 65). Indeed eosinophils may be more numerous in biopsies from children who have IBD compared to biopsies from children with allergic conditions (66), and elevated eotaxin-1 levels are reported in rectosigmoid biopsies from children with ulcerative colitis (64). The presence of acute inflammatory cells in colon biopsies showing chronic changes and excess eosinophils, especially those lacking sheets of eosinophils, should raise suspicion for IBD with excess eosinophils as the correct diagnosis. More abundant co-localization of IgE deposits with tryptase deposits in perineural locations may distinguish EC biopsies from biopsies of patients who have IBD with excess eosinophils (67).

The role of eosinophils in IBD is not clear. Recently, however, increased mucosal expression of genes that mediate type 2 and type 17 immune responses were shown to distinguish UC at baseline from colon-only Crohn disease at diagnosis, and high IL-13 expression was found in patients who subsequently exhibited improved clinical outcome (68).

Increased numbers of eosinophils may be found in colon biopsies of immunosuppressed patients, especially those receiving tacrolimus, who have had an organ transplant (69–71) which may resolve with food restriction.

Few studies quantify mast cells in the colon (56, 72), but colonic biopsies that show apparently increased numbers of mast cells, either as part of mastocytic enterocolitis (73, 74) or as part of systemic mastocytosis (75, 76), also show increased numbers of eosinophils.

TABLE 2 | Underlying diseases associated with gastrointestinal (GI) mucosal eosinophilia.

Underlying disease	Affected GI site
Food allergy	Any site
Gastroesophageal reflux disease	Esophagus
Inflammatory bowel disease	Small intestine, colon
Parasitic infections	Any site, e.g., stomach, small intestine (Anasakis, *Helicobacter pylori*), proximal small intestine (*Strongyloides stercoralis*, *Giardia*), small intestine, colon (*Cryptosporidium)*, small intestine (*Ascaris lumbricoides*), colon (*Entamoeba histolytica*, *Dientamoeba fragilis*, *Blastocystis* species, *Balantidium coli*, *Trichuris trichiura*), distal small intestine (*Angiostrongylus costaricensis*), proximal colon, and appendix (*Enterobius vermicularis*)
Drug reactions	Any site including medication-induced ("pill-induced") esophagitis
Systemic mastocytosis	Small and large intestine
Neoplasm, e.g., leiomyomatosis, granular cell tumor	Esophagus
Vasculitis e. g., eosinophilic granulomatosis with vasculitis (Churg-Strauss syndrome), granulomatosis with polyangiitis, microscopic polyangiitis	Any site
Connective tissue disease (e. g., systemic sclerosis)	Any site
Hypereosinophilic syndrome	Any site
Celiac disease	Esophagus, duodenum
Organ transplant	Any site

Allergic colitis of infancy has been diagnosed if >20 eosinophils/HPF are found in rectal biopsies (77) which may be in a patchy distribution (78), which resolves clinically following removal of the offending antigens, typically cow's milk, from an affected infant's diet. Since this condition is so easily, and apparently permanently, treated by withdrawal of a single food substance from the diet, classification as an EGID is considered inappropriate by some experts.

The differential diagnosis for increased eosinophil density in colon mucosa is more extensive than discussed above, and includes parasitic infections, hypereosinophilic syndrome, etc. Primary EC is a diagnosis made only after all known causes for increased mucosal colon eosinophils have been eliminated (58) (**Table 2**).

EOSINOPHILIC GASTROENTERITIS (EGE)

This term is used in multiple ways and unfortunately may indicate excess eosinophils in one or more than one part of the GI tract. Use of site-specific terminology, such as EG for eosinophilia restricted to the stomach, would help to bring greater clarity than using a term that is less specific.

In children with allergic EGE defined as excess eosinophils in either gastric or duodenal mucosa, significantly greater numbers of mast cells were found in duodenal but not gastric biopsies from patients who had associated protein-losing enteropathy compared to those who did not (79). All patients responded well to amino-acid-based formula, but food hypersensitivities did not completely resolve.

FUTURE DIRECTIONS

Many aspects of EGID diagnosis, pathogenesis, therapy, etc., remain to be determined. Recently, the NIH funded the Consortium of Eosinophilic Gastrointestinal Researchers (CEGIR) to facilitate studies of these rare diseases. CEGIR conducts observational and interventional studies of EGID subjects that include correlative studies of clinical, pathological, molecular, genetic, and microbiomic components of these diseases. These studies will hopefully yield basic science and clinical knowledge that will lead to novel and effective therapies.

AUTHOR CONTRIBUTIONS

MC, KC, and G-YY: substantial contribution to conception and design of work; drafting and revising for intellectual content; final approval of version to be published; and agreement to be accountable for all aspects of the work.

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REVIEW
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Clinical Applications of the Eosinophilic Esophagitis Diagnostic Panel

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Eosinophilic esophagitis (EoE) is a recently recognized upper gastrointestinal allergic disorder characterized by esophageal dysfunction (e.g., dysphagia) and esophageal eosinophilia of ≥15 eosinophils/high-power field in patients who have persistent esophagitis even on proton pump inhibitor (PPI) therapy. The histologic method is the gold standard of EoE diagnosis. However, EoE clinical symptoms do not always correlate with histology, and the histologic method has sensitivity and specificity issues due to the patchiness of EoE and the subjective nature of the method. The "EoE transcriptome" was initially discovered in 2006, which led to the invention of the EoE diagnostic panel (EDP). In addition to providing a definitive EoE diagnosis with high accuracy, the EDP has been useful in elucidating several key elements about the disease including the efficacy of specific drugs such as swallowed glucocorticoids and anti-IL-13 humanized antibody therapy, the relationship between EoE and PPI-responsive esophageal eosinophilia, and predicting the disease course and responsiveness to therapy. The EDP's long-term potential arises from its plasticity to incorporate new genes and uncover novel disease pathogenesis. We expect that the EDP will be increasingly helpful for personalized medicine approaches and improved diagnostics and disease monitoring.

Keywords: eosinophilic esophagitis, reflux, EoE diagnostic panel, transcriptome, eosinophils, T helper type 2, histology, molecular profiling

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A molecular revolution swept through the fields of clinical diagnosis and predictive medicine at the turn of the century. Though advances in technology are affecting how basic research and clinical practice are performed, molecular diagnosis of diseases has been largely limited to cancer and genetic disorders. Conditions such as allergy have been underexplored in regard to the modern technical platform of molecular diagnosis.

Eosinophilic esophagitis (EoE) is a chronic, T helper type 2 (Th2)-associated immune disorder (1, 2) that involves food hypersensitivity. Typically, milk, egg, wheat, soy, corn, nuts, and fish represent the six food allergens that most commonly trigger disease activity in EoE (3). Diet elimination and steroid intervention are the two most effective therapies to quell disease activity and are frequently used together. At the cellular level, the inflammation is a well-concerted process orchestrated by local lymphocytes (primarily Th2), mast cells, and eosinophils within the esophagus and contributes to the clinical symptoms. Clinically, EoE is characterized by esophageal dysfunction (e.g., dysphagia) and is historically defined by tissue biopsy eosinophilia ≥15 eosinophils/high-power field (EOS/HPF), a cut-off agreed to by a panel of experts and referred to as a consensus recommendation (CR) (4, 5). Both histology and clinical symptoms, as well as an 8- to 12-week proton pump inhibitor (PPI) trial, have been historically required for diagnosis of EoE but it is now

becoming apparent that PPI responsiveness does not necessarily differentiate esophageal eosinophilia into distinct clinical entities. Typically, as mentioned in the CR, five to six biopsy samples are required to reach a satisfactory sensitivity, with one biopsy yielding only ~55% sensitivity (4, 6).

Thus far, histologic examination is the only widely accepted EoE diagnostic test. Yet, the disadvantages of the conventional histologic method are its subjectivity, time requirement, reliance on experts, and expense. The histologic analysis of esophageal specimens can be confounded by other variables, including inter-observer variation, size differences in microscopic HPF among multiple microscope manufacturers, and the patchiness of the disease (7). Moreover, the histologic detection of tissue eosinophilia is non-specific to EoE, as there are several other diseases sharing similar esophageal eosinophilia, and cannot identify specific exposure to medications (such as glucocorticoids) nor differentiate patients with EoE remission from patients who do not have EoE (both have no eosinophilia). Therefore, the only available "gold standard" method has limitations, which a next-generation diagnostic method could overcome.

The EoE transcriptome is 1610 genes dysregulated at differing magnitudes and bidirectionally in the esophagus and was identified by Blanchard et al. in 2006 (8) together with subsequent studies (9, 10). This transcriptome serves as the foundation for the molecular differentiation of EoE from other disorders, such as gastroesophageal reflux disease (GERD). With having the raw threshold cycle (Ct) values of quantitative PCR (qPCR), the critical next step was to develop a way to interpret the results in a way that general physicians and patients could comprehend and use. One of the unique features of the EoE diagnostic panel (EDP, US Patent pending 47108-510N01US) is the novel dual algorithm based on the raw qPCR Ct values (11). Briefly, the first algorithm is a clustering analysis based on the Pearson correlation of a 77-gene/dimension hypothetical space. With 50 upregulated genes and 27 downregulated genes, the bidirectional dysregulation provides a pronounced contrast for signature recognition. The dendrogram (hierarchical tree) is derived on the basis of the inter-sample distance metrics aided by commercial analysis softwares such as GeneSpring (Agilent Inc.). The first branch of the dendrogram tree serves as a diagnostic bifurcation point. The "EoE score" algorithm performed in parallel is essentially a mathematical summing-up of the relative Ct value change of each gene to the housekeeping gene (*GADPH*) considering the bidirectional changes (+ and − vectors). The end read-out of this algorithm is an absolute integer providing definitive EoE diagnosis and linearly correlated with the disease severity. This direct output allows the physicians to readily assess the disease status and to plan for corresponding therapies. It is worthwhile to mention that the unique qPCR array design combined with the practical scoring algorithm can be readily applied to the diagnosis of other allergy inflammatory diseases with minimal modifications, such as other eosinophilic gastrointestinal (GI) disorders (EGIDs) and atopic dermatitis (12).

Of note, the EDP's compatibility with formalin-fixed, paraffin-embedded (FFPE) samples provides a valuable and unique opportunity to retrospectively study archived paraffin samples (11). Importantly, this crucial feature has been validated externally by at least two independent groups (13, 14). Whereas FFPE RNA is known to be highly degraded as a function of time, the EDP signature acquisition has been intentionally designed with short amplicons, so that it can be readily used with paraffin-archived samples (highly degraded/fragmented RNA) that are usually not compatible with RNA sequencing. One can imagine how many clinical questions can be answered with a vast storage of FFPE-archived pools and the ample amount of associated information regarding clinical outcome.

Importantly, since the original publication of the EDP by Wen et al. (11), multiple studies successfully reproduced the diagnostic merit at external institutions with independent samples, using the same molecular platform and array design. Notably, Dellon et al. performed a well-controlled EDP study at University of North Carolina using both FFPE and matching RNAlater™ samples (13). A total of 72 samples, representing paired FFPE and RNAlater™ specimens from 9 EoE cases and 3 GERD controls were analyzed by EDP (13). A robust correlation was demonstrated between paired FFPE and RNAlater™ samples. Moreover, by the reported EDP score, EoE was well distinguished from control GERD samples without overlap and with excellent diagnostic merit (13). The second external validation was led by Drs. Genta and Lash from Miraca Life Sciences (TX, USA), a large-scale GI disorder diagnostic corporation based in the USA (14). This study encompassed a relatively large cohort of 265 FFPE samples randomly selected from their sample archive, which repeatedly showed an equally excellent diagnostic merit of the EDP (11, 14). Collectively, these studies show that the EDP is a highly reliable and reproducible molecular procedure to distinguish EoE from normal and GERD tissues.

Besides providing definitive EoE diagnosis, the EDP's clinical utility has been demonstrated in multiple registered clinical trials. In a double-blinded, placebo-controlled fluticasone clinical trial, the EDP was used as a molecular gauge to evaluate the treatment efficacy and remission status of EoE (15). Of note, the EDP-derived EoE score, a readily available and interpretable parameter, was directly used in this clinical trial to assess EoE molecular severity and intervention efficacy in a quantified fashion. It was also found that certain embedded genes were able to predict fluticasone responders vs. non-responders, suggesting a potential for predictive medicine in the field of Th2 allergic GI disorders (15). Likewise, in a recent anti-IL-13 humanized antibody clinical trial (QAX576, Novartis) (16), a similar transcriptome analysis was used to monitor EoE activity following this anti-Th2 cytokine intervention. Using transcriptome analysis, there was a remarkable reversibility of nearly all EoE-associated genes, including reduction in expression of the cardinal eosinophil chemoattractant (*CCL26*), mast cell signature genes (e.g., *CPA3*), and increased levels of the barrier gene *DSG1*. Notably, the molecular improvements were even more impressive than the histologic improvement, which did include reduced levels of esophageal eosinophilia and a trend for improvement in dysphagia. The improved molecular signature is likely providing early insight into the potential positive impact that anti-Th2 cytokine therapy may have in EoE.

The EDP as a molecular platform also represents a tool for identifying the patients whose disease is most likely to respond

to anti-Th2 cytokine treatment (16). This personalized medicine approach is potentially applicable to not only the management of EoE but also other Th2 disorders, such as asthma. Anti-Th2 cytokine intervention (anti-IL-5) has been proven to be quite efficient in eosinophilic asthma and was recently approved by the FDA (17, 18). We envision that anti-IL-13 therapy, guided by the personalized assessment of EDP, will likely follow a similarly successful path to clinical utility.

The EDP is also useful in defining new disorders and disease subentities or in molecularly analyzing comorbidities, such as in defining the molecular signature of PPI-responsive esophageal eosinophilia (PPI-REE) (16). PPI-REE, a large dilemma and confounding factor in the field of EoE, was recently identified in patients with endoscopic and symptomatic features of EoE but who respond well to PPI mono-therapy (4, 19, 20). It remained unclear whether PPI-REE represented an independent clinical disorder or is a subentity of either EoE or GERD (20). The initial EDP study demonstrated that the signature of PPI-REE largely overlapped with that of EoE, suggesting that they share the same disease process and may represent a continuum (11). This is consistent with other studies using alternative methods showing PPI-REE and EoE are indistinguishable (20, 21). However, with the genome-wide screening between PPI-REE and EoE now being readily available, we hope that the next version of the EDP would have a component to prospectively distinguish PPI-REE and EoE before the PPI therapy, which could save significant clinical resources and improve the clinical care quality (e.g., reducing time to effective treatment). The EDP is not a fixed design in terms of molecular composition. With the same quantification algorithm and technical platform, any new leads that have potential in predictive and personalized medicine, as well as those genes helpful to solve diagnostic dilemmas, could be readily incorporated to enhance the value of this molecular panel.

As for the *bona fide* feasibility of distinguishing EoE and PPI-REE, it is now generally accepted that EoE and PPI-REE are very similar subentities and that PPI-REE may not have a pathogenesis initiated by GERD (11, 19). The PPI-REE transcriptome study (16), as well as a recent genome-wide transcriptome study (22), directly demonstrated an overlapping transcriptome between EoE and PPI-REE. There is only a marginal difference between the two entities, with a potassium channel (KCNJ2/Kir 2.1) potentially being differentially expressed. A collaborative effort is ongoing to validate the utility of this gene in EoE vs. PPI-REE differential diagnosis before PPI therapy. A genome-wide screening with a large cohort number and a sufficient sequencing depth will have the potential to identify some low-abundance differential genes, serving as the foundation for future diagnostic tests.

Another useful application of the EDP is deciphering disease endotypes. Recently, it has become increasingly clear that many human diseases are complex disorders made up of several clinical and pathologic variants with disparate underlying etiology. For example, in asthma, it is now appreciated that there are multiple endotypes including eosinophil-high, neutrophil-high, and Th2 cytokine-high subgroups. Unlike asthma, there is no such approach being explored in the field of human allergic GI disorders. Of note, there is a considerable amount of variability among the EoE cohort in terms of molecular scoring and expression pattern (11). It is conceivable that EoE also has different molecular manifestations due to distinct causes and responses, even though all samples meet the >15 EOS/HPF histologic diagnostic standard. By molecular profiling, it is hoped that a better understanding of EoE subtypes will promote a more personalized medicine approach to EoE management.

Distinguishing GERD and EoE can be clinically difficult due to several confounding factors, including symptom overlap, histologic similarity, and the presence of PPI-REE (4). Though several tests can be used for this differential diagnosis [e.g., impedance (23), pH probe (24), endoscopy (25), and histology (26)], the molecular diagnostic panel excels in distinguishing EoE and GERD. In the original EDP study (11), using FFPE samples, Wen et al. showed that the EDP is able to distinguish impedance/pH-selected patients with GERD from patients with EoE, as the transcriptome of the former is more similar to normal controls. Although most of the GERD [>98% (27)] cases do not have as great a degree of eosinophilia as EoE does (>15 EOS/HPF), the caveat herein was whether the EDP would distinguish EoE from atypical GERD samples with high eosinophilia. In this same study, Wen et al. showed a similar transcriptome between EoE and EoE + GERD comorbidity, suggesting that the EoE signature is dominant. Though there is little doubt that the EDP is able to distinguish EoE from GERD without a high eosinophil level, it remains unclear whether the EDP can dissect out the GERD components to differentiate GERD with high eosinophilia from GERD with comorbid EoE, as the high eosinophilia may be mimicking the dominant EoE signature. Notably, this type of differential diagnosis is of less clinical importance because it is more significant to distinguish GERD with low eosinophilia from EoE due to distinct management.

It is worthwhile to mention that human disease development and remission are usually a progressive and dynamic process rather than an abrupt "all or none" change. Many common disorders, such as diabetes and hypertension, have ambiguous areas between normal and diseased cutoffs, indicating a borderline state and equilibrium between promoting and inhibitory factors. Following food allergen exposure, EoE development is a linear course regulated by a series of counterbalanced components. Therefore, a "black and white" threshold may not truly exist. In this sense, any EoE score value around the 333 cutoff represents a "snapshot" of the equilibrium reached between pro-EoE factors (e.g., food allergen presence, Th2 cytokine production, mastocytosis, inflammatory cell proliferation) and anti-EoE factors (e.g., elemental diet, steroid exposure, anti-Th2 therapy). One can imagine that such an integer score can be used to monitor disease status and responses to therapy intervention. Indeed, the aforementioned Dellon et al. study demonstrated the key function of the EDP in assessing the efficacy of EoE clinical management (9), an external validation study with 265 independent samples. Therefore, multiple lines of evidence show that the EDP serves well as an EoE status monitoring tool with prognostic values, a personalized quantification that can barely be achieved by histologic analysis.

With the non-invasive trend sweeping through the diagnosis of other disorders, the field of EoE has an urgent

need for a non-invasive or less invasive method to replace the biopsy-based examination (28). It is conceivable that the current molecular platform of the EDP, when combined with its associated algorithms, could be used for a non-invasive diagnosis of EoE. Burgeoning progress has been made regarding the circulating biomarkers for EoE, including miRs (29) and certain cytokine signaling molecules (30). In the next 5 years, it is also possible for the EDP to incorporate some genetic components from the results obtained from some promising non/less invasive platforms, such as oral cytobrush, esophageal string test (31), and swallowed sponge test (32), or even more biomarkers from the patients' blood (30). The recently reported increase in levels of circulating eosinophil progenitors (33) in patients with EoE may render value in embedding certain eosinophil progenitor markers in the EDP, which could be potentially useful on biological samples not derived from esophageal biopsy. One of the primary goals of a future version of the EDP is to embed a non-invasive or less invasive component to facilitate improved diagnosis and monitoring and quality of life of patients with EoE.

The EoE transcriptome has been deposited online by the Rothenberg group for public use (Microarray 2006 GEO

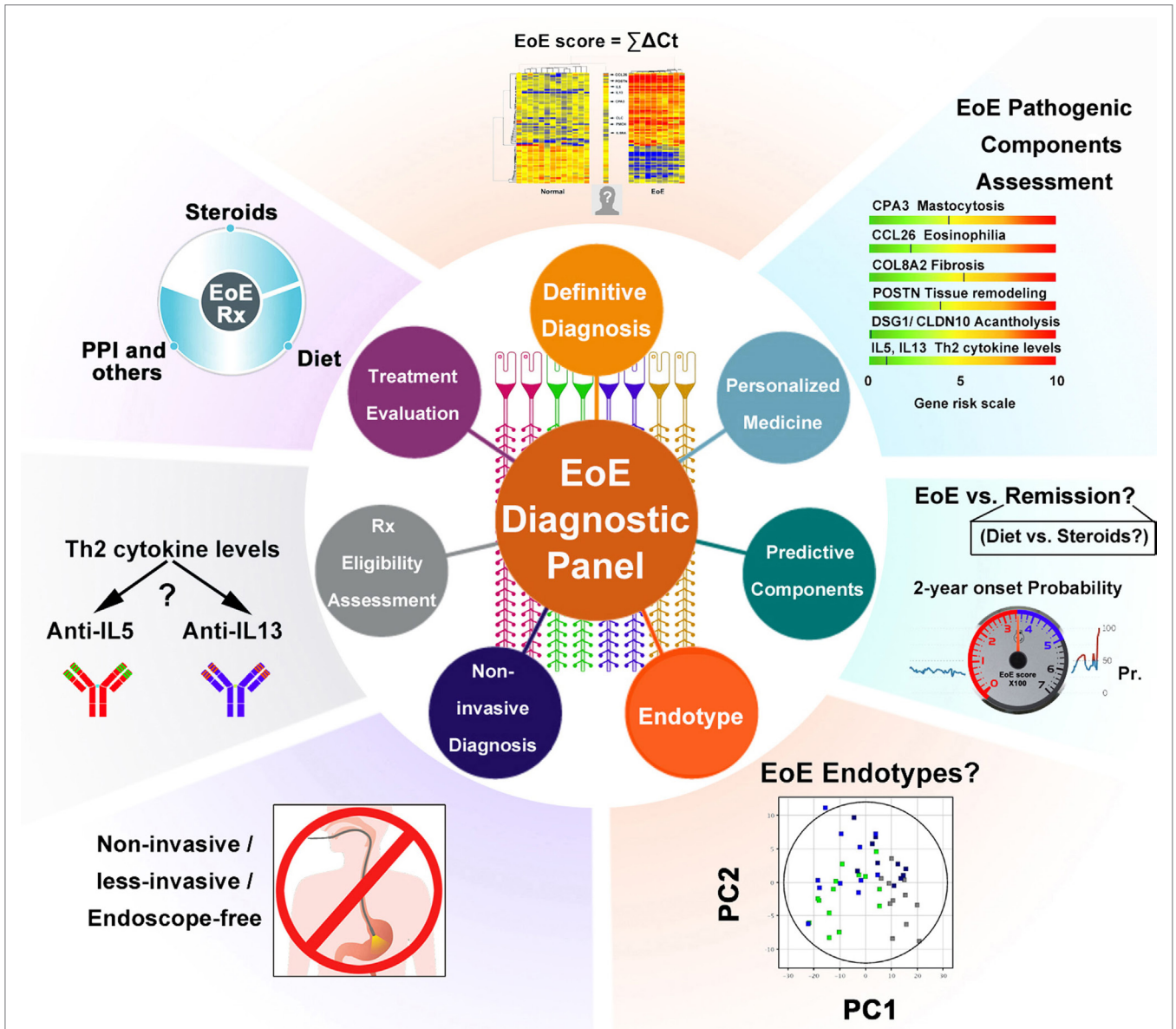

FIGURE 1 | Schematic summary of the EDP's multiple functions. Multiple components of the EDP are graphically summarized to illustrate the applicable functions of the molecular panel. Some are in clinical practice, whereas others are potentials for future diagnostic utilities. Abbreviations: PPI, proton pump inhibitor; Th2, T helper type 2; Rx, treatment; EoE, eosinophilic esophagitis; PC, principal component; ΣΔCT, EoE score = Σ (Ct. downregulated gene – Ct. GAPDH) – Σ (Ct. upregulated gene – Ct. GAPDH) (refer to Wen et al. study); Pr., probability; 2-year onset probability, the estimated possibility of EoE relapse for patients who currently have EoE in remission.

GSE8853; RNA sequencing 2014 GEO GSE58640). Utilizing modern bioinformatics is a key approach to identifying new information from old data to guide basic research. We propose several questions and unmet areas that we think are meaningful undertakings to better guide EoE translational/clinical research:

1. What is the overlap between the EoE transcriptome and those of other Th2 human disorders, including GI (EGIDs) and non-GI allergic disorders (asthma, atopic dermatitis)? Within the overlap, what functional elements are shared by EoE and other Th2 disorders?
2. Since the EoE miRome (small RNA transcriptome) was also elucidated by the Rothenberg group (29, 34), what is the functional network between the EoE transcriptome and the corresponding miRome considering the robust roles of miRs in regulating gene expression? Can the dysregulated miRome explain or regulate the dysregulated EoE transcriptome? If so, what is the miR–gene interaction network? Which is the cause, which is the consequence, or are both consequences of further upstream factors?
3. With more and more cell type-specific data deposited in GEO, are we able to perform a "deconvoluted" bioinformatic dissection (35) to determine which part of the EoE transcriptome is contributed by each cell type?
4. In the same line of thinking as question 3, this field urgently calls for a single-cell analysis to decipher the contribution of each cell type and their developmental relationship in parallel. The recently published Drop-seq technique seems to be well suited for this purpose (36).
5. With the EoE genome-wide association study (GWAS) data published by the Rothenberg group (37), it remains to be determined what percentage of the EoE transcriptome is regulated at the genomic DNA level. If EoE is a known multifactor genetic disorder, how could an isolated single-nucleotide polymorphism difference explain the vast dysregulated transcriptome in EoE? Are the mutations identified by the GWAS family specific? If yes, at what point do they converge? The causal links between the GWAS findings and EoE transcriptome need to be established.

In summary, the EDP and associated dual algorithm represent a cutting-edge approach poised to advance EoE and related inflammatory disorders to a 21st century molecular and precision medicine level (see **Figure 1** summarizing the utilities of the EDP in studying the EoE signature). Moreover, the transcriptome study also offers strong potential for future personalized medicine practice with prognosis-predicating values. In the next 5 years, we predict that new components embedded in the EDP will distinguish PPI-REE from EoE, define EoE endotypes, and hopefully suggest the best treatment or predict the effectiveness of a certain regimen. This proposed achievement will be greatly facilitated by genome-wide sequencing (10) and genomic DNA variant studies such as GWAS and whole-exome sequencing (37). Though the EDP is the first to carry molecular signature interpretation from the field of cancer to that of digestive tract disorders, we also envision that the same success could be reproduced in the diagnosis, monitoring, and clinical management of other allergic inflammatory disorders, such as eosinophilic gastritis and eosinophilic colitis.

AUTHOR CONTRIBUTIONS

TW and MR collaboratively studied the topic and wrote the review together.

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Conflict of Interest Statement: TW and MR are coinventors of the EDP, a patent owned by CCHMC. There is no commercial interest to disclose at the moment.

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Clinical and Biological Markers in Hypereosinophilic Syndromes

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Hypereosinophilic syndromes (HES) are rare, heterogeneous syndromes characterized by markedly elevated eosinophil counts in the blood and/or tissue and evidence of eosinophil-associated pathology. Although parasitic infections, drug hypersensitivity, and other disorders of defined etiology can present as HES (associated HES), treatment is directed at the underlying cause rather than the eosinophilia itself. A number of additional subtypes of HES have been described, based on clinical and laboratory features. These include (1) myeloid HES—a primary disorder of the myeloid lineage, (2) lymphocytic variant HES—eosinophilia driven by aberrant or clonal lymphocytes secreting eosinophil-promoting cytokines, (3) overlap HES—eosinophilia restricted to a single organ or organ system, (4) familial eosinophilia—a rare inherited form of HES, and (5) idiopathic HES. Since clinical manifestations, response to therapy, and prognosis all differ between HES subtypes, this review will focus on clinical and biological markers that serve as markers of disease activity in HES (excluding associated HES), including those that are likely to be useful only in specific clinical subtypes.

Keywords: eosinophil, biomarkers, hypereosinophilic syndrome, eosinophilic esophagitis, eosinophilic granulomatosis with polyangiitis, eosinophilia, eosinophilic disorders

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INTRODUCTION

Hypereosinophilic syndromes (HES) are defined by the presence of hypereosinophilia [absolute eosinophil count (AEC) > 1,500/μL or marked tissue eosinophilia] and eosinophil-associated clinical manifestations. Various clinical subtypes of HES have been described based on the etiology of the eosinophilia (primary, secondary, or unknown) and clinical features (systemic or organ-restricted) (1). Although HES can occur in the context of defined disorders, such as drug hypersensitivity, helminth infection, and neoplasia, for which specific treatment of the underlying secondary cause leads to resolution of the eosinophilia (associated HES), for the purposes of this review, HES refers to all clinical subtypes of HES with the exception of associated HES.

The development of standardized clinical assessments of disease activity, such as patient-reported outcomes (PROs) and clinician-reported outcomes (ClinROs), that can be used to guide treatment and serve as clinical trial endpoints has been complicated in HES due to the heterogeneity of disease across HES subtypes and organ systems and the rarity of the disorder itself. Although significant progress has been made in the development of these tools in organ-restricted eosinophilic disorders, such as eosinophilic esophagitis (EoE) (2–5), these subtype-specific PROs and ClinROs are not broadly applicable to the overall HES population. This has, in turn, hampered the development of surrogate markers of disease activity in HES.

Given the central role of eosinophils in HES, quantification of eosinophil numbers in the blood or tissue would seem the most logical method to monitor disease activity in HES and is, in fact,

the most common biomarker used in clinical practice. Despite this, AEC has not been widely accepted as a surrogate of disease activity in clinical trials of HES, particularly those involving novel therapies that specifically target eosinophils but may or may not affect clinical outcomes. Clearly, additional biological markers are needed. This review is divided into two parts. The first section will focus on data pertaining to biomarkers related to eosinophilia and eosinophil activation as general indicators of disease activity in HES. This will be followed by a discussion of biomarkers relevant to selected subtypes of HES, but unlikely to be generalizable to HES as a whole.

GENERAL BIOMARKERS OF DISEASE ACTIVITY IN HES

Eosinophils are characterized by the presence of eosin-avid secondary granules containing cationic granule proteins [major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN)] and a wide array of cytokines and chemokines. When released into the tissues by activated eosinophils, these mediators, together with reactive oxygen species and lipid mediators, can lead to tissue damage and the end organ manifestations of HES. As mentioned earlier, a major controversy in clinical trial endpoint design in HES has been whether reduction in AEC is an appropriate surrogate marker of disease activity. The fact that some individuals with hypereosinophilia (AEC > 1,500/μL) are asymptomatic and do not develop end organ manifestations (6) has been cited as evidence that biomarkers of eosinophil activation or tissue infiltration might be more useful in this regard. Available data addressing this question are summarized below.

Absolute Eosinophil Count

The association between elevated peripheral eosinophil counts and clinical pathology was first noted at the turn of the century by Loeffler who described a characteristic form of endomyocardial fibrosis in association with blood eosinophilia (7). Subsequent case series, using persistent AEC > 1,500/μL as a defining criterion for HES, noted an association between extremely elevated AEC (white blood cell counts >100,000/μL) and poor prognosis (8, 9). Consistent with these findings, patients with *PDGFRA*-positive myeloid neoplasm, one of the most aggressive forms of HES, have higher AECs than patients with other clinical subtypes of HES and dramatic resolution of clinical manifestations following normalization of the AEC with imatinib therapy (10).

Despite these findings and the large body of circumstantial evidence from case reports, case series and clinical practice documenting an association between the resolution of clinical manifestations of HES and normalization of the AEC, assessment of the AEC as a surrogate marker of disease activity has not been studied directly in the context of clinical trials to date. That said, the efficacy of mepolizumab as a steroid-sparing agent was associated with reduction of AEC in two placebo-controlled, double-blind trials in HES [one in subjects with steroid-responsive HES (11) and the second in subjects with eosinophilic granulomatosis with polyangiitis (EGPA) (12)], suggesting that AEC is a useful marker of disease activity. The results of ongoing and recently completed trials (NCT02130882; NCT02101138) in HES using agents that selectively target eosinophils should provide additional support for the utility of the AEC as a biomarker of response.

Tissue Eosinophilia

Although tissue eosinophilia would seem to be a more specific indicator of disease activity in HES, the utility of eosinophil quantification in tissue biopsies to monitor disease activity is hampered by the difficulty in obtaining samples, the patchy nature of eosinophilic tissue infiltration, and the fact that intact eosinophils may be absent despite clear evidence of their involvement by immunohistochemical staining for eosinophil granule proteins (EGPs) (13–16). To date, the best data associating tissue eosinophil numbers with clinical symptomatology come from EoE where the numbers of eosinophils in normal tissue have been defined (17), and suppression of tissue eosinophil counts has been associated with improved long-term prognosis (18). Unfortunately, despite encouraging data from a small open-label trial (19), randomized placebo-controlled trials using anti-IL-5 antibody therapy (mepolizumab and reslizumab) have not demonstrated an association between reduction in tissue eosinophilia and improved symptoms (20–22). Potential explanations for the lack of symptomatic improvement include incomplete depletion of tissue eosinophilia, involvement of other cell types and/or structural changes due to fibrosis and remodeling that may require a longer time frame for resolution.

Eosinophil Granule Proteins

Released during eosinophil activation and deposited in tissue in sites of eosinophilic inflammation, EGPs are attractive candidate biomarkers for the monitoring of disease activity in HES (23). They can be detected and quantified in the blood (24–26), body fluids (27), and tissue (13–15, 28–31) using various immunoassays, and blood and/or body fluid levels have been shown to correlate with tissue deposition of EGP in a wide range of HES, including EoE in the absence of peripheral eosinophilia (24).

There are a several biologically relevant differences between EDN, EPO, MBP, and ECP. MBP is the predominant protein in the core of the eosinophil secondary granule. It exists as two highly basic homologs, MBP-1 and MBP-2 (26), both of which circulate as neutral pH pro-proteins. Of note, most immunoassays do not distinguish between pro-MBP and MBP. Whereas EPO is quite specific for the eosinophil lineage, MBP-1, EDN, and ECP are also present in neutrophils and/or basophils albeit at lower levels (32, 33). This does not appear to affect their ability to be used as a proxy for eosinophil-associated tissue pathology in most settings but deserves mention.

Immunohistochemical staining of tissue for EGP has been extremely useful in clarifying the role of eosinophils in the pathogenesis of HES when intact eosinophils are not detectable. Moreover, EGP staining has been shown to correlate with disease activity in some settings. For example, in one study, serial skin biopsies from patients with episodic angioedema with eosinophilia demonstrated EGP staining only when symptoms were present (15, 34). Unfortunately, the utility of EGP tissue staining

as a biomarker of disease activity is limited by the need for serial tissue sampling. To address this issue in EoE, a number of novel and less invasive techniques have been developed. These include the esophageal string test (35) and the cytosponge (36). Both techniques involve swallowing a string (in the case of the cytosponge, this is attached to a gelatin capsule containing a mesh) from which EGP can be eluted and quantified. Good correlation between eluted EGP levels and immunohistochemical staining of matched biopsies has been confirmed for both techniques (37). Finally, ultrasound visualization of granule protein density using MBP-1 labeled-insulin particles has been demonstrated in *ex vivo* monkey esophagi and may ultimately provide a third non-invasive tool for the measurement of tissue EGP in EoE (38). The applicability of these or similar techniques to other tissues remains to be seen.

The utility of measuring circulating levels of EGPs to monitor disease activity in HES has been somewhat controversial in large part due to the lack of standardization of sample collection (eosinophil lysis could lead to falsely elevated levels) and differing assay parameters between studies. Nevertheless, there are some data to suggest that circulating EGP levels have value in the monitoring of disease activity in HES. An interesting observation in this regard has been the association of elevated EDN and EPO levels with clinical manifestations rather than peak eosinophil count in patients with episodic angioedema and eosinophilia (24). A similar association between clinical disease and elevated EDN levels was seen in a study comparing subjects with asymptomatic familial eosinophilia to subjects with active HES (39).

Data from clinical treatment trials have been more difficult to interpret. Whereas decreases in serum EDN levels were reported in subjects who received active drug in a placebo-controlled trial of mepolizumab in HES (11) and ECP levels decreased in response to mepolizumab therapy in three patients with HES and eosinophilic dermatitis (40), AEC also decreased in both studies making it difficult to assess the added benefit of measuring EGP levels. Moreover, in a recent study of non-invasive biomarkers of EoE, AEC, but not serum levels of ECP, was predictive of residual disease following topical steroid therapy (41).

Measurement of EGP in body fluids, such as urine, that do not normally contain eosinophils, has the theoretical advantage of eliminating false positive results due to eosinophil lysis. Although there are no studies examining urine levels of EGP in HES to date, several small studies in atopic dermatitis (42, 43) demonstrated a correlation between urine levels of ECP and clinical disease severity. By contrast, no such relationship was noted in children with asthma (44).

Eosinophil Surface Receptors

A wide variety of eosinophil surface markers are reported to be up- or downregulated on activated eosinophils (23, 45), Many of these, including IL-5Rα, CD69, and CD44, have been shown to have altered expression on eosinophils from patients with HES, but also in patients with HE_{US} (39). Despite this, there are little longitudinal data assessing changes in expression of these activation markers in response to therapy in patients with HES. In a single study in EoE, expression of activation markers on blood eosinophils was unchanged by topical steroid therapy, although the effect of therapy on esophageal eosinophilia was not reported (46).

Serum Cytokines, Chemokines, and Soluble Receptors

Despite its clear role in the production, activation and regulation of eosinophils, IL-5 has been disappointing as a biomarker of disease activity in HES. Although IL-5 levels correlate with AEC overall, serum IL-5 is undetectable in some patients with untreated HES [**Figure 1**, unpublished data from Ref. (47)]. In this regard, serum IL-5 levels do not contribute additional information when the AEC is known. In addition, increased serum IL-5 levels have been reported in the setting of clinical and hematologic remission following administration of several different biologics designed to target eosinophils, including mepolizumab and benralizumab (48, 49). The reasons for this are likely multifactorial and include measurement of IL-5/anti-IL-5 immune complexes (mepolizumab) and antibody blocking of IL-5 binding to its receptor (benralizumab). Soluble IL-5R is measurable in the serum of most, if not all, patients with HES, and levels are correlated with serum IL-5 levels (47). Whether this would provide a better biomarker of disease activity, owing to its reliable detection in serum in contrast to IL-5, remains to be seen. Finally, a number of studies have looked at other serum cytokines and chemokines as biomarkers of disease activity in HES. Of these, mediators of potential interest have been identified mostly in EGPA and include IL-25 (50), serum CCL17/thymus and activation-regulated chemokine (TARC) levels (51, 52), and CCL26/eotaxin-3 (53, 54). Interestingly, despite tissue data implicating CCL26/eotaxin-3 in the pathogenesis of EoE, serum levels of these mediators were not increased in EoE patients and were not altered by therapy (55). Finally, although some authors have reported elevated IL-3 in the plasma of patients with eosinophilia in conjunction with intracellular staining in CD8+ T cells (56), IL-3 is not universally detected in serum (57) of patients with HES, and the role of IL-3 as a biomarker in HES remains to be explored.

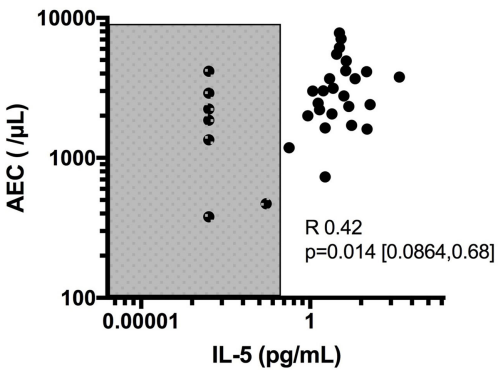

FIGURE 1 | Correlation of absolute eosinophil count (AEC) with IL-5 levels and demonstration of subjects with undetectable IL-5 despite elevated eosinophil counts. Shaded area denotes values below the limit of detection of IL-5 (0.1 pg/mL) in the assay used.

Omics

An exciting advance in biomarker development has been the use of molecular profiling techniques to identify patterns of expression that can be used to follow disease activity. This approach has been used successfully in EoE, where patterns of gene expression in esophageal biopsies have led to the development of an EoE molecular diagnostic panel (EDP) (58, 59) which is further discussed in the accompanying review, and a microRNA signature (60) that correlate with disease activity and response to therapy.

CLINICAL SUBTYPE-SPECIFIC BIOMARKERS IN HES

Over the past decade, it has become increasingly apparent that there are distinct clinical subtypes of HES that differ in their etiologies, clinical manifestations, and responses to therapy. These are more extensively discussed in the companion article by Lefevre (61). Whereas the biomarkers discussed herein are relevant to eosinophilic disorders and HES in general, additional markers have been described that have utility restricted to a particular HES clinical subtype.

Lymphocytic Variant HES (LHES)

LHES is defined by the presence of a clonal or aberrant phenotypic T cell that secretes type 2 cytokines driving the eosinophilia and elevated serum IgE levels seen in this clinical variant (62, 63). Whereas the most common aberrant immunophenotype is CD3−CD4+, various aberrant immunophenotypes have been described (64), and some patients have cytokine-secreting clonal T-cell populations despite an apparently normal immunophenotype. Dermatologic manifestations, including angioedema, nodules, eczematous dermatitis, and erythroderma, are common in patients with LHES (65), and aberrant T cells can often be detected in skin biopsies from affected areas (66). Patients with LHES are often glucocorticoid responsive but typically require moderately high doses (67). Consequently, glucocorticoid-sparing agents with effects on T cells, such as interferon-alpha and cyclosporine, are frequently used. Although LHES is considered a benign lymphoproliferative disorder, a small proportion of LHES patients eventually develop a lymphoid malignancy, often heralded by expansion of the aberrant clonal T-cell population (62, 68). Conversely, regression of the aberrant T-cell population can be seen in response to effective therapy (**Figure 2**). Elevations in serum CCL17/TARC are more frequent in patients with LHES (69, 70), but information is lacking on the use of CCL17/TARC levels to monitor disease activity.

Myeloid HES (MHES)

Myeloid HES refers to the subgroup of patients with HES in the setting of a primary myeloid disorder. Although most of these patients have detectable molecular abnormalities (most commonly the fusion gene *FIP1L1-PDGFRA*), others have a similar clinical phenotype of unknown cause. Clinical and laboratory features associated with MHES include dysplastic eosinophils, anemia and/or thrombocytopenia, elevated serum tryptase and B12 levels, and bone marrow features suggestive of a myeloid neoplasm (10). Before the availability of imatinib (a tyrosine kinase inhibitor with activity against *PDGFRA* and *PDGFRB*), mortality rates in patients with MHES were extremely high, due primarily to endomyocardial fibrosis and thromboembolic events. Currently, remission rates on imatinib therapy approach 100% in patients with *PDGFR*-associated disease and up to 50% in patients with other forms of MHES.

Although the AEC normalizes with effective therapy in MHES and can be used to monitor disease activity, data from chronic myelogenous leukemia and drug interruption trials in *PDGFRA*-associated HES suggest that molecular monitoring is preferable when possible since molecular relapse may precede hematologic (and clinical) relapse by several months (71). This is particularly important in view of recent data demonstrating sustained remission after imatinib discontinuation in some patients (72–74).

Overlap HES

Overlap HES includes single organ and/or defined disorders that are characterized by eosinophilia and eosinophil-associated

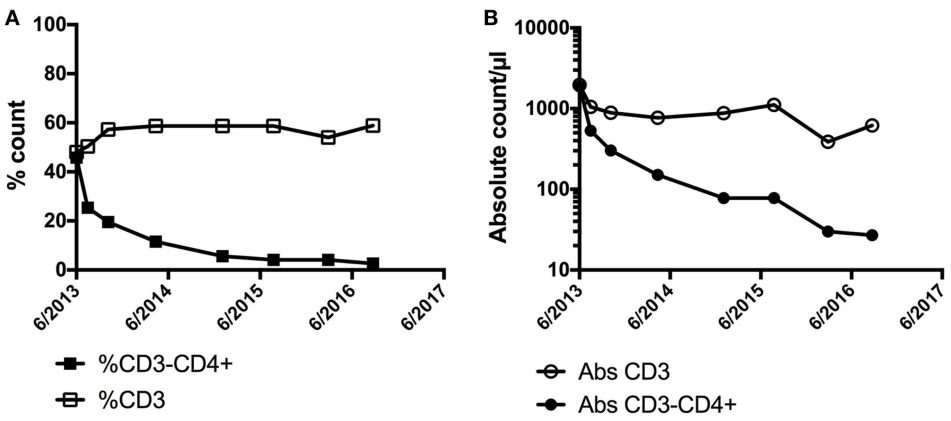

FIGURE 2 | Demonstration of a preferential decline in the **(A)** % of CD3−CD4+ and **(B)** absolute count of CD3−CD4+ cells in a patient with LHES after initiation of interferon-alpha with associated clinical improvement in skin involvement. No significant change was noted in the % or absolute counts of CD3+ cells overall.

pathogenesis, including eosinophilic gastrointestinal disorders and EGPA. These disorders have distinct clinical presentations and complications and, for this reason, are often approached differently than other forms of HES. Although potential biomarkers for these conditions include the previously discussed general markers of eosinophilia and eosinophil activation, additional disease-specific issues are discussed below.

Eosinophilic Esophagitis

Despite significant advances in the development of biomarkers for EoE, the lack of correlation between the number of eosinophils in tissue and clinical symptoms remains a problem, particularly with regard to clinical trial design. Consequently, there has been increasing interest in the development of additional objective measures to assess improvement of long-term sequelae. One such tool is EndoFLIP® (endolumenal functional lumen imaging probe), an inflatable balloon that measures the cross-sectional area and intraluminal pressure of the esophagus while under distension (as if a solid bolus was present). Using this technique, reduced distensibility was demonstrated in patients with dysphagia or a history of impaction as compared with healthy controls (75). Interestingly, decreased distensibility did not correlate with mucosal eosinophilia (75) but did correlate with ring severity and impactions (76).

Eosinophilic Granulomatosis with Polyangiitis

Several studies have examined the use of standard laboratory markers of inflammation, including erythrocyte sedimentation rate and C-reactive protein, in EGPA. Although these markers have been shown to be elevated in active disease at the population level, a longitudinal study using a validated ClinRO as the gold standard found that they were affected by disease severity and treatment status, limiting their success in predicting disease activity and relapse at the individual patient level (77).

CONCLUSION

With the advent of targeted therapies that reduce blood eosinophilia but may have varied effects on tissue eosinophilia and eosinophil-related end organ manifestations, there is an increasing need for reliable, non-invasive markers of disease activity in HES. Although some progress has been made in select subtypes of HES, including EoE and *PDGFRA*-positive myeloid neoplasm, generally applicable, validated biomarkers in HES are lacking. This is likely due, at least in part, to the heterogeneity of clinical manifestations, lack of understanding of the factors driving the varied HES subtypes and paucity of longitudinal studies addressing this issue. Although not validated as a surrogate marker for disease activity in HES, the AEC remains a key laboratory test that is used by experts to assess disease activity and response to therapy in all HES subtypes. Soluble mediators that correlate with active disease, including serum levels of EGP, have been identified, although increased predictive value compared to the AEC has not been demonstrated in most cases and none have been validated in prospective double-blind clinical trials to date. Finally, tissue-based markers, including tissue eosinophilia, granule protein deposition, and transcriptome analysis, have demonstrated utility in monitoring disease activity in some settings but are limited by the availability of appropriate tissue samples. While the development of novel non-invasive sampling methods and global approaches to biomarker discovery ("omics") are exciting, carefully designed clinical trials are clearly needed to validate existing and novel biomarkers for accurate monitoring and assessment of therapeutic interventions.

ETHICS STATEMENT

The data presented in this manuscript were collected under research protocol NCT00001406. This study was carried out in accordance with the recommendations of the Belmont Report with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the NIAID Institutional Review Board.

AUTHOR CONTRIBUTIONS

AK, PK, and MM each contributed to the writing of the manuscript.

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REVIEW
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Targeting the Interleukin-5 Pathway for Treatment of Eosinophilic Conditions Other than Asthma

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Improved understanding of the contribution of eosinophils to various chronic inflammatory conditions, most notably allergic asthma, has encouraged development of monoclonal antibodies specifically targeting mediators and surface receptors involved in eosinophil expansion and activation. The pivotal role of interleukin-5 (IL-5) in eosinophil biology, its high specificity for this leukocyte subset, and its involvement in the majority of eosinophilic conditions make it a very enticing target for treatment of eosinophil-mediated disorders. Two types of antibodies have been developed to target eosinophils: antibodies against IL-5 (mepolizumab and reslizumab), and an antibody against the IL-5-receptor-alpha-chain (IL-5Rα) (benralizumab). Both types of antibodies prevent IL-5 from engaging its receptor and in addition, anti-IL-5Rα antibodies induce target-cell lysis. They have been shown to reduce circulating eosinophil counts rapidly in humans with various disorders. Herein, a brief overview of the role of IL-5 in eosinophil biology will be presented, followed by a description of the development and characteristics of antibodies targeting IL-5 or its receptor. Results of clinical trials evaluating the efficacy and safety of these new antibodies in diseases (other than eosinophilic asthma) with prominent tissue eosinophilia are reviewed, followed by safety considerations and potential future applications.

Keywords: benralizumab, eosinophilic esophagitis, eosinophilic granulomatosis with polyangiitis, hypereosinophilic syndrome, interleukin-5, mepolizumab, nasal polyposis, reslizumab

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INTRODUCTION

Improved understanding of the contribution of eosinophils to various chronic inflammatory conditions, most notably allergic asthma, has encouraged development of monoclonal antibodies specifically targeting mediators and surface receptors involved in eosinophil expansion and activation. Interleukin-5 (IL-5) is a key mediator acting at many levels of eosinophil biology. Importantly, this cytokine has a very narrow set of cellular targets as, in humans, only eosinophils, basophils and a subset of mast cells are known to express the IL-5Rα (CD125) chain. The pivotal role of IL-5 in eosinophil biology, as well as its high specificity for this leukocyte subset, makes it a very enticing target for treatment of eosinophil-mediated disorders.

Two types of antibodies have been developed to target eosinophils: antibodies against IL-5 (mepolizumab and reslizumab), and an antibody against the IL-5Rα chain (benralizumab). Anti-IL-5 antibodies bind to IL-5 and interfere with occupation of the IL-5R, whereas anti-IL-5Rα antibodies bind to the membrane-expressed receptor, and both inhibit signaling and induce cell lysis. Both types of antibodies have been shown to rapidly reduce eosinophil counts in peripheral blood in humans.

Herein, a brief overview of the role of IL-5 in eosinophil biology will be presented, followed by a description of the development and characteristics of antibodies targeting IL-5 or its receptor. Results of clinical trials evaluating the efficacy and safety of these new antibodies in diseases (other than eosinophilic asthma) with prominent tissue eosinophilia are reviewed, followed by safety considerations and potential future applications.

EOSINOPHILS AND IL-5

Eosinophils derive from a myeloid multipotent progenitor in bone marrow, with GATA-1, PU-1, and c/EBP acting as key transcription factors for their differentiation (1). The importance of GATA-1 for eosinophil lineage commitment is reflected by the complete absence of eosinophils in mice following deletion of the high-affinity GATA binding site in the GATA-1 promoter [delta dblGATA eosinophil-deficient strain (2)]. Human eosinophil progenitors express CD34, CD38, and CD125 (IL-5Rα). They pursue their maturation and proliferation in response to transcription and growth factors, including most notably IL-5. As they mature, eosinophils produce eosinophil cationic protein (ECP), major basic protein, eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN) that are stored in cytoplasmic granules. These cationic proteins account for eosinophil avidity for the acidic dye eosin. The specificity of EPO expression by eosinophils has been exploited to generate the transgenic PHIL eosinophil-less mouse strain, wherein the EPO promoter drives expression of diphtheria toxin A (3). Mature eosinophils also produce a multitude of cytokines, growth factors, chemokines, and lipid mediators.

Among the factors contributing to eosinophil maturation, IL-5 is the most specific. This cytokine functions as a homo-dimer and its receptor (IL-5R) is a hetero-dimer, with a ligand-binding alpha-subunit, and a non-ligand-binding signal transducing beta-subunit (4). The IL-5Rα chain is expressed only by eosinophils, basophils, and mast cells (with highest expression levels on the former) in humans. The common beta chain is also involved in intracellular signaling in response to IL-3 and granulocyte macrophage colony stimulating factor (GM-CSF), and in contrast to IL-5Rα, the ligand-binding receptor components for IL-3 and GM-CSF are shared by diverse cell types.

Interleukin-5 acts on eosinophils at multiple functional levels and time points during their life-span (5). Besides stimulating proliferation, differentiation and maturation of IL-5Rα-expressing eosinophil-committed progenitors in the marrow, IL-5 contributes to eosinophil egress from the marrow toward the intravascular compartment. When produced in tissues, this cytokine also synergizes with chemotactic factors such as eotaxin-1 (CCL11) to attract eosinophils (homing), and primes these cells for activation in response to various mediators. Finally, IL-5 prolongs eosinophil survival in concert with other anti-apoptotic factors. Thus, increased IL-5 production induces (hyper)eosinophilia (i.e., blood eosinophil count above 1.5 G/L and/or increased presence of eosinophils/eosinophil granule proteins in tissue), both by stimulating eosinophoiesis and by reducing peripheral apoptosis. Interestingly, however, IL-5 over-expression alone appears to be insufficient for induction of eosinophil-mediated damage, as evidenced in IL-5 transgenic mice that have marked eosinophilia in blood and certain tissues, without associated organ dysfunction (6). Furthermore, eosinophil maturation may occur independently of IL-5, as suggested by presence of eosinophils in blood and tissues in IL-5 knock-out mice (7). Indeed, these mice fail to mount hypereosinophilia in the setting of a Th2 immune response (8), but homeostatic eosinophils remain detectable. Recent studies confirm that homeostatic eosinophils have different response patterns and functions depending on their localization; resident eosinophils home to lungs and survive independently of IL-5, contrasting with those in adipose tissue (9). Furthermore, peripheral survival of mature eosinophils may be supported by IL-3 and/or GM-CSF through induction of bcl-xl expression (10). Thus, although IL-5 clearly plays a central role in eosinophil biology, it appears neither entirely necessary nor sufficient for certain eosinophil functions.

Most human diseases accompanied by hypereosinophilia are associated with increased IL-5 production (4). The most common source of IL-5 is "type 2" $CD4^+$ helper T cells, either in the setting of an immune response to an environmental agent or pathogen (e.g., allergy and helminthiasis), or in the setting of T cell lymphoma (e.g., Sezary syndrome). In these conditions, IL-5 is often co-expressed with other cytokines including IL-4 and IL-13, resulting in associated increased vascular permeability, smooth muscle contractility, and IgE production. Other less common sources of IL-5 include transformed epithelial cells (e.g., cervical, colorectal, or non-small-cell lung cancer), and Reed-Sternberg cells in Hodgkin's lymphoma. More recently, type 2 innate lymphoid cells have been shown to represent a source of IL-5 (11). These cells reside in the skin, lungs, and gastrointestinal tract and are activated in presence of alarmins (IL-33 and TSLP) and IL-25 (a.k.a. IL-17E). They contribute to eosinophilic inflammation in murine models of allergic asthma and are increased in sputum from patients with severe allergic asthma where they represent the predominant source of IL-5 (12).

Whatever the source of IL-5 may be, this cytokine selectively and broadly affects eosinophil biology in humans and is involved in the majority of diseases mediated by eosinophils. As such, IL-5 represents an appealing therapeutic target for hypereosinophilic conditions.

HUMANIZED MONOCLONAL ANTIBODIES TARGETING IL-5 AND ITS RECEPTOR

Three anti-IL-5 pathway therapies have been developed for clinical use (**Table 1**) (13, 14). Mepolizumab and reslizumab, both anti-IL-5 antibodies, bind to and neutralize soluble IL-5, thereby interfering with its ligation to IL-5Rα. Benralizumab is directed against the membrane-expressed IL-5Rα chain, and thereby recognizes (and binds) eosinophils directly. All three have been evaluated in asthmatic patients in large-scale clinical trials, from which most of the pharmacokinetic/dynamic data that follows has been derived. Studies in eosinophilic conditions other than asthma, which are the focus of this review, have been published only for mepolizumab and reslizumab so far. Abundant data is available about effects of treatment on

TABLE 1 | Antibodies targeting IL-5 and its receptor.

	Mepolizumab	Reslizumab	Benralizumab
Trade name	Nucala	Cinquair (USA), Cinquaero (EU)	Fasenra
Other names	SB-240563	SCH55700	MEDI-563
Company	GlaxoSmithKline	Teva	AstraZeneca/Medimmune
Regulatory approval	2015: FDA 4 Nov, EMA 2 Dec (asthma)	2016: FDA 23 Mar, EMA 16 Aug	2017: FDA Nov 14
Vial strength	100 mg	25 and 100 mg	30 mg (pre-filled syringe)
Route of administration	SC (formerly IV)	IV	SC
Dosing (approved in asthma)	100 mg SC/4 wks	3 mg/kg IV/4 wks	30 mg/4 wks (first 3 doses), then /8 wks
Dosing (other)	EGPA, HES: 300 mg	–	–
Type of Ig	IgG1, kappa humanized	IgG4, kappa humanized	IgG1, kappa humanized
Mechanism of action	Neutralizes free IL-5: prevents binding to IL-5Rα	Neutralizes free IL-5: prevents binding to IL-5Rα	Binds to IL-5Rα: interferes with binding of IL-5 and induces ADCC
Time to response, blood eos	1 day	1 day	1 day
Elimination half-life	SC: 16–26 days, IV: 28 days	24 days	15 days

ADCC, antibody-dependent cellular cytotoxicity; EGPA, eosinophilic granulomatosis with polyangiitis; EMA, European Medicines Agency; eos, eosinophils; FDA, Federal Drug Administration; HES, hypereosinophilic syndrome; Ig, immunoglobulin; IL, interleukin; IL-5, interleukin-5; IV, intravenous; SC, subcutaneous; wk, week.

blood eosinophilia, whereas only a few of the earlier small-scale studies have assessed bone marrow and tissue eosinophil responses.

Anti-IL-5 Antibodies

Mepolizumab (Nucala®) is a fully humanized, IgG1-type antibody with high affinity and specificity for IL-5 (15). It has been administered intravenously (IV) and subcutaneously (SC) at various doses in a number of clinical trials conducted in eosinophil-mediated diseases and is currently approved (as first-in-class) for use as add-on therapy for patients with severe eosinophilic asthma, at the dose of 100 mg SC every 4 weeks. The route of elimination is unknown, but like other immunoglobulins, it is probably degraded by proteolytic enzymes. The dose need therefore not be adapted in patients with kidney or liver impairment. The bioavailability of SC mepolizumab is roughly 80%, with dose-proportional pharmacokinetics over a range of doses, and a median time to maximal concentration of 6–8 days post-dosing (compared with 30 min for IV) (16). When three consecutive doses are administered SC, the accumulation ratio is 1.7. The ratio between maximal mepolizumab concentrations reached in blood following monthly SC versus IV administration (when doses are normalized) is 42% after the first dose, and 54% after the third dose. The elimination half-life of SC mepolizumab is 16–22 days and slightly longer (28 days) for the IV route.

Pharmacodynamic and/or clinical studies have shown that the effect of mepolizumab on blood eosinophil levels is rapid and dose-dependent. Reduced eosinophilia is observed in blood already 24 h after administration (SC or IV), although levels continue to decline, with a peak reduction in asthma seen at 4 weeks (17). To determine the optimal dosing regimen in asthma, the extent of eosinophil depletion was quantified over a range of SC doses after three consecutive monthly injections; a 90% maximal reduction was achieved with a dose of 99 mg SC, whereas 11 mg only reached 50% of the maximal effect (15). In this line, posttreatment eosinophil levels were higher in asthmatic patients receiving 12.5 mg SC than in those treated with 125 mg SC, 250 mg SC, and 75 mg IV (16). The duration of the effect on eosinophils is also dose-dependent, in keeping with prolonged detection of mepolizumab in plasma as the dose increases (18). Depending on the dose, route of administration, and disease, the return of blood eosinophilia to baseline values varies. In patients with normal or marginally increased eosinophil counts, the effect of 100 mg SC or more lasts roughly 3 months (16). In patients with hypereosinophilic syndrome (HES, defined on the basis of blood eosinophilia of at least 1.5 G/L, i.e., 1,500/μL) in whom higher doses have been tested (750 mg IV) the duration of eosinophil depletion is variable, ranging from 3 to 37 weeks, with a median interval between infusions of 12.8 weeks (19, 20). This variability is likely related to the amount of endogenously produced IL-5 in this heterogeneous disease.

While eosinophil counts drop in mepolizumab-treated subjects, serum IL-5 levels have been shown to increase over time (16, 21). One group showed that most of the IL-5 detected during treatment is part of a complex, bound to an immunoglobulin (20) (most likely mepolizumab), and it has been hypothesized that the half-life of complexed IL-5 is prolonged. The biological significance and fate of these complexes remain unknown.

The effects of mepolizumab on bone marrow eosinophils have been examined in asthma and other eosinophilic disorders. One study with asthmatic patients showed a 70% decrease in mature eosinophil counts compared with placebo but no effects on $CD34^+$ cells expressing the IL-5Rα receptor (early eosinophil progenitors) following mepolizumab administration, indicating that treatment leads to maturational arrest of the eosinophil lineage (22). Despite this observation, no major concerns have been raised with mepolizumab regarding enhanced eosinophil maturation once treatment is interrupted (see Safety of Therapeutic Antibodies Targeting IL-5 and Its Receptor).

Effects of mepolizumab on tissue eosinophils will be developed in detail below (see Clinical Trials Evaluating Antibodies That Target IL-5 or Its Receptor in Mucosal Eosinophilic Disorders besides Eosinophilic Asthma and Clinical Trials Evaluating Antibodies That Target IL-5 or Its Receptor in Systemic HESs). In asthmatic patients, bronchial mucosal eosinophils decrease by roughly 50% at maximal dosing (750 mg IV), regardless of the duration of treatment [similar findings after 3 (17) or 12 (23) monthly infusions].

Reslizumab (Cinquaero® or Cinquair®), previously known as SCH55700, is a fully humanized, IgG4-type antibody with high affinity and specificity for IL-5 (24). It has been administered IV in clinical trials so far, and this route of administration has recently been approved in the USA and Europe for use as add-on maintenance therapy in adult patients with severe eosinophilic asthma. The SC route of administration is currently being assessed for treatment of asthma. Repeated dosing of reslizumab results in 1.5- to 2-fold accumulation relative to a single dose. The half-life of this antibody has been estimated at 24 days, and like mepolizumab, proteolytic degradation is the presumed mechanism of elimination.

The effects of reslizumab on blood eosinophil counts are dose-dependent, which probably explains the low (50%) response rate observed in an early study conducted in patients with HES treated with 1 mg/kg (25). Subsequent trials with higher dosing in patients with lower baseline eosinophil levels have confirmed the rapid and profound eosinophil-depleting effect, similar to mepolizumab. Effects on bone marrow eosinophilia have not been evaluated in asthma, but one study conducted on four patients with HES showed unchanged bone marrow cellularity and eosinophilia (25). Maturational arrest was not observed in aspirates from this small cohort of patients with markedly increased bone marrow eosinophilia and low-dose anti-IL-5 treatment.

Like mepolizumab, reslizumab increases the serum IL-5 level one month posttreatment in patients with HES; it remains unknown whether this represents free or complexed IL-5 (26). Culture-medium-containing serum from reslizumab-treated patients was shown to prolong eosinophil survival *in vitro*, leading investigators to hypothesize that anti-IL-5 may not only prolong half-life but also actually potentiate IL-5 activity in certain conditions.

Anti-IL-5R Antibody

Benralizumab (Fasenra®) is a fully humanized, afucosylated IgG1-type anti-IL-5Rα antibody (27). This antibody binds to the IL-5Rα expressed by eosinophils and basophils, close to the site that binds IL-5, thereby hindering access of IL-5 to its receptor (and neutralizing its effects) and inducing target-cell depletion through natural killer cell-mediated antibody-dependent cellular cytotoxicity (ADCC). Benralizumab has just been approved by the FDA as add-on maintenance therapy for children (12 years and older) and adults with severe asthma and an eosinophilic phenotype. Afucosylation of this antibody results in marked enhancement of its affinity for the FcγRIIIa receptor on natural killer cells, thereby competing with non-specific endogenous IgGs, and making benralizumab a highly efficient cytotoxic antibody. Importantly, because of its mechanism of action, benralizumab can destroy IL-5Rα-expressing cells, regardless of their relative dependency on IL-5 or other mediators for their growth or survival. Moreover, ADCC is not significantly affected by the density of target antigen, so benralizumab is capable of destroying cells even if they display low-level expression of the IL-5Rα chain (13). Finally, the efficacy of benralizumab should theoretically not be decreased in presence of high-level endogenous IL-5 production, in contrast to anti-IL-5 antibodies.

Pharmacokinetic studies with benralizumab have shown a linear relationship between dosing and concentration. The volume of distribution exceeds that of the intravascular compartment, indicating potential binding to blood cells and/or access to the extravascular compartment (28). Benralizumab's mean elimination half-life is roughly 18 days. The bioavailability of subcutaneous dosing is roughly 50%, and this route has pharmacokinetic/dynamic properties similar to IV dosing.

The depleting effect of benralizumab on peripheral blood eosinophils is particularly rapid and pronounced. At doses of 0.3 mg/kg IV and above, the maximal effect is observed at 24 h, at which time eosinophils are barely detectable (close to the limit of detection in healthy subjects and patients with asthma) (28, 29). Basophils also rapidly become undetectable with benralizumab, but this has been studied less extensively and the clinical relevance is unknown (30). One group investigated eosinophil biomarkers in benralizumab-treated asthmatic patients (3 monthly SC doses) to determine whether uncontrolled and potentially detrimental release of toxic eosinophil-derived mediators occurs at treatment initiation when eosinophils are destroyed (31). Serum levels of EDN and ECP were shown to decrease compared with baseline levels. Innocuity of eosinophil destruction by ADCC is further supported by the fact that none of the clinical trials in asthmatic patients have reported disease worsening at treatment initiation. Single-dosing studies have shown that eosinophil depletion is prolonged after administration of various doses of benralizumab, lasting at least 12 weeks for doses ranging from 0.3 to 3 mg/kg IV. At lower doses, the effect is less long-lasting (28). Because of the rapidity and duration of eosinophil depletion in response to benralizumab, it was tested in patients with acute asthma attacks presenting to the emergency department (32). Administration of a single dose of IV benralizumab (0.3 or 1 mg/kg) within 7 days in addition to standard of care reduced the frequency of subsequent exacerbations by 50%, and hospitalizations by 60%, over a period of 12 weeks compared with placebo.

Bone marrow eosinophils (precursors and mature cells) in asthmatic patients treated with a single IV (1 mg/kg) or 3 monthly SC (100 mg) doses of benralizumab are completely suppressed 4 weeks after dosing (30). Immunohistochemical staining of lung biopsies from asthmatic patients has shown that benralizumab stains more than 90% of eosinophils (33), indicating that effects on tissue eosinophilia could be dramatic, provided the antibody can access inflamed tissue. Bronchial biopsies obtained during a relatively small-scale placebo-controlled clinical trial before and after IV or SC benralizumab treatment showed that airway mucosal eosinophils decreased in 82% patients receiving active treatment, with a 96% median reduction after three consecutive SC doses (100 or 200 mg) (30). The effect was dose-dependent, with a less profound reduction following a single IV dose (1 mg/kg). The effects on tissue eosinophils with the dosing regimens used in the most recent clinical trials (30 mg SC at 4- or 8-week intervals) have not been assessed.

Similar to anti-IL-5 antibodies, treatment with benralizumab is followed by an increase in serum IL-5 levels as well as eotaxin-1 and -2 (but not eotaxin-3) (31). Presumed mechanisms include IL-5 accumulation in serum as a result of depletion of

target receptors, and disruption of a negative autoregulatory loop whereby eosinophils inhibit IL-5 production.

General Considerations

Overall, antibodies targeting IL-5 reduce blood eosinophil counts in a dose-dependent manner, with dramatic reductions observed at sufficient dosing. The mechanisms of eosinophil depletion have not been fully elucidated. Maturational arrest has been demonstrated in the bone marrow of mepolizumab-treated asthmatics. The rapidity of the drop in blood eosinophil counts suggests additional peripheral mechanisms that may include apoptosis through cytokine deprivation. The effects of these antibodies on tissue eosinophils are less pronounced, often closer to a twofold reduction. This may be explained by restricted access of these antibodies to tissues, the contribution of mediators other than IL-5 to eosinophil survival in tissue, and/or shedding of the IL-5Rα chain by activated tissue-infiltrating eosinophils (34). Whether residual tissue eosinophilia accounts for some of the disappointing clinical responses observed with anti-IL-5 treatment remains unknown (35).

Anti-IL-5R antibodies have been shown to deplete tissue eosinophils more profoundly in asthmatic subjects. Future clinical trials with anti-IL-5R may finally clarify the true role played by eosinophils in organ damage and dysfunction in other eosinophilic conditions, such as eosinophilic esophagitis (EoE). A potential limitation to efficacy of benralizumab in disorders with marked expansion of activated eosinophils may be enhanced membrane cleavage of IL-5Rα with shedding of its soluble form (sIL-5Rα) and/or alternative splicing of IL-5Rα mRNA (36). Indeed, serum levels of sIL-5Rα have been shown to rise with increasing eosinophilia, while membrane expression decreases, in subjects with hypereosinophilia. The soluble form may intercept benralizumab before it can access target cells. The results of an ongoing placebo-controlled clinical trial evaluating efficacy of benralizumab in patients with HES, who commonly have increased serum sIL-5Rα (36), should shed some light on this question.

A BRIEF HISTORICAL PERSPECTIVE ON THE DEVELOPMENT OF IL-5 TARGETED THERAPY FOR HUMAN DISEASES

Well before the development of therapeutic antibodies targeting the IL-5 pathway, numerous observations in humans and mice established the eosinophil as a key player in the pathogenesis of allergic airway disease. In asthmatic humans, blood and airway eosinophil counts were shown to increase with clinical severity, and histopathological studies showed that bronchial epithelial shedding was associated with close proximity of degranulated eosinophils (37). Furthermore, in murine models of experimental asthma, either genetic manipulation of IL-5 expression or pretreatment of mice with anti-IL-5 antibodies abolished blood and airway eosinophilia, prevented development of airway damage, and reduced airway hyperreactivity, confirming the key role both of eosinophils and IL-5 in this disease (8, 38).

Two companies (GlaxoSmithKline and Schering-Plough) developed anti-IL-5 antibodies (mepolizumab and SCH55700/reslizumab, respectively) at the same period, and a third company subsequently developed an anti-IL-5Rα antibody (AstraZeneca-Medimmune, benralizumab), with the intention of improving asthma control and reducing the need for poorly tolerated anti-inflammatory agents such as oral corticosteroids (OCS). Establishing the efficacy of eosinophil-depleting antibodies in asthma turned out to be challenging, with a particularly long interval between the first clinical trial (published in 2000) and regulatory approval of the first anti-IL-5 antibody for severe eosinophilic asthma in 2015. Indeed, initial trials with anti-IL-5 antibodies enrolled "all-comer" asthmatic patients regardless of disease severity, phenotype or endotype (13, 39), and although blood and sputum eosinophils decreased significantly, no improvement in lung function was observed. It took several years to identify the ideal candidates for IL-5 targeted therapy, based on a better understanding of asthma heterogeneity.

In the meantime, the two companies producing anti-IL-5 approached an entirely different medical community to seek validation of the concept that neutralizing IL-5 results in eosinophil depletion, and control of eosinophil-mediated disease. HESs compose a heterogeneous group of diseases characterized by a marked increase in blood and/or tissue eosinophils, associated with organ dysfunction and damage for which no cause other than eosinophil toxicity can be detected. Mepolizumab was administered to a handful of patients with HES in two short mono-centric open-label studies (40, 41), the clinical results of which were so encouraging that orphan drug status was granted, and an international placebo-controlled double-blind randomized clinical trial was undertaken to assess efficacy in this rare disease in 2004 (42) (see Clinical Trials Evaluating Antibodies That Target IL-5 or Its Receptor in Systemic HESs). Although the results of this trial confirmed that mepolizumab was an effective CS-sparing agent for patients with HES, regulatory authorities judged that the trial design was flawed and requested additional data supporting use of anti-IL-5 in this indication. Indeed, (1) physicians were not blinded to eosinophil counts, and were therefore practically speaking not blinded to treatment, given the clear-cut eosinophil-depleting effect of mepolizumab, (2) disease was controlled with maintenance OCS treatment at baseline, and the fact that disease control was maintained despite significant OCS tapering in the active treatment arm was not considered a valid surrogate for a clinical response to mepolizumab, and (3) patients in the placebo arm had significantly shorter exposure to drug than patients in the active treatment arm, because the trial design permitted early withdrawal and open-label access to mepolizumab after the first two study-drug infusions. GlaxoSmithKline withdrew its marketing authorization application for mepolizumab in HES in 2009, and a long effort toward designing a trial that would address regulatory concerns began.

Notwithstanding, proof of concept was clearly achieved in HES, and with improved characterization of asthma phenotypes, a more accurate picture of the type of patient most likely to benefit from therapeutic eosinophil-targeting emerged. Two pilot studies were undertaken to evaluate mepolizumab

versus placebo in patients with severe asthma and persistent eosinophilic inflammation (a factor known to be associated with asthma exacerbations) despite high-dose inhaled CS use (23, 43). As expected, these studies showed rapid normalization of blood and sputum eosinophil counts in the active treatment arm, but more importantly, a significant reduction in the exacerbation rate in mepolizumab-treated compared with placebo-treated patients was observed. Patients requiring long-term OCS treatment to maintain disease control before inclusion were better able to lower their OCS dose in the active-treatment arm. The two landmark studies were published back-to-back in 2009 and were followed by a series of large-scale placebo-controlled trials that consistently confirmed the added value of anti-IL-5(R) treatment in severe eosinophilic asthma, with decreased exacerbation rates relative to placebo, improved ability to taper OCS, increased forced expiratory volume, all reflected by better clinical asthma scores. The trials involved in establishing the efficacy of IL-5 pathway targeting in asthma, leading to regulatory approval, have recently been reviewed (44).

As for patients with HES, more than 10 years after the first large-scale clinical trial, mepolizumab is now being tested in a randomized placebo-controlled trial that will be pivotal in seeking regulatory approval for this rare disorder. The trial has been designed to truly assess the clinical efficacy of anti-IL-5 and should guarantee double-blinding. Indeed, the primary endpoint is related to disease flares, and physicians will be blinded to eosinophil counts.

The long story of anti-IL-5(R) development illustrates nicely how rare diseases, with homogenous (and occasionally well delineated) pathogenic mechanisms, represent powerful tools to establish proof of concept for the development of highly targeted therapeutic compounds (45). Thus, patients with rare diseases are finally offered opportunities to access efficacious treatment through clinical trial participation, followed by open-label long-term access programs and regulatory approval. In turn, biomarker data collected during these studies can be used to improve selection of patients for large-scale clinical trial implementation in the setting of more common, but also more heterogeneous, illnesses (46).

CLINICAL TRIALS EVALUATING ANTIBODIES THAT TARGET IL-5 OR ITS RECEPTOR IN MUCOSAL EOSINOPHILIC DISORDERS BESIDES EOSINOPHILIC ASTHMA

Eosinophilic asthma is one of several disorders wherein eosinophils participate massively to inflammatory infiltrates in mucosal tissue; these include EoE and chronic rhinosinusitis, especially in presence of nasal polyps (CRSwNP). Like asthma, blood eosinophilia is often mild (if present) in these disorders, and pathogenic mechanisms likely include allergic sensitization, and numerous cell types and mediators beyond eosinophils and IL-5. Targeting IL-5 in eosinophilic asthma has nonetheless been shown to improve certain disease components and reduce the need for OCS. Effects of anti-IL-5 antibodies in EoE and CRSwNP have been evaluated in several clinical trials (**Table 2**).

Eosinophilic Esophagitis

Eosinophilic esophagitis is a Th2-mediated inflammatory disease involving the esophagus, characterized by symptoms of esophageal dysfunction, increased eosinophil counts in esophageal biopsies (>15/high-power field) with epithelial hyperplasia, and lack of response to treatment directed against gastro-esophageal reflux disease (53). Frequent sensitization to food allergens has provided rationale for treatment strategies based on elimination of the most common food allergens (six-food elimination diet), and often impracticable amino acid-based diets. Other approaches include swallowing inhaled CS, and OCS therapy, with variable efficacy and significant long-term toxicity. In severe disease, endoluminal dilatation, enteral feeding, or parenteral nutrition may be required. EoE is therefore potentially a profoundly debilitating disease for which classical therapeutic options are difficult to adhere to and/or tolerate.

Animal models and translational research on large patient cohorts have led to a better understanding of pathogenesis, and to elaboration of targeted strategies. IL-5 is a key mediator in murine models of allergen- and IL-13-induced EoE, as evidenced by abolished esophageal eosinophilia and reduced remodeling in IL-5-deficient or anti-IL-5-treated mice (54, 55).

Anti-IL-5 treatment was first assessed in EoE in a single adult patient with refractory disease, in the setting of a small open-label study evaluating efficacy of monthly mepolizumab infusions (40). Biological, clinical (dysphagia and vomiting), and histopathological improvement of disease was observed, encouraging the same group to evaluate three additional patients with long-standing symptomatic EoE (47). This pilot study confirmed that 3 monthly infusions of mepolizumab reduced clinical manifestations, increased quality of life scores, and improved endoscopic appearance (narrowing and strictures) although esophageal thickening and furrowing persisted in one patient. A significant reduction of esophageal eosinophilia was observed in all four subjects (mean ninefold), but peak residual counts remained above 20/hpf. The clinical findings were deemed sufficiently promising to design several randomized double-blind trials with anti-IL-5 antibodies in adults and children with EoE.

One group evaluated mepolizumab (750 mg) versus placebo in 11 adults with treatment-refractory symptomatic EoE, using a very stringent primary endpoint: peak esophageal eosinophilia <5/hpf after 2 weekly infusions of study-drug (48). Because none of the patients reached this endpoint, two additional infusions of high-dose mepolizumab (1,500 mg) or placebo were administered at 4-week intervals. Biopsies showed a roughly threefold (65%) reduction in peak/mean eosinophil counts only in mepolizumab-treated patients. Findings were similar after the second and fourth infusions, indicating that the maximal histological (eosinophilic) response to 750 mg IV mepolizumab is achieved rapidly, with no further dose–response. Endoscopic appearance of the esophagus was not significantly improved by active treatment, and clinical benefit was marginal (both treatment groups experienced a reduction in the proportion of days

TABLE 2 | Clinical trials evaluating anti-IL-5 antibodies in mucosal eosinophilic disorders other than asthma.

Reference Drug Clinicaltrials.gov ID	• Study design • Dose • Route • # Injections • Interval	Patients			Response to *active* treatment[a]				
		Age (yrs)	Nbr	Baseline disease characteristics	Primary EP	Blood EOS	Tissue EOS	Clinical (symptoms/signs)	Other endpoints/ findings
Eosinophilic Esophagitis									
Stein et al. (47) MEPO NCT00266565	• Open label • 10 mg/kg (max 750) • IV • 3× • 4 wks	18–41	4	Sympt >9 yrs Strictures (3/4) CS resistant (2/4) EsoEOS > 24/hpf	–	Mean sixfold decrease (444 to 69.5/mm^3) Still low 12 wks after last infusion	Mean ninefold decrease Resolution of eos microabsc (2/2)	Variable symptom improvement (4/4): reduced dysphagia, vomiting, food impactions, pain, and diet advancement	Endoscopy improved in 3/4 (persistent thickening and furrowing in 1)
Straumann et al. (48) MEPO NCT00274703	• RDB PC • 750 (2×) then 1,500 (2×) mg • IV • 4× • d0, d7, wk5, wk9	>18, mean 33	11	Peak EsoEOS > 20/hpf Mean peak eos at BL 200/hpf At inclusion: dysphagia present, off all EoE Tx History: poor response to topical/oral CS, food impactions	Peak esoEOS < 5/hpf 4 wks after 2× 750 mg: 0/5 patients	Decreased at wk1, and 12 wks after last infusion (up to 10-fold) Return to BL 34 wks after last infusion	No patients below 15 eos/hpf 65–72% reduction peak/mean eos counts at wks 4 + 13 (twofold to threefold)	No significant difference btw MEPO and PLAC Days with dysphagia decreased by 20/30% (wks 9–13/13–17) with MEPO and by 20/18% with PLAC	Endoscopy: 3/5 showed improvement with MEPO versus 2/6 with PLAC No significant differences in endoscopic response btw groups
Assa'ad et al. (18) MEPO NCT00358449	• RDB (no PLAC) • 0.55, 2.5, and 10 mg/kg (3 arms) • IV • 3× • 4 wks	2–17, mean 10.4	59	Peak EsoEOS ≥ 20/hpf At inclusion: 19% asymptomatic, no info on ongoing EoE Tx History: poor response/ tolerance to prior Tx	Peak EsoEOS < 5/hpf 4 wks after 3rd infusion: 8.8% patients (no dose response)	Decreased at d1, wk12 More rapid recurrence in 0.55 mg/kg group No rebound	4 wks after 3rd infusion: – Peak EsoEOS fell to <20/hpf in 32% subjects (all 3 doses) – Peak/Mean EsoEOS decreased threefold/ fourfold 16 wks after 3rd infusion: Peak/Mean EsoEOS remained below BL; lowest in 10 mg/kg group	No significant changes in symptoms Low symptom scores at BL; study not powered to detect changes	Endoscopy: reduced erythema, vertical lines, furrows in PR (EsoEOS < 20/hpf), and CR (EsoEOS < 5/hpf) *Predictor reduction mean eos count: higher epithelial BL eos count*
Spergel et al. (49) RESLI NCT00538434	• RDB PC • 1, 2, and 3 mg/kg (4 arms) • IV • 4× • 4 wks	5–18	226	Peak EsoEOS ≥ 24/hpf Median peak EsoEOS 80/hpf At inclusion: ≥1 active symptom (moderate severity or worse), no topical or oral CS, diet (maintained)	1. % Change peak EsoEOS count: twofold reduction 2. Physicians EoE GAS: no significant difference	Not reported	Few patients had <5 EsoEOS/hpf at end of study: 2 PLAC, 8 RESLI (1 and 2 mg/kg arms) (all CR had peak BL EsoEOS < 60/hpf) Fold reduction in peak EsoEOS slightly higher in the RESLI 3 mg/kg arm	Symptomatic improvement in all groups (PLAC and RESLI): physician GAS, patient predominant EoE symptom score	Endoscopy not reported Followed by open-label extension study NCT00635089 evaluating long-term safety and efficacy (no published results)

(*Continued*)

TABLE 2 | Continued

Reference Drug Clinicaltrials.gov ID	• Study design • Dose • Route • # Injections • Interval	Patients			Response to *active* treatment[a]				
		Age (yrs)	Nbr	Baseline disease characteristics	Primary EP	Blood EOS	Tissue EOS	Clinical (symptoms/signs)	Other endpoints/ findings
Chronic rhinosinusitis with nasal polyposis									
Gevaert et al. (50) RESLI	• Phase I, PoC, RDB PC • 1 and 3 mg/kg (3 arms) • IV • 1×	18–63	24	Severe CRSwNP, with bilateral grade 3/4 polyps, or recurrence after surgery At inclusion: no local Tx or oral CS	Nasal polyp score at wk12: significant decrease only in the RESLI 1 mg/kg arm	Reduced at d1, wk8 Back to BL at wk12 Rebound in 10/16 patients, at wk24 (1 mg/kg) and 32 (3 mg/kg)	Not assessed	No improvement in symptom scores or nasal PIF No rebound after treatment	50% patients had ≥1-point reduction in NP score (up to wk4)
Gevaert et al. (51) MEPO	• RDB PC • PLAC $n = 10$, MEPO $n = 20$ • 750 mg • IV • 2× • 4 wks	Mean 48	30	Severe CRSwNP with grade 3/4 polyps, or recurrence after surgery, refractory to topical CS At inclusion: no local Tx or oral CS	Change from BL in total NP score 4 wks after 2nd infusion: −1.30 (PLAC 0.00, $P = 0.028$)	Reduced at wk1 (NS), wk4, wk8 Reduced to <200 in all subjects at wk8 No rebound	Not assessed	No significant improvement in symptom scores or nasal PIF *Improved olfaction, postnasal drip, congestion (not rhinorrhea) at wk8 (NS) Increased nasal PIF—(NS) Improved olfaction very durable (11 mo) when present*	60% patients had ≥1-point reduction in NP score at wk8 (versus 10% PLAC) > 50% had improved CT findings (versus <20% PLAC) In responders (≥1-point reduction NP score), effect maintained 36 wks after last infusion
Bachert et al. (52) MEPO NCT01362244	• RDB PC • 750 mg • IV • 6× • 4 wks	18–70	105	Severe NP requiring surgery (nasal polyp score ≥3 + VAS symptom score >7) At inclusion: topical CS (standardized dose) History: refractory to SOC Tx, ≥1 prior surgery	Need for surgery 4 wks after 6th infusion: 30% reduction (PLAC 10%, $P = 0.006$)	Reduced from GM 500 to 80/mm^3 after 1 wk, 50/mm^3 4 wks after 6th infusion	Not assessed	Improvement in VAS score for NP severity (-1.8 after 6 infusions) Improvement in VAS score for rhinorrhea and nasal blockage (delay 4 wks), mucus and anosmia (delay 8 wks) Improved SNOT-22 score after 6th infusion Nasal PIF improved after 6th infusion	Sixfold increased probability of having improved NP score after the 2nd infusion 50% patients had ≥1-point reduction NP score (versus 27% PLAC) No association btw baseline eos counts and reduction of NP score

[a]*For placebo-controlled trials, the reported findings concern statistically significant (unless mentioned otherwise) differences observed between the active treatment arm and the placebo arm; the response rate to active treatment arm is reported first, followed by response to placebo. Several non-significant differences judged worth underlining are mentioned as well.*

BL, baseline; btw, between; CR, complete response(ders); CRSwNP, chronic rhinosinusitis with nasal polyposis; CS, corticosteroid; d, day; EoE, eosinophilic esophagitis; eos, eosinophil; EP, endpoint; EsoEOS, esophageal epithelial eosinophils; GAS, global assessment score; GM, geometric mean; hpf, high-power field; IL-5, interleukin-5; IV, intravenous; MEPO, mepolizumab; mo, month; Nbr, number; NP, nasal polyposis; NS, non-significant; PIF, peak inspiratory flow; PLAC, placebo; PoC, proof of concept; PR, partial responders; RDB PC, randomized double-blind placebo-controlled; RESLI, reslizumab; SC, subcutaneous; SNOT, sinonasal outcome test; SOC, standard of care; Tx, treatment; VAS, visual analogy scale; wk, week; yr, year.

with dysphagia, that was slightly more substantial at later time points in the active treatment arm).

Effects of anti-IL-5 on pediatric EoE were assessed in two large-scale multicenter studies published shortly thereafter, one with mepolizumab (18), the other with reslizumab (49). Three doses of each drug were tested, but only the reslizumab trial included a placebo arm. Histological findings were comparable to adults: although very few children experienced complete remission (i.e., peak eos <5/hpf), a partial response, with an overall twofold to threefold reduction in peak eosinophilia, was observed in many cases. In the mepolizumab trial, most eosinophilic microabscesses disappeared and had not recurred at the long-term follow-up visit 16 weeks after the third dose, although tissue eosinophilia was increasing. Furthermore, treatment responders also displayed endoscopic regression of erythema, vertical lines and furrows. Neither of the studies showed substantial clinical benefit with anti-IL-5: the mepolizumab study enrolled patients who were largely symptom-free at enrollment and was therefore not powered to detect significant improvements, and in the reslizumab trial, symptomatic improvement was observed in all groups including the placebo arm.

Overall, clinical trials in EoE strongly support a role for IL-5 in eosinophil accumulation in the esophageal epithelium, as most patients receiving active treatment (mepolizumab and reslizumab) experience a roughly 50–60% reduction in esophageal eosinophilia. However, only a minority of patients have a complete histological response (peak eosinophilia <5/hpf), with peak eosinophilia often remaining above the 15/hpf threshold defining EoE. The maximal effect of anti-IL-5 on esophageal eosinophilia appears to reach a plateau within weeks, at which point no further improvements can be achieved by increasing the dosing regimen (48). Furthermore, the effect of treatment on symptoms is inconsistent.

There are a number of potential explanations for the disappointing clinical response to anti-IL-5 in these trials. First, the residual tissue eosinophilia observed in the majority of treated patients may perpetuate disease activity and symptoms. Unfortunately, no data is available on individual clinical responses in the few patients who did normalize their esophageal eosinophil counts with anti-IL-5. Second, subepithelial fibrosis (remodeling) may contribute to symptom burden and be less amenable to reversal with therapy, especially in adult patients who often have long-standing disease. One group has shown, however, that short-term treatment with mepolizumab led to decreased esophageal expression of tenascin C and TGFβ, both of which are involved in remodeling (48). Clinical trials in EoE conducted so far may have been too short (only 3–4 monthly doses) for reversal of fibrosis and its functional consequences. Perhaps more prolonged reduction of eosinophilic inflammation is required to translate clinically into symptomatic improvement. One group recently reported their experience with a small cohort of children treated for up to 9 years with reslizumab (56) [enrolled in a open-label extension study then a compassionate use program, following participation in a randomized trial (49)], showing clear-cut symptom improvement and absence of disease progression despite a relatively unrestricted diet and no topical CS during this prolonged observation period. Third, cell types other than eosinophils and mediators other than IL-5 may contribute to EoE symptomatology. Indeed, the relationship between esophageal eosinophilia and symptoms is poor across various clinical conditions and therapeutic strategies (49, 57). Pathogenic mechanisms of EoE also involve alarmins (TSLP), IL-13 and its transcriptional targets, epithelial barrier dysfunction, and mast cells (58). One study on pediatric EoE for example has shown that in patients whose mast cell counts decrease most with mepolizumab treatment, baseline mast cell (but not eosinophil) counts are correlated with severity of pain (59), suggesting that mast cells may specifically contribute to this clinical manifestation.

Future trials with eosinophil-targeting compounds that consistently induce more profound tissue eosinophil depletion, such as benralizumab, should provide insight on whether eosinophils are central players in EoE symptoms once and for all. Should this prove not to be the case, combined therapy may yield better results, with topical CS potentially enhancing effects of antibodies targeting eosinophils and key cytokines such as IL-13 and/or chemokines.

Chronic Rhinosinusitis with Nasal Polyposis

Among patients with chronic rhinosinusitis, those with nasal polyposis (CRSwNP) experience a particularly debilitating disease course, with refractory disease that recurs after surgery and a frequent association with severe asthma (60). Clinical manifestations impact quality of life significantly, with nasal obstruction, anosmia, nasal discharge, and headache. Treatment generally associates topical CS, and repeated courses of antibiotics and OCS to alleviate symptom exacerbation. This condition is associated with a Th2-type immune response in Caucasians, associating eosinophilic inflammation and elevated IL-5 levels in nasal secretions and tissue. When cultured nasal polyps are subjected to various *in vitro* treatments, only antibodies directed against IL-5 (but not IL-3 or GM-CSF) induce eosinophil apoptosis and deplete tissue eosinophils (61). These observations have provided rationale for clinical trials evaluating efficacy of antibodies targeting the IL-5 pathway in CRSwNP (**Table 2**). This disease offers the advantage of easy non-invasive access to tissue (polyps) and secretions for assessment of treatment effects on eosinophilia and soluble biomarkers.

An early phase 1 placebo-controlled clinical trial assessed the safety and efficacy of a single dose of reslizumab (1 or 3 mg/kg) in patients with severe (grade 3 and 4) bilateral nasal polyps (50). Although blood eosinophilia decreased in reslizumab-treated patients, the nasal polyp score decreased in only half of these subjects, and no significant improvements were noted in symptom scores or peak nasal flow. Patients whose nasal polyp score decreased had significantly higher baseline IL-5 levels in nasal secretions than non-responders. In fact, an IL-5 level above 40 pg/mL was the only predictive marker for a clinical response to reslizumab. Rebound blood eosinophilia was observed in two-thirds of anti-IL-5-treated patients, but posttreatment nasal polyp scores did not worsen.

The same group subsequently assessed the efficacy of 2 monthly mepolizumab infusions (750 mg) in patients with CRSwNP in a double-blind placebo-controlled study with a prolonged observation period (48 weeks) (51). An early and durable reduction in the endoscopic nasal polyp score was observed in 60% of mepolizumab-treated patients versus 10% in the placebo arm. The extent of the improvement was more pronounced than that observed in clinical trials with topical CS. Sinus CT findings were also significantly better after active treatment, and a trend toward symptom improvement was observed. Interestingly, the increased sense of smell experienced by certain patients was prolonged, contrasting with other symptoms (congestion and postnasal drip) that recurred more rapidly. Because patients were not allowed to use rescue intranasal therapy for the first 2 months, early withdrawals were numerous. The facts that the time to dropout was significantly longer in the mepolizumab-treated group, and that more placebo-treated patients required OCS therapy or surgery after withdrawal, provide additional indirect support for efficacy of mepolizumab in this disease. Rebound eosinophilia was not observed in this trial, and in contrast to the prior study with reslizumab, the level of IL-5 in baseline nasal secretions was not predictive of endoscopic improvement.

More recently, a large-scale trial focusing on the clinical outcome of patients with severe CRSwNP has shown a beneficial effect of mepolizumab on the requirement for surgery (52). In this double-blind placebo-controlled study, patients fulfilling endoscopic and symptomatic criteria for surgery were randomized to receive 6 monthly infusions of 750 mg mepolizumab or placebo. A higher proportion of mepolizumab- than placebo-treated patients no longer required surgery 4 weeks after the sixth infusion (30 versus 10%, respectively). Symptom scores and the quality of life SNOT-22 score improved. Interestingly, the time-to-improvement of individual symptoms varied; rhinorrhea and nasal obstruction regressed more rapidly (4 weeks) than anosmia (8 weeks). Among patients receiving active treatment, two achieved the primary endpoint only after the sixth infusion, suggesting that longer treatment duration may further increase the beneficial effect on requirement for surgery. Effects persisted well after treatment cessation, but too few patients entered the posttreatment extension phase for accurate assessment.

Chronic rhinosinusitis with nasal polyposis may be observed in patients with asthma and eosinophilic granulomatosis with polyangiitis (EGPA), and as such, has also been taken into consideration in clinical trials testing efficacy of anti-IL-5 in these disorders. Interestingly, one trial evaluating efficacy of reslizumab in severe eosinophilic asthma has shown that only patients with associated nasal polyposis experience significant clinical improvement (62) (asthma control questionnaires), suggesting that eosinophils may contribute more to the symptomatic burden and/or pathogenesis of asthma in this patient sub-group (63). In the recent study evaluating mepolizumab in EGPA (see Clinical Trials Evaluating Antibodies That Target IL-5 or Its Receptor in Systemic HESs), 94% of enrolled subjects had sinonasal abnormalities (64). Active treatment induced a significant reduction in the SNOT-22 score compared with placebo, and in the occurrence of EGPA relapses involving worsening of sinonasal symptoms, indicating that CRS associated with more complex systemic disorders may also benefit from IL-5 targeted therapy.

In summary, anti-IL-5 has the capacity to reduce the size and number of nasal polyps in patients with CRSwNP, and to reduce the need for surgery. Although this treatment option may seem unreasonably expensive for a disease that does not target vital organs, other financial considerations like the need for repeated surgery and decreased work productivity should be taken into account. IL-5 targeting appears to have prolonged effects and could be administered intermittently to forestall surgery. Costs may be further limited by dose reduction, an option that is currently being evaluated in a trial with monthly subcutaneous injections of 100 mg mepolizumab.

CLINICAL TRIALS EVALUATING ANTIBODIES THAT TARGET IL-5 OR ITS RECEPTOR IN SYSTEMIC HESs

Hypereosinophilic syndromes are rare and often debilitating chronic inflammatory disorders characterized by blood and tissue eosinophilia, with associated eosinophil-mediated organ damage and/or dysfunction. These disorders are currently classified on the basis of underlying molecular and immunological defects and the spectrum of target organ damage (65, 66) [see Kahn in this research topic (67)]. Although the mechanisms resulting in eosinophil expansion remain unknown in the majority of patients ("idiopathic" HES variant), the role played by eosinophils in tissue damage is undeniable and targeting the IL-5 pathway makes sense. The one disease variant for which IL-5 targeting should not be considered an option is chronic eosinophilic leukemia (CEL) with well-documented underlying cytogenetic rearrangements, most commonly the FIP1L1/PDGFRA (F/P) fusion gene (68). Even though mepolizumab did actually reduce blood eosinophilia in one patient with F/P^+ CEL (19), such patients respond exquisitely well to low-dose imatinib mesylate that selectively targets the molecular default that drives disease and may even offer the prospect of cure. This section will focus on studies evaluating efficacy of IL-5-targeted treatment in systemic HES (namely, idiopathic and lymphocytic variants) and EGPA (**Table 3**). Only mepolizumab has been assessed repeatedly and in large-scale trials so far.

Idiopathic HES

Patients with idiopathic HES may present with single (organ-restricted) or multiple (complex) organ involvement. Single-organ disorders comprise most commonly chronic eosinophilic pneumonia, gastroenteritis, and dermatitis. In complex HES, two or more organs/systems are affected (skin, lungs, digestive tract, heart and/or blood vessels, central and/or peripheral nervous system, and coagulation), and certain complications may be life-threatening. Reduction of blood and tissue eosinophilia is critical to prevent and reverse organ damage, and together with control of disease complications, represents the major goal of treatment (72). Most patients with HES respond to systemic CS, but many patients require second-line CS-sparing agents, none of which are fully safe and/or effective. The most commonly used

TABLE 3 | Clinical trials evaluating anti-IL-5 antibodies in systemic hypereosinophilic disorders.

Reference Drug Clinicaltrials.gov ID	• Study design • Dose • Route • # Injections • Interval	Patients			Response to *active* treatment[a]				
		Age (yrs)	Nbr	Baseline Disease Characteristics	Primary EP	Blood EOS	Tissue EOS	Clinical (symptoms/signs)	Other endpoints/findings
Hypereosinophilic Syndrome									
Plotz et al. (40) MEPO	• Open-label • 750 mg • IV • Variable (2, 8, and 10) • d1, wk2, then monthly	60, 62, and 82	3	OCS-resistant eosinophilic dermatitis Patient 2: +fever and abd pain BL blood EOS > 1 G/L	–	Reduced at d1 post-Tx 4- to 37-fold reduction 1 wk after 2nd infusion	Disappearance of skin EOS 1 wk after 2nd infusion Twofold to eightfold reduction skin ECP^+ cells	Resolution of pruritus and skin lesions (delay 3 d to 3 wks)	Prolonged 17-month remission after 2 infusions in 1 patient
Garrett et al. (41) MEPO	• Open-label • 10 mg/kg (max 750) • IV • 3× • 4 wks	40, 48, and 55	3[b]	Systemic F/P^- HES Run-in period: reduction of HES Tx (EOS increase twofold or >0.75 G/L)	–	Reduced in all 3 patients from wk2 to 12 wks after 3rd dose	Not assessed	Improved in all patients: skin, nasal congestion Improved FEV1, polyposis, and exercise tolerance	
Klion et al. (25) RESLI NCT00017862	• Open-label • 1 mg/kg • IV • Single dose; +5 doses if response • 4 wks	32–52	4	Systemic HES 1 F/P^+ patient BL blood EOS > 2.5 G/L despite maintenance treatment	–	Rapid reduction in 3 patients Duration: 7 d in 1 case, >30 d in 2 cases	Not assessed	Improved in 2 patients: rash, mucosal ulcerations, angioedema, and arthromyalgia	F/P^+ patient: no biological or clinical response +5 doses to 2 patients with biological (>30 d) and clinical response: magnitude and duration of response decreased Rebound at 6–8 wks BM 4 wks post-Tx: no effect on eosinophilia and cellularity
Stein et al. (19) MEPO NCT00266565	• Open-label • 10 mg/kg (max 750) • IV • 3× • 4 wks	19–57	19[c]	Systemic HES 1 imatinib-resistant F/P^+ patient Run-in: reduction of BL therapy to achieve twofold increase of EOS or EOS > 0.75 G/L	Evaluation of impact on immune function (**Table 4**)	Reduced in 18 patients 4 wks after 3rd dose Responders: 26-fold reduction, duration 3 mo in 10/14 assessed	Not assessed	Not assessed	3 cohorts on the basis of % reduction in BL HES Tx during study: A 0%, B 25%, and C 50% Rebound HE only observed in cohort C F/P^+ patient responded to MEPO, EOS counts normal until wk28 The only non-responder had highest IL-13 levels in PHA-stimulated-PBMC supernatants

(*Continued*)

TABLE 3 | Continued

Reference Drug Clinicaltrials.gov ID	• Study design • Dose • Route • # Injections • Interval	Patients			Response to *active* treatment[a]				
		Age (yrs)	Nbr	Baseline Disease Characteristics	Primary EP	Blood EOS	Tissue EOS	Clinical (symptoms/signs)	Other endpoints/findings
Rothenberg et al. (42) MEPO NCT00086658 MHE100185	• RDB PC (OCS tapering) • 750 mg • IV • 12× • 4 wks	Adults, mean 48.1	85	Systemic F/P⁻ OCS-responsive HES (Chusid's definition) Run-in: stabilized with PDN monotherapy (20–60 mg/d), EOS < 1 G/L	PDN dose ≤10 mg for ≥8 wks: 84 versus 43%	Eos < 0.6 G/L for ≥8 wks: 95 versus 45% If BL PDN > 30 mg: 100 versus 8%	Not assessed	Not assessed Ability to taper down OCS while maintaining disease stability considered surrogate for clinical response	difference in primary EP achievement btw MEPO and PLAC more pronounced in patients requiring > 30 mg PDN at BL: 77 versus 8% daily PDN dose decreased from roughly 30 mg at BL (mean in all patients) to 6.2 mg with MEPO and 21.8 mg with PLAC tapered off OCS until study completion: 47 versus 5%
Roufosse et al. (20) MEPO NCT00097370 MHE100901	• Open-label (extension of MHE100185) • 750 mg • IV • 5 yrs • 4 wks (stage 1), then variable (stages 2 and 3)	18–75, median 50	78	Eligible if participated in MHE100185 (completed or received at least 2 doses of study Tx)	Long-term safety: safety confirmed, No recurrent drug-related AEs/ SAEs leading to Tx interruption	Mean EOS < 0.5 G/L in all but 1 patient in stage 2 (1 non-responder, EOS count unchanged)	Not assessed	5 withdrawals due to lack of efficacy on HES symptoms/signs 54 continued until end of study PDN ≤ 10 mg end of study: 83% PDN-free during study: >50%	Study design with 3 stages: (1) tapering of background Tx to minimal effective dose, assessment of EOS response, (2) determination of optimal dosing interval in responders (re-dosing if EOS > 0.6 G/L or disease manifestations present), (3) dosing at fixed intervals Dosing interval end stage 2: >12 wks in 50% patients; median 12.8 wks; range 21–37 wks
Eosinophilic granulomatosis with polyangiitis									
Kim et al. (69) MEPO NCT00527566	• Open-label (OCS tapering) • 750 mg • IV • 4× (28-wk FU) • 4 wks	28–62, mean 45	7	OCS-depend EGPA, PDN ≥ 10 mg (ANCA status unknown) At baseline: mean daily PDN 12.9 mg; IS (MTX) 3/7, disease stable, mean EOS 3.4%	PDN dose reduction: mean 4.6 mg 4 wks after 4th dose (64% reduction)	Reduced mean EOS to 0.8% at end of active Tx	Not assessed (no change in FeNO)	Decreased exacerbation rate during active Tx (compared with washout and FU) Decreased ACQ Unchanged FEV1	Prolonged PDN dose reduction: mean 5 mg 12 wks after 4th dose Return to 15.7 mg 28 wks (7 mo) after last dose
Moosig et al. (70) MEPO NCT00716651	• Open-label (OCS tapering) • 750 mg • IV • 9× • 4 wks	43–78, mean 62	10	Relapsing/refractory EGPA despite PDN ≥ 12.5 mg and IS (ANCA status unknown) At inclusion: median daily PDN 19 mg, no IS, BVAS ≥ 3, active organ involvement	BVAS 0 with daily PDN <7.5 mg: achieved by 8/10 patients	Mean 0.026 at end of active Tx phase (for the 9/10 patients that completed)	Not assessed	No exacerbations during active Tx period No change in FEV1 at end of active Tx	PDN dose reduction: From median daily PDN dose 19 mg at BL to 4 mg at time of 9th dose [d]Follow-up study with MTX 0.3 mg/kg as maintenance Tx (9 patients): 3 had prolonged remission (median FU 22 mo), 6 relapsed (delay 4.5 mo to >2 yrs)

(*Continued*)

TABLE 3 | Continued

Reference Drug Clinicaltrials.gov ID	• Study design • Dose • Route • # Injections • Interval	Patients			Response to *active* treatment[a]				
		Age (yrs)	Nbr	Baseline Disease Characteristics	Primary EP	Blood EOS	Tissue EOS	Clinical (symptoms/signs)	Other endpoints/findings
Wechsler et al. (64) MEPO NCT02020889 MEA115921	• RDB PC • 300 mg • IV • 8× • 4 wks	Mean 48.5	136	Daily PDN dose required to control EGPA 7.5–50 mg History: asthma + EOS > 1 G/L + 2 criteria typical of EGPA 19% ANCA+, 75% required IS	*Remission (BVAS 0 with PDN ≤ 4 mg)* – Accrued REM ≥ 24 wks: 28 versus 3% – REM at wks 36 + 48: 32 versus 3%	Significant decrease in active Tx arm only (not detailed)	Not assessed	Twofold lower relapse rate in MEPO arm (1.14 versus 2.27) Higher proportion of patients experienced REM in MEPO arm (53 versus 19%)	PDN dose reduction: Higher proportion of patients at PDN dose ≤4 mg for the last 4 wks (44 versus 7%) Higher proportion of patients able to stop PDN during trial (18 versus 3%) Treatment benefit significant only in patients with BL blood EOS > 0.15 G/L: 33 versus 0% had REM ≥ 24 wks

[a]*For placebo-controlled trials, the reported findings concern statistically significant (unless mentioned otherwise) differences observed between the active treatment arm and the placebo arm; the response rate to active treatment arm is reported first, followed by response to placebo. Several non-significant differences judged worth underlining are mentioned as well.*

[b]*One patient in this study had EoE and was also included in Ref. (47) [see Stein et al. (47),* ***Table 2****]; not included in this table.*

[c]*Three patients with HES were already reported in Ref. (41) [see Garrett et al. (41),* ***Table 3****]; 6 patients in this cohort with EoE are not included in this table.*

[d]*Extended post-Tx follow-up was reported for 9 of the 10 patients in a separate publication (71).*

abd, abdominal; ACQ, asthma control questionnaire; (S)AE, (serious) adverse event; ANCA, antineutrophil cytoplasmic antibody; BL, baseline; BM, bone marrow; btw, between; BVAS, Birmingham vasculitis activity score; (O)CS, (oral) corticosteroid; d1, day 1; ECP, eosinophil cationic protein; EGPA, eosinophilic granulomatosis with polyangiitis; EOS, eosinophil; EP, endpoint; FeNO, fractional exhaled nitric oxide; FEV1, forced expiratory volume in 1 s; F/P, FIP1L1–PDGFRA; FU, follow-up; HES, hypereosinophilic syndrome; IL-5, interleukin-5; IS, immunosuppressor; IV, intravenous; MEPO, mepolizumab; mo, month; MTX, methotrexate; Nbr, number; OCS, oral corticosteroids; PBMC, peripheral blood mononuclear cells; PDN, prednisone; PE, primary endpoint; PHA, phytohemagglutinin; PLAC, placebo; RDB PC, randomized double-blind placebo-controlled; REM, remission; RESLI, reslizumab; SAE, serious adverse events; SC, subcutaneous; Tx, treatment; wk, week; yr, year.

TABLE 4 | Biological effects of IL-5(R) targeted therapy other than eosinophil depletion in diseases other than asthma.[a]

	Eosinophils	T cells	Mast cells	Remodeling	Serum/mediators	Tissue/mediators
Eosinophilic esophagitis						
Stein et al. (47) MEPO Esophagus	No change in CCR3 expression by blood EOS		Twofold decrease Eso MC (in 3 out of 4 patients)	Decreased epithelial hyperplasia (in 3 out of 4 patients)		
Straumann et al. (48) MEPO Esophagus	Unchanged expression of IL-5Rα by blood EOS	No effect on Eso CD3 T cells	No effect on Eso MC (tryptase$^+$)	Reduced epithelial TGFβ1 + tenascin C (effect delayed, most marked 4 wks after 4th dose)	Decreased ECP + EDN Increased eotaxin	Reduced EDN$^+$ cells and extracellular EDN deposition >60% reduction eotaxin-1,2,3 and IL-5 positive cells in esophagus Unchanged Eso epithelial expression of eot-3 and TNFα
Otani et al. (59) MEPO Esophagus *Responders*: <15 EsoEOS/hpf (40%)*	Reduced Eso EOS degranulation and clusters Reduced IL-9$^+$ EOS in Eso epithelium		Decreased Eso MC in 77% patients after 3rd infusion Responders*: threefold decrease Eso MC, sixfold decrease EOS/MC couplets, correlation btw MC and EOS counts			Responders*: Reduced epithelial IL-9$^+$ cells (NS reduction non-EOS IL-9$^+$ cells) *Ccl: eos induce/sustain MC through IL-9 production, or MC are directly targeted by anti-IL-5*
Chronic rhinosinusitis with nasal polyposis						
Gevaert et al. (50) RESLI Nasal polyps					Reduced sIL-5Rα and ECP Eotaxin unchanged	Nasal secretions: Reduced sIL-5Rα, ECP Reduced IL-5 only in responders Eotaxin unchanged
Gevaert et al. (51) MEPO Nasal polyps					Reduced sIL-5Rα and ECP	Nasal secretions: Reduced sIL-5Rα, IL-6, IL-1β (*impact on tissue neutrophils not investigated*) ECP, IL-5, IgE unchanged
Hypereosinophilic syndrome						
Plotz et al. (40) MEPO Dermatitis		Modest reduction in skin T cells (CD4 and CD8) Reduced production IL-4/5/13 by PHA-stimulated PBMC (in 2 out of 3 patients)			Reduced ECP, IL-5, TARC, eotaxin	
Kim et al. (26) RESLI HES—EGID	Unchanged survival *in vitro*, in medium ± IL-5 *Indirectly indicates unchanged IL-5Rα expr.*	Unchanged % of IL-5, IL-3, GM-CSF, IFN-γ-expressing PMA/iono-stimulated T cells			Unchanged IL-2, IL-3, IL-8, IL-15, GM-CSF, IFNγ, TNFy IL-5 decreased 2–3 d post-Tx (3 patients), then increased at 1 mo (5/6 patients)	

(*Continued*)

TABLE 4 | Continued

	Eosinophils	T cells	Mast cells	Remodeling	Serum/mediators	Tissue/mediators
Stein et al. (19) MEPO HES	Increased expression of IL-5Rα (unchanged CCR3) by blood EOS Reduced shape change in response to eotaxin-1,2,3	Increased % IL-5 expressing PMA/iono-stimulated T cells in 8 (CD4) and 7 (CD8) out of 12 patients Unchanged % IL-4/IL-13/IFNγ/TNFα$^+$ T cells			Increased IL-5 in 60% subjects: high MW, precipitated by protein A/G (complexed with immunoglobulin)	Increased production IL-13 by PHA-stimulated PBMC in 13 out of 20 patients (no change in IL-4, IL-5, IFNγ, IL-10, AND GM-CSF)
Rothenberg et al. (42) MEPO HES					Decreased EDN	

[a]*This table summarizes posttreatment findings (active treatment). See* ***Tables 2*** *and* ***3*** *for details on the clinical trials during which these observations were made, except for Otani et al. (59) [this was a follow-up biomarker study performed with biopsy samples obtained in the clinical trial published by Assa'ad et al. (18)].*

btw, between; ccl, conclusion; d, day; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EGID, eosinophilic gastrointestinal disease; EOS, eosinophil; Eot, eotaxin; Eso, esophageal epithelial; FC, flow cytometry; GM-CSF, granulocyte macrophage colony stimulating factor; gp, group; hpf, high-power field; IL-5, interleukin-5; iono, ionomycin; MC, mast cell; MEPO, mepolizumab; MPO, myeloperoxidase; MW, molecular weight; NS, non-significant; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; RESLI, reslizumab; TARC, thymus- and activation-regulated chemokine; TNF, tumor necrosis factor; Tx, treatment; wk, week.

agents are hydroxyurea and interferon-alpha (IFN-α), followed by other cytotoxic or immunosuppressive drugs, and by stem cell transplantation for the most refractory cases. Because of their ability to deplete eosinophils rapidly and specifically, IL-5 pathway-targeting antibodies represent attractive therapeutic options for these disorders. Among the three available antibodies, only mepolizumab has been proven effective in HES in the setting of a randomized placebo-controlled clinical trial. Past and ongoing studies with reslizumab and benralizumab are summarized in **Tables 3** and **5**.

Efficacy of mepolizumab in patients with HES was first tested in two small open-label pilot studies. In the first (40), three patients with OCS-resistant eosinophilic dermatitis and blood eosinophil counts above 1.5 G/L received IV mepolizumab infusions (750 mg), resulting in normalization of blood eosinophilia within 1 day, and rapid improvement of cutaneous manifestations. In contrast to the initial asthma trials, tissue eosinophils were practically undetectable in posttreatment hematoxylin–eosin-stained biopsies (although ECP staining was reduced but not abolished). In the second study (41), 3 monthly mepolizumab infusions were administered to three patients with complex HES, after an initial run-in period during which their maintenance therapy was tapered to a level such that blood eosinophil levels increased at least twofold or rose above 0.75 G/L. Treatment resulted in profound and prolonged eosinophil depletion, symptom improvement, reduced nasal polyp volume, and increased FEV1.

These promising results encouraged the conception of the first randomized double-blind placebo-controlled trial ever conducted in subjects with F/P-negative HES to date (MHE100185) (42). Before randomization, patients had to be clinically stable and have eosinophil counts below 1 G/L with OCS monotherapy (daily prednisone-equivalent dose at least 20 mg, but no more than 60 mg). One week after study-treatment commenced, prednisone was tapered according to a predefined algorithm based on clinical manifestations and blood eosinophilia. Patients in the active treatment arm were significantly more likely to achieve the primary endpoint (prednisone dose 10 mg or less for a period of at least eight consecutive weeks), and the difference with placebo was particularly marked in those who needed more than 30 mg at baseline. Other secondary/exploratory CS-sparing endpoints establishing superiority of mepolizumab over placebo included a significant reduction of the mean daily prednisone dose at the end of the study (mepolizumab 6.2 mg and placebo 21.8 mg), and a higher proportion of patients able to taper off OCS treatment completely until the end of the trial (47 versus 5%). Although blood eosinophilia is much higher in this disease than in asthma, EoE, and CRSwNP (defining criteria >1.5 G/L), mepolizumab-treated patients were more likely to maintain counts below 0.6 G/L than placebo-treated patients, despite the fact that the OCS dose was lower in the former group during treatment. Although effects on HES-related complications were not evaluated in this study (patients were stabilized at baseline), clinical deterioration requiring a major escalation in treatment (i.e., treatment failure) was experienced by 21% of mepolizumab-treated versus 69% placebo-treated subjects, and time to treatment failure was significantly shorter in the placebo

TABLE 5 | Ongoing and planned clinical trials in eosinophilic disorders other than asthma using IL-5 targeted therapy.

Drug	Dosing	Design	Primary endpoint Secondary endpoints	Comments	Clinicaltrials.gov identifier
Eosinophilic esophagitis (pediatric)					
Reslizumab	1–3 mg/kg IV every 4 wks	Open-label	Long-term safety and efficacy	Study completed, not published 112/190 enrolled subjects completed the study; 28/78 withdrawals due to lack of efficacy	NCT00635089
Chronic rhinosinusitis with nasal polyposis					
Mepolizumab	100 mg SC every 4 wks (13 doses)	Phase 3, RDB PC Severe bilateral NP Add-on maintenance therapy	Endoscopic nasal polyp score Nasal obstruction VAS score	6-mo extension study for half of the patients	NCT03085797
Reslizumab	3 mg/kg IV every 4 wks (6 doses)	Phase 3, RDB PC	Change in NP CT score (imaging)		NCT02799446
Benralizumab	Not available Tx period 24 wks	Phase 2, RDB PC	Change in NP score (endoscopy)		NCT02772419 Japan
Hypereosinophilic syndrome					
Mepolizumab MHE104317	Initially 750 mg IV (250 mg vials), variable interval Currently, 100 mg vials; sponsor recommendation: 300 mg IV every 4 wks	1. Compassionate use program for life-threatening HES with documented failure of/intolerance to ≥3 standard Tx 2. Long-term access program for patients who participated in a previous HES study	Support provision of mepolizumab until commercially available for HES	Subjects ≥12 yrs Regular evaluation of risk:benefit ratio to support continued treatment More than 200 patients enrolled	NCT00244686
Mepolizumab MID200622	300 mg SC every 4 weeks (9 doses)	Phase 3, RDB PC History of 2 flares 12 mo before enrollment, EOS ≥ 1 G/L on stable Tx at inclusion	Proportion of patients who experience a flare	Adolescents ≥12 yrs eligible Followed by 20-wk open-label study 205203	NCT02836496
Benralizumab	30 mg SC every 4 wks (total study duration 1 yr)	Phase 2, RDB PC (3 mo) followed by active Tx in all Refractory HES (EOS > 1 G/L despite Tx)	50% reduction blood EOS on stable HES background Tx at wk12	Study completed, results awaited	NCT02130882
Eosinophilic granulomatosis with polyangiitis					
Mepolizumab MEA116841	300 mg SC every 4 wks	Open-label	Systemic CS use Adverse events	Long-term access program for MEA115921 participants who require ≥5 mg PDN	NCT03298061
Benralizumab	30 mg SC, 5 injections over 32 wks	Open-label	Safety Change in OCS dose and exacerbation rate		NCT03010436
Reslizumab	3 mg/kg IV every 4 wks (7 doses)	Open-label	Safety CS-sparing effect		NCT02947945

(O)CS, (oral) corticosteroid; EOS, eosinophil; IL-5, interleukin-5; IV, intravenous; mo, month; NP, nasal polyposis; OCS, oral corticosteroids; PDN, prednisone; RDB PC, randomized double-blind placebo-controlled; SC, subcutaneous; Tx, treatment; VAS, visual analog scale; wk, week; yr, year.

arm. Most (84%) mepolizumab-treated patients completed the trial, whereas only 36% of placebo-treated patients did, the main reason for withdrawal being lack of efficacy.

Patients who participated in the MHE100185 trial were eligible for enrollment in an open-label extension study (MHE100901) designed to assess the long-term safety and optimal dosing interval of 750 mg IV mepolizumab in HES (20). This study included three stages (see **Table 3**) and lasted 5 years. During stage 1, mepolizumab was administered monthly, and background HES therapy was tapered off, or down to the minimal dose required for disease control. During stage 2, mepolizumab infusions were spaced, and administered only when blood eosinophilia (>0.6 G/L) and/or clinical manifestations recurred. More than half the patients were CS-free by week 48, and the proportion off CS remained constant until the end of the study (63%). The median average daily prednisone dose during the entire study was 1.8 mg, and only three patients required addition of other immunosuppressive medications for HES control. The optimal dosing interval between infusions (median 12.8 weeks) was relatively reproducible for each individual over the prolonged observation period, with half of the patients requiring re-treatment after more than 12 weeks. This study was not designed

to evaluate efficacy, but did provide indirect confirmation that mepolizumab benefits patients with HES, since only 6 of the 78 enrolled patients withdrew because of lack of efficacy (persistent blood hypereosinophilia in 1, and HES-related symptoms in 5), and 54 were still receiving treatment when the study ended more than 5 years later. Practically speaking, for a meaningful proportion of CS-dependent patients with long-standing HES, this trial resulted in replacement of daily OCS absorption by a visit to the hospital every 3 months for a 30-min mepolizumab infusion. After study termination, patients were given the opportunity to continue treatment on a compassionate use basis and many are still receiving mepolizumab at time of writing.

The compassionate use program (MHE104317) is also open to subjects aged 12 or more with life-threatening HES and documented failure to at least three standard therapies (e.g., CS, hydroxyurea, IFN-α, and imatinib mesylate). Patient and disease characteristics, exposure to mepolizumab, and safety data are being collected in this cohort (73). Case reports showing efficacy of mepolizumab for severe treatment-refractory idiopathic HES have been published, including one patient with recurrent arterial thrombosis in extremities (74) and another with eosinophilic myocarditis and pericardial effusion (75). In children, toxicity of classical HES therapies is a major concern, contrasting with the favorable safety profile of mepolizumab, explaining that some children with severe HES enter this program as soon as they are 12 (76).

Despite the observed efficacy of mepolizumab in HES, this agent has not yet been approved in this indication. One reason is that clinical efficacy has not yet been formally proven (see A Brief Historical Perspective on the Development of IL-5 Targeted Therapy for Human Diseases). An ongoing clinical trial in HES has derived some useful lessons from asthma studies, choosing exacerbation rate reduction as primary endpoint (**Table 5**), and should provide more insight on how mepolizumab impacts disease manifestations. Although there is some concern that efficacy may be lower than in the previous placebo-controlled trial because of reduced dosing (300 mg SC rather than 750 mg IV) in patients whose eosinophil levels can be very high, data from the compassionate use program suggest that many patients continue to do well when the IV dose is lowered.

Lymphocytic-Variant HES (L-HES)

In L-HES, hypereosinophilia is driven by a clonal population of activated T cells that over-produce IL-5 in vivo (77). In the majority of cases, these cells can be distinguished in peripheral blood on the basis of their abnormal surface phenotype ($CD3^{-}CD4^{+}$) by lymphocyte phenotyping. Patients with L-HES have elevated serum levels of thymus-and- activation-regulated chemokine (TARC), a chemokine that presumably reflects *in vivo* production of Th2 cytokines (78).

Although anti-IL-5 efficacy has not been evaluated specifically in patients with L-HES, a biomarker sub-study was conducted during the MHE100185 trial to identify these patients at baseline, and to compare their treatment response to that of patients with a normal T cell profile (79). Among patients with appropriate testing, 13/63 were classified as L-HES on the basis of T cell phenotyping (predominantly $CD3^{-}CD4^{+}$), and 33/81 had elevated serum TARC levels. In the active-treatment arm (monthly mepolizumab 750 mg infusions), patients with phenotypically abnormal T cells were as likely as those with a normal T cell profile to achieve the CS-sparing endpoints, as were patients with elevated versus normal serum TARC values. However, the ability to maintain eosinophil counts below 0.6 G/L throughout the trial was significantly lower in patients with T cell-driven HES: (1) 71% patients with an abnormal phenotype, versus 100% patients with a normal phenotype and (2) 73% patients with serum TARC > 1,000 pg/mL versus 100% patients with TARC. During the MHE100901 open-label study that immediately followed this trial, treatment response was compared between these patient sub-populations as well (personal observation). No statistically significant differences were observed in terms of long-term CS-sparing. However, the interval between mepolizumab infusions (750 mg) in patients with $CD3^{-}CD4^{+}$ L-HES was roughly half that of subjects with phenotypically normal T cells, and mean eosinophil levels 4 weeks after each infusion during stage 2 were significantly higher, although still within normal limits. It remains unclear whether these subtle differences in treatment response between patients with L-HES and non-T cell driven HES have clinically relevant consequences, as neither trial was designed to explore the efficacy of mepolizumab on disease manifestations. It is noteworthy that a complete clinical and biological (eosinophils <0.5 G/L) response to mepolizumab (dosing not detailed) was observed in four of five patients with L-HES in the largest L-HES cohort published to date (80).

Altogether, these observations suggest that in some patients with T cell-driven HES, higher and/or more frequent dosing of anti-IL-5 may be required to neutralize the large amounts of IL-5 produced *in vivo*. Patients with L-HES will certainly be enrolled in the ongoing clinical trial evaluating mepolizumab in HES, and their response to the 300 mg SC dosing regimen will be evaluated and compared with that of patients with idiopathic HES in the setting of a biomarker sub-study. Notwithstanding, provided dosing is sufficient, anti-IL-5 treatment does allow for CS tapering in many L-HES patients and represents an extremely well tolerated alternative to the high-dose CS maintenance treatment they often require. Until T cell targeted treatment directed against pathogenic cells has been developed for this HES variant, anti-IL-5 antibodies fulfill a strong unmet need.

EGPA (Formerly Churg–Strauss Syndrome)

Eosinophilic granulomatosis with polyangiitis is a complex disorder combining peripheral blood hypereosinophilia, severe asthma, eosinophil-rich and granulomatous inflammation in lungs and other organs, and small/medium-vessel necrotizing vasculitis (81, 82). Classically, disease develops in three consecutive stages: (1) progressive adult-onset asthma, often associated with chronic rhinosinusitis, (2) peripheral blood hypereosinophilia with eosinophilic infiltrates in lungs and possibly other organs, and (3) vasculitis. The disease is heterogeneous, with underlying pathogenic mechanisms that presumably differ in patient subgroups. Patients with positive ANCA serology for example are more likely to develop purpura, glomerulonephritis, pulmonary hemorrhage, and mononeuritis multiplex than ANCA-negative subjects. An operational approach to diagnosis requires presence of asthma, sinusitis and/or rhinitis, pathological confirmation of

vasculitis or clinical surrogates highly evocative of vasculitis in at least two organs, and blood eosinophilia above 1.5 G/L. The vasculitic component often responds durably to immunosuppressive drugs such as cyclophosphamide, rituximab, and azathioprine, but the majority of patients remain OCS-dependent because of asthma exacerbations and chronic rhinosinusitis. Methotrexate or azathioprine may be required as maintenance therapy for CS-sparing purposes.

Although it is unclear whether eosinophils contribute directly to the vasculitic features of EGPA, they do infiltrate lungs and account for dyspnea in stage 2 disease. At this stage, ANCA-negative EGPA is often indistinguishable from chronic eosinophilic pneumonia (single-organ HES) or idiopathic HES (83, 84) (when organs other than lungs are affected as well). Because HES patients with lung involvement responded well to mepolizumab in the MHE100185 trial, and because anti-IL-5 is efficacious in severe eosinophilic asthma (which is a key feature of EGPA), it is not surprising that IL-5-targeted treatment has been tested in EGPA.

The first attempt to treat EGPA with mepolizumab was published in 2010 as a case report (85). A patient with multiorgan ANCA-negative EGPA resistant to high-dose prednisolone and various immunosuppressive and cytotoxic agents experienced a clear-cut response to monthly mepolizumab infusions (750 mg) with regression of blood eosinophilia, lung infiltrates and asthma, and normalization of pulmonary function tests. An attempt to increase the interval between doses led to an exacerbation, which was controlled when monthly infusions were resumed.

This observation was closely followed by two pilot single-center open-label studies in the United States (69) and in Germany (70). The first study enrolled OCS-dependent patients whose disease was controlled with stable background therapy at inclusion. During the active treatment period, 4 monthly mepolizumab infusions (750 mg) were administered, and the mean dose of prednisone required to maintain disease control was significantly lowered, from 12.9 mg at baseline to 4.6 mg 4 weeks after the fourth dose. The CS-sparing effect was prolonged an additional 2 months, but the dose had to be increased thereafter. The exacerbation rate was significantly lower during the active treatment phase compared with the washout and follow-up periods. The second study included patients with more severe EGPA, whose disease was active at baseline (BVAS 3 or more) despite more potent background therapy (daily OCS dose at least 12.5 mg combined with an immunosuppressive agent). Besides lung and sinus involvement, other organs were affected in most subjects (heart, digestive tract, and peripheral nervous system). Nine monthly mepolizumab infusions (750 mg) were administered after tapering off immunosuppressant(s), and 8/10 patients experienced a clinical remission (BVAS 0) with a prednisone dose below 7.5 mg. A significant reduction of the median prednisone dose was observed (19 mg at baseline versus 4 mg on the day of the ninth infusion), and no disease exacerbations were observed during treatment. Thus, during 9 months, disease activity was abrogated despite cessation of maintenance immunosuppressive therapy and decreased prednisone dosing in the majority of these patients with severe EGPA.

These very encouraging pilot studies led the European Commission to grant orphan designation to mepolizumab for the treatment of EGPA in 2013, and a large-scale placebo-controlled clinical trial was designed to evaluate efficacy in patients with relapsing or refractory EGPA requiring daily prednisone intake (between 7.5 and 50 mg) with or without concomitant immunosuppressive agent(s) to stabilize disease (MEA115921) (64). Treatment (mepolizumab 300 mg or placebo SC) was administered every 4 weeks for 52 weeks, and investigators were blinded to eosinophil counts to guarantee double blinding. The two primary efficacy endpoints related to remission, which was defined as a BVAS of 0 with a PDN dose of 4 mg or less. There was a statistically significant difference in favor of mepolizumab-treated patients, who were more likely to experience an accrued remission of 24 weeks or more (28 versus 3%), and to be in remission at weeks 36 and 48 (32 versus 3%). Like in asthma, treatment benefit on the relapse rate was significant only in patients with blood eosinophil counts above 0.15 G/L at inclusion. There was a twofold lower relapse rate in the mepolizumab group (1.14 versus 2.27), although a higher proportion of these patients had tapered the prednisone dose to 4 mg or less at the end of the trial (44 versus 7% in the placebo arm), and some even completely discontinued OCS treatment during the trial (18 versus 3%). Globally, IL-5 targeted therapy maintained disease control despite OCS tapering in roughly half of subjects with EGPA. A supplemental Biologics License Application seeking approval for mepolizumab as add-on therapy to OCS for EGPA has been submitted to the FDA.

Compared with patients with HES enrolled in the MHE100185 trial, the response rate in EGPA is disappointing. This may be due to the reduced dosing, and/or key involvement of additional IL-5-independent pathogenic mechanisms in this complex disease. The dose chosen for the EGPA trial (300 mg SC), although higher than for eosinophilic asthma (100 mg SC), was one-third of that used for HES (750 mg IV). Patients with EGPA often have markedly increased blood eosinophilia, similar to patients with HES. Serum TARC levels are often elevated, and CRTH2-postive cells have been detected in nasal tissue, suggesting pathogenic involvement of Th2 cells. Furthermore, the first patient with EGPA who responded to monthly high-dose mepolizumab experienced a relapse when the dosing interval was increased. It is therefore conceivable that, like for L-HES, certain patients with EGPA may require higher dosing to neutralize higher endogenous IL-5 production. The contribution of pathogenic complexity to variable treatment responses in EGPA is difficult to assess on the basis of data collected during clinical trials. Neither histological findings before inclusion showing vasculitis nor ANCA status were reported in the two pilot studies. In the placebo-controlled trial, baseline characteristics indicate that both patients with vasculitic disease (ANCA-positivity, alveolar hemorrhage, palpable purpura) and patients with stage 2 EGPA (asthma, blood eosinophilia at least 1 G/L, pulmonary infiltrates, sinonasal abnormalities) were enrolled, but the small size of clinical sub-groups precluded statistical comparisons in response rates.

Likewise, no conclusions can be formally drawn regarding the effects of mepolizumab on the different components of EGPA:

asthma, sinonasal disease, and vasculitis. In the recent placebo-controlled trial, benefit of active treatment was slightly greater on relapses that were defined as worsening asthma or rhinosinusitis, although relapses considered as both vasculitic and asthma/sinonasal were also reduced. In the pilot study conducted by the German group, there were no flares during the active treatment phase although these patients had been tapered off immunosuppressive therapy and OCS dosing was reduced, whereas after treatment cessation, six out of nine patients with extended follow-up relapsed, including two patients who developed progressive neuropathy and alveolar hemorrhage respectively (71), suggesting that vasculitic manifestations of EGPA may have been controlled during treatment with anti-IL-5. Alternatively, these findings may reflect natural disease course, with protracted relapses occurring in patients who had been tapered off azathioprine for the purpose of the clinical trial.

The ongoing NIH-funded biomarker sub-study conducted on biological material obtained from a subset of patients enrolled in MEA115921 may identify disease characteristics, subsets, and biomarkers that are predictive for treatment response and disease exacerbations.

ANTI-IL-5 TREATMENT IN OTHER INFLAMMATORY DISORDERS ASSOCIATED WITH BUT NOT EXCLUSIVELY DRIVEN BY HYPEREOSINOPHILIA

Several complex inflammatory diseases associated with symptomatic hypereosinophilia have been shown to benefit from treatment with mepolizumab, the only anti-IL-5 antibody available for such indications through the compassionate use program, or insurance company approval for use of commercialized 100 mg vials.

Chronic Inflammatory Disorders with Hypereosinophilia

In some chronic inflammatory and/or indolent hematological diseases with accompanying eosinophilia, (partial) symptomatic improvement may be obtained by targeting eosinophils, even if the underlying condition does not warrant (or respond to) specific therapy. Given the excellent safety profile of mepolizumab, this holds especially true if the toxicity of disease-modifying treatment exceeds the anticipated benefit.

Elderly patients with bullous pemphigoid, for example, may not tolerate immunosuppressive therapy at doses required to stabilize disease. Eosinophils are present in skin lesions and are often increased in peripheral blood, and recent studies indicate that they contribute to pathogenesis (86). Targeting eosinophils may therefore represent a future therapeutic alternative for this disease (87). A 3-month phase 2 placebo-controlled trial evaluating the efficacy of monthly infusions of mepolizumab (750 mg) in adult patients with active bullous pemphigoid has recently been completed and results should be available shortly. Drug reaction with eosinophilia and systemic symptoms (DRESS), a potentially life-threatening eosinophil-mediated inflammatory disorder triggered by an inappropriate immune response to certain therapeutic agents [see Musette and Janela in this topic (88)], may also be an interesting indication for short-term anti-IL-5 treatment in patients with particularly CS-refractory or recurrent manifestations (89).

Eosinophils may also contribute to symptoms and complications in certain indolent hematological disorders, such as mastocytosis and cutaneous T cell lymphoma, that need not necessarily be treated aggressively. One patient with unrecognized cutaneous mastocytosis associated with hypereosinophilia responded to prolonged treatment with mepolizumab (750 mg IV), before the correct diagnosis was made more than 10 years after initial presentation (90). Eosinophil counts normalized and she experienced symptomatic improvement (pruritus, erythematous eruptions, and chronic cough) with repeated mepolizumab infusions. It remains unclear whether symptoms were due to eosinophils themselves, or to mast cells that may have been directly (if they expressed the IL-5R) or indirectly [abolished production of an eosinophil-derived mediator involved in mast cell growth or activation, like IL-9 (91)] targeted by treatment. Although anti-IL-5(R) may indeed offer some relief in chronic hematological conditions like this, it should be kept in mind that the role played by eosinophils on natural disease course remains elusive, and that eosinophil depletion may jeopardize negative regulatory pathways acting on clonal cells.

Combined Therapy with Other Monoclonal Antibodies

Mepolizumab has successfully been administered concomitantly with other monoclonal antibodies to treat complex immune-mediated diseases driven by more than one cell type and/or mediator.

Mepolizumab improved disease course in a patient with atypical hemolytic uremic syndrome (aHUS), who responded poorly to eculizumab (anti-C5a) alone (92). Monthly mepolizumab injections were initiated because of associated blood and tissue (colon) eosinophilia, resulting in normalization of eosinophil and platelet counts, increased ADAMTS13 activity, and regression of digestive and neurological manifestations. This observation suggests that hypereosinophilia and aHUS may enhance one another, with C3a and C5a enhancing eosinophil activation, and conversely, eosinophil-induced endothelial damage exacerbating thrombotic microangiopathy. The authors conclude that complement dyscrasias with an eosinophilic component may benefit from anti-IL-5 therapy.

Monthly administration of mepolizumab significantly improved disease course in a wheelchair-bound CS- and oxygen-dependent patient with severe allergic bronchopulmonary aspergillosis who responded only partially to omalizumab (93). She was able to discontinue OCS and oxygen, and to resume activities of daily living for the first time in years after addition of mepolizumab. These interesting case reports provide insight on new pathogenic roles played by eosinophils in complex disorders.

A Word of Caution

Although the often dramatic effect on eosinophilia and excellent safety profile of anti-IL-5 treatment understandably rouse enthusiasm, the priority should be given to compounds that specifically target disease-inducing defects when available, or to other less expensive options if their efficacy and toxicity are satisfactory. For example, lymphomatoid papulosis associated with symptomatic hypereosinophilia was shown to (transiently and partially) respond to mepolizumab in one patient (94). However, this condition may be observed in subjects with F/P^+ CEL, and should be treated with imatinib mesylate when this is the case. Another group reported a young woman with ulcerative colitis and marked blood hypereosinophilia who responded to combined mepolizumab and infliximab (95). She had initially failed to respond clinically to mepolizumab, although blood eosinophilia regressed. Repeat biopsies showed active colitis with cryptitis, infliximab was administered, and together, these monoclonals resulted in clinical and biological remission. However, no attempt to discontinue mepolizumab was reported, to determine whether infliximab alone would have sufficed to control disease and resolve hypereosinophilia.

It is also important to judge whether eosinophils are indeed contributing to organ damage or dysfunction at all (i.e., "is there a hypereosinophilic *syndrome*"?), and not to administer eosinophil-targeted therapy if this is not the case. For example, mepolizumab was administered to a subject with blood and (sub) cutaneous eosinophilia in the setting of angiolymphoid hyperplasia with eosinophilia (ALHE, or epitheloid hemangioma) (96). Hypereosinophilia and local pruritus disappeared, but the subcutaneous nodule regressed only slightly. Pathogenesis of ALHE is not well delineated, but many cell types are involved, and it is unlikely that eosinophils represent the predominant driving force.

In summary, treatment with anti-IL-5 antibodies is expensive and justified only when other therapies fail and/or have a negative impact on health or quality of life, and there is reasonable evidence that the role played by eosinophils in organ damage or dysfunction is significant. Isolated reports showing efficacy in conditions for which other safe treatment options exist should not encourage physicians to squander health care resources and resort to anti-IL-5 treatment whenever eosinophils are present.

PREDICTORS OF RESPONSE/ RESISTANCE TO IL-5 TARGETED THERAPY

Little is known about disease characteristics that are predictive of a response to anti-IL-5 treatment in patients with hypereosinophilic conditions.

Clinical Presentation

Clinical trials evaluating anti-IL-5 in patients with HES are few and have given no clear indication on specific disease manifestations whose presence may predict treatment response. In the MHE100185 trial, patients with active cutaneous involvement at enrollment had a slightly lower response rate to mepolizumab (69%) than those with active respiratory, gastrointestinal, or cardiac manifestations (90–100%) (42). However, this finding may reflect disease severity, as 72% of patients requiring more than 30 mg prednisone at baseline had active cutaneous involvement (versus only 37% of those requiring 30 mg or less). In the same trial, patients with L-HES receiving active treatment were less likely to maintain eosinophil counts below 0.6 G/L than patients with normal T cells (79) and required more frequent dosing in the follow-up dosing-interval study MHE100901. Their requirement for higher dosing is likely related to higher endogenous production of IL-5 by the dysregulated T cells that drive disease.

The type of eosinophil-mediated complications more likely to regress with treatment have not been studied either, as clinical outcome has not been a major endpoint so far. In L-HES, part of the clinical manifestations may actually be related to T cell overproduced cytokines other than IL-5, such as IL-2, IL-4, IL-13, and TNF-α.

Eosinophil Counts

Patients with eosinophilic asthma who were most likely to respond to IL-5 targeted treatment in the placebo-controlled trials that led to FDA approval were those with higher baseline blood eosinophil counts. For mepolizumab, a relationship was observed between exacerbation rate reduction and baseline eosinophilia (97), and for reslizumab and benralizumab, clinical and functional responses were observed in patients with baseline eosinophilia at or above 0.4 and 0.3 G/L, respectively (13). Likewise, in the EGPA mepolizumab-versus-placebo trial, a reduction in the exacerbation rate was observed only in patients whose baseline eosinophilia was 0.15 G/L or more (64).

In patients with other HES, the relationship between eosinophil counts before treatment and treatment response has not been evaluated, because in most studies, baseline eosinophilia was controlled with maintenance OCS therapy. In one open-label study where maintenance therapy was tapered down so that eosinophil counts were increasing at the time mepolizumab was initiated, the extent of the decrease in eosinophil counts in response to treatment was most marked in the patient with the highest baseline eosinophilia (>1.5 G/L) (19).

Serum Biomarkers of Eosinophil Activation

Eosinophil expression of membrane IL-5Rα may decrease, and soluble IL-5 receptor (sIL-5Rα) levels may increase, in tissue and body fluids from patients with eosinophilic inflammation, as a result of alternative splicing and/or shedding (36, 50). Incubation of eosinophils *in vitro* in presence of IL-5, IL-3, and GM-CSF results in decreased IL-5Rα expression, and a correlation has been shown between serum IL-5 and sIL-5Rα levels in subjects with HES (36). It has been hypothesized that sIL-5Rα may bind IL-5 and trap anti-IL-5 antibodies; it may also be recognized by the anti-IL-5R antibody benralizumab and prevent it from binding to target cells. The impact of increased pretreatment serum sIL-5Rα levels on efficacy of anti-IL-5 treatment has only been assessed in one study evaluating reslizumab in CRSwNP, showing no relationship (50).

IL-5 Production

Both eosinophils and T cells have the capacity to produce IL-5 and release it in serum. Baseline serum IL-5 levels were measured in patients with HES that participated in several trials with anti-IL-5, and no correlation with treatment response was observed (25, 41, 42). Background therapy at baseline probably lowered the serum IL-5 level, making it impossible to determine the utility of this marker. Moreover, IL-5 levels in serum may reflect imperfectly the degree of IL-5 production at sites of inflammation and in lymphoid tissue. IL-5 was measured in nasal secretions in mepolizumab-treated subjects with nasal polyposis to address this issue, initially demonstrating that levels above 40 pg/mL were predictive of a clinical response (50), although this was not confirmed in a subsequent larger-scale study conducted by the same group (51). Demonstration of local IL-5 expression in biopsies from eosinophil-rich tissues would represent more convincing evidence of the role played by IL-5 in inflammation. Very few biopsy studies have been conducted in the setting of anti-IL-5 clinical trials for hypereosinophilic conditions. The ongoing NIH-funded biomarker sub-study on patients who participated in the mepolizumab/EGPA trial may provide insight on the association between local Th2 inflammation and a specific treatment response profile.

Another means of quantifying the Th2 response *ex vivo* is to investigate cytokine production by PBMC. This was undertaken by one group who evaluated the efficacy of open-label mepolizumab in subjects with HES (19). Interestingly, the single patient who did not respond to treatment was the one whose PBMC secreted the highest amounts of IL-13 in culture supernatants, suggesting that patients with marked *in vivo* Th2 activation may not respond as well to treatment, like patients with L-HES. Evaluation of the cytokine profile of *in vitro*-stimulated PBMC as a means of detecting Th2-driven disease in patients with HES is not routine or standardized, however, and requires expertise.

Serum Biomarkers of Th2-Driven Disease

Measurement of TARC in serum may be a more reliable means of detecting Th2-driven inflammation in tissues, where TARC is produced by resident cells. Elevated levels have been observed in subjects with T cell lymphoma, and atopic dermatitis amongst others. In the MHE100185 trial, HES patients with serum TARC levels above 1,000 pg/mL were less likely to maintain blood eosinophil counts below 0.6 G/L than those with low TARC levels, among those receiving active treatment (78). So far, this is the only standardized biomarker shown to be associated with a suboptimal treatment response. In this era of precision medicine, measurement of serum TARC may help identify patients less likely to respond to anti-IL-5, and/or more likely to require higher or more frequent dosing. The predictive value of baseline and peak serum TARC levels for response to mepolizumab will be evaluated prospectively in a biomarker sub-study within the ongoing MID200622 clinical trial.

Like TARC, eotaxins could potentially reflect local Th2 inflammation. In one study, plasma eotaxin-3 levels were not predictive of a response to mepolizumab (19).

INDIRECT EFFECTS OF IL-5 TARGETING ON CELL TYPES OTHER THAN EOSINOPHILS

Besides eosinophils, cell types expressing the IL-5R in humans and thereby predictably targeted by anti-IL-5(R) treatment include basophils, and certain mast cell subsets. In addition, eosinophils entertain numerous interactions with their environment (98), and it is legitimate to explore indirect effects of IL-5-targeted treatment, resulting from eosinophil depletion, on other cell types *in vivo*. Overall, no changes in blood leukocyte counts (besides eosinophils) have been observed in the numerous trials with anti-IL-5 for asthma and eosinophilic disorders. Pre- versus posttreatment lymphocyte sub-populations were enumerated in mepolizumab-treated subjects, showing no significant changes in blood CD3, CD4, CD8, CD19 (B cell), CD16/56 (NK cell), or γ/δ cell counts (19, 40, 99). Benralizumab depletes basophils in addition to eosinophils, and the mild and transient decrease in other white blood cells (lymphocytes, monocytes, and neutrophils) initially observed with rapid intravenous administration of this compound (28) has not been reported subsequently with subcutaneous dosing.

Effects of IL-5-targeted treatment on numbers and functional properties of several relevant cell types and on inflammatory mediators in patients with hypereosinophilic conditions other than asthma are detailed in **Table 4**.

SAFETY OF THERAPEUTIC ANTIBODIES TARGETING IL-5 AND ITS RECEPTOR

Adverse Events (AE) in Clinical Trials

The most obvious means of evaluating safety is to compare AE recorded in patients receiving active treatment to patients receiving placebo in the setting of clinical trials. The safety data from severe eosinophilic asthma trials, and in less common diseases such as EoE, HES and EGPA, has been reviewed in detail and will not be developed herein (44, 100–102). No major concerns have been raised so far, after more than 10 years of data collection with anti-IL-5; the occurrence of AE has been overall similar to placebo, both in number and nature (most commonly headache, nasopharyngitis, and upper respiratory tract infection). Some of the reported AE are related to tapering of background treatment (20) (e.g., symptoms/signs of adrenal insufficiency, unmasking of unrelated CS-responsive inflammatory conditions such as rheumatoid arthritis), and/or specific disease components responding less well to eosinophil-targeted therapy (e.g., persistent airway hyperreactivity in patients with severe asthma). None of the clinical trials conducted with mepolizumab or reslizumab have raised any major concerns with regard to anaphylaxis, or infusion/injection site reactions. The acute infusion-related AEs that were initially observed with intravenous benralizumab were easily resolved by increasing the duration of the infusion (28) and have no longer been an issue with subcutaneous dosing. Although benralizumab induces eosinophil cell death extremely rapidly, there is no evidence that degranulation products are released massively at treatment initiation. Indeed, serum levels

of eosinophil-derived granule proteins actually decrease early after treatment initiation in asthmatic patients (31). No specific clinical AE have repeatedly been considered by investigators as drug-related with any of these antibodies.

The limitation of clinical trials for obtaining information on safety is their short duration, with the exception of the MHE100901 study during which no major safety concerns were raised in more than 50 patients receiving mepolizumab for HES, despite the fact that mean exposure in those receiving more than one infusion was almost 5 years (20). Cough, fatigue, headache, upper respiratory tract infection, and sinusitis were the most common AE in roughly one-third of subjects each. Patients who participated in the pivotal placebo-controlled trials evaluating efficacy of mepolizumab in asthma (101) and EGPA were given the opportunity to receive open-label treatment in the setting of long-term access programs that have not raised major safety concerns although safety data collection has spanned prolonged periods.

Consequences of Prolonged Eosinophil Depletion

Now that anti-IL-5 antibodies have been commercialized and widespread use in patients with severe asthma is anticipated, phase 4 safety data may provide some insight on effects of prolonged eosinophil depletion on human biology in terms of host defense against infections, cancer, and possibly other immune and non-immune mechanisms. Indeed, besides causing damage in inflammatory disorders, eosinophils contribute to protective immune responses directed against selected parasites, and there is accumulating evidence that they are involved in defense against viral, bacterial, and fungal pathogens (5). Experimental findings indicate a possible role in anti-tumor immunity, although their increased presence in cancer does not necessarily correlate with favorable outcome. Eosinophils also contribute to maintenance of a healthy immune response (e.g., crucial for the survival of long-lived plasma cells), and it is believed they represent critical regulators of local immunity and remodeling/repair in both health and disease (103). Their presence in various tissues in healthy subjects [i.e., homeostatic eosinophils (9)] is increasingly being explored at the functional level, and experimental evidence indicates that they regulate an array of biological functions including control of glucose homeostasis, protection against obesity, regulation of mammary gland development, and preparation of the uterus for pregnancy. It is therefore logical to question whether prolonged therapeutic eosinophil depletion may impact some of these processes.

This concern was addressed by a group of experts in eosinophil biology, who reviewed clinical and experimental observations made in humans (case reports on eosinopenic subjects) and murine strains devoid of eosinophils (104). Eosinopenia was not associated with increased susceptibility to infections, cancer or any other major abnormalities in homeostasis impacting global health. They concluded that, although eosinophils do contribute to many immune and physiological processes, they appear to be dispensable, thanks to the existence of other eosinophil-independent pathways and are not critical for maintenance of homeostasis in mammals.

Safety data from clinical trials is in agreement with this interesting review, namely, with regard to susceptibility to infections. The rare occurrence of herpes zoster infections that were considered serious in patients receiving mepolizumab (23) (open-label extension studies) or benralizumab (105) may eventually justify vaccination before treatment initiation. Helminthic infections are uncommon in industrialized countries in the Northern hemisphere where anti-IL-5(R) antibodies have been administered so far. Patients who travel regularly or who reside in countries where helminths are endemic should be followed carefully now that anti-IL-5 treatment is widely available for severe asthma. There is no data suggesting increased susceptibility to opportunistic infections.

Regarding tumor surveillance, the majority of neoplasms that have developed during clinical trials with anti-IL-5 are those that are most common in the general population (100) (e.g., basal cell carcinoma, prostate cancer, and squamous cell carcinoma). Their incidence appears not to be meaningfully higher than expected on the basis of the SEER database, taking into account the close observation of patients during clinical trials. The only exception is T cell lymphoma. Indeed, the incidence of lymphoma in mepolizumab-treated patients with HES was higher than expected in a control population (20). However, it is well known that HES may precede diagnosis of T cell lymphoma, either because occult lymphoma is not detected at the time a patient presents with paraneoplastic hypereosinophilia, or because the eosinophil-driving disease itself progresses to T cell lymphoma (e.g., L-HES). Thus, occurrence of lymphoma in an anti-IL-5-treated patient with HES is more likely to reflect progression of underlying disease (or its unmasking, following reduction of background treatment) than a treatment-effect on clonal T cells. This is supported both by the fact that the two patients who developed T cell lymphoma in the MHE100901 study had markedly elevated serum TARC levels (a marker of underlying T cell disease) before initiation of treatment with mepolizumab (20), and by the fact that lymphoma has not occurred in the many anti-IL-5(R)-treated patients with asthma, a disease that has no inherent relationship with the development of this malignancy (100).

As for homeostatic functions, it appears that eosinophils in certain compartments are less dependent on IL-5 for their persistence than inflammatory eosinophils. For example, homeostatic lung eosinophils that have been shown to suppress Th2-driven allergic airway responses do not express the IL-5R (9). Likewise, resident duodenal eosinophils do not consistently express IL-5Rα, and they are not affected in mepolizumab-treated patients with EoE (106), which together with unchanged mast cells and T cells, suggest preserved local host defense.

Finally, it should be noted that the effects of anti-IL-5 antibodies on eosinophil depletion in tissues is partial (often roughly 50%), so eosinophils likely continue to play their physiological roles. The more pronounced effect of benralizumab on tissue eosinophils may affect certain functions more profoundly. Long-term safety data is being collected in patients with severe asthma.

Effects of IL-5 Targeting on Eosinophil Activation

Treatment with antibodies against soluble ligands is known to potentially impact receptor-bearing cells at a functional level. For example, omalizumab decreases the expression level of the IgE receptor by mast cells, and this is believed to contribute to clinical efficacy. IL-5 is known to prime eosinophils *in vivo* for trafficking and activation in response to other inflammatory mediators, so neutralizing this cytokine may render the eosinophils that remain less reactive to other stimulatory pathways and less prone to perpetuate inflammation. One study has shown that eosinophils isolated from blood of mepolizumab-treated patients were indeed less responsive *in vitro* to eotaxins in terms of eosinophil shape change compared with pretreatment eosinophils (19) (**Table 4**). However, another group recently showed that in mepolizumab-treated asthmatic subjects subjected to allergen challenge, airway eosinophils express an activated phenotype similar to that observed before treatment, suggesting that this eosinophil subset does conserve its functional properties in these conditions despite anti-IL-5 treatment (107).

Increased membrane expression of the IL-5α receptor chain by eosinophils has been observed after treatment with anti-IL-5 (19), raising concern about a potentially enhanced response to endogenous IL-5 after treatment cessation, with rebound hypereosinophilia (i.e., posttreatment eosinophilia higher than baseline level), and/or eosinophil degranulation. Rebound eosinophilia and disease manifestations have not been observed with mepolizumab, despite the fact that it has been evaluated extensively in asthma and several other eosinophilic conditions, including studies with prolonged follow-up (48, 51). By contrast, rebound hypereosinophilia and severe symptom exacerbations occurred between 60 and 90 days after treatment in six of six patients with HES or eosinophilic gastroenteritis whose eosinophil levels initially decreased in response to a single dose of 1 mg/kg reslizumab (26). A similar phenomenon was observed in asthma and nasal polyposis trials, but was clearly related to lower dosing regimens (50, 108). It has been suggested that at low molar antibody/cytokine ratios, biologically active cytokines may be released from their complexed form and/or may retain their capacity to bind to and activate their receptor (109, 110). This phenomenon may contribute to rebound eosinophilia and/or disease worsening with anti-IL-5 treatment. In this line, rebound hypereosinophilia was observed only in HES patients treated with reslizumab (1 mg/kg), who received a substantially lower dose of antibody than those treated with mepolizumab (10 mg/kg). With the currently approved dose of reslizumab (3 mg/kg), no major concerns about rebound eosinophilia have been raised in diseases with low baseline eosinophil counts, such as asthma or EoE.

Potential Consequences of Reduced Dosing Regimens

Over time, the dosing regimen of mepolizumab has progressively decreased in clinical trials, passing from 750 mg IV in the first asthma and HES studies, to 100 mg SC in asthma and 300 mg SC in HES. The consequences of administering low-dose anti-IL-5 treatment are an increasing subject of concern (111, 112), for several reasons besides potential rebound hypereosinophilia. These include reduced efficacy, and the possible development of an immune complex-mediated inflammatory response in the setting of excess antigen. Indeed, as the antibody:antigen ratio decreases, there is an increased likelihood that both Fab sites of each antibody will be occupied, leading to the formation of immune complexes that are more likely to precipitate and activate complement (113). One group has recently reported the case of a prednisone-dependent asthmatic patient whose airway disease worsened dramatically while treated with mepolizumab 100 mg daily (114). The authors hypothesized that local immune complex deposition, activation of complement, and recruitment of immune cells may have caused uncontrolled inflammation. Increased sputum IL-5 levels (bound to antibody) and eosinophilia were observed as lung function deteriorated, suggesting that mepolizumab prolonged the half-life of biologically active IL-5, resulting in maturation of eosinophil progenitors in the airways. This observation further underscores the likelihood that OCS-dependent asthmatic subjects have a more severe disease (115), with higher needs in terms of anti-IL-5 dosing, to reach the airways and effectively neutralize the target cytokine locally in addition to the intravascular compartment (112).

FUTURE PERSPECTIVES AND CLOSING REMARKS

Specific targeting of eosinophils has become possible and is now implemented in daily clinical practice, with antibodies directed against IL-5 and its receptor. For eosinophil-driven chronic inflammatory conditions, targeting IL-5-dependent pathways enables a much-needed shift from global immunosuppression to precision medicine, with improved safety and tolerance. How this translates into clinical improvement of complex diseases depends on how central eosinophils are in pathogenesis. For several HES variants, the benefit has been outstanding, whereas for diseases driven by several mediators and cell types with various phenotypes and endotypes, such as severe asthma and EoE, IL-5 targeting has variable efficacy. Improved understanding of pathogenic disease mechanisms on one hand, and more profound eosinophil depletion in tissues in ongoing and future clinical trials with benralizumab on the other hand, will help gain insight on the extent and nature of eosinophil involvement in various complex inflammatory states and determine which disorders will benefit most from treatment with anti-IL-5(R).

With increasing use of these antibodies for hypereosinophilic conditions in clinical practice, optimal dosing regimens should be the focus of future attention. Indeed, dosing should be tailored to patient needs in a more personalized approach to treatment than that implemented in clinical trials, taking into account both efficacy and tolerance. Ideally, similar to other immune-modulatory agents, the minimal clinically efficacious dose should be tailored to each patient/condition, as the potential negative impact of long-term eosinophil depletion on the quality of global immune responses remains unknown. The amount

of antibody administered at once, and/or the interval between doses may be titrated to this end. The likelihood that sustained IL-5 targeting eventually reverses natural disease course is low, as confirmed in asthmatic patients whose eosinophilia and symptoms recurred after discontinuation of mepolizumab (116) and in most patients with EGPA who were shifted to maintenance treatment with methotrexate after 9 monthly infusions (71). In patients with idiopathic HES, disease may exceptionally regress over time, either spontaneously, or as the result of prior immunosuppressive/cytotoxic treatment, which may explain that prolonged remission after cessation of anti-IL-5 has been reported in a few cases (20, 40, 71). It may be worthwhile to carefully taper selected patients off anti-IL-5 treatment in the future, to explore this possibility. On the other hand, patients in whom endogenous IL-5 production is particularly intense (e.g., L-HES and subsets of patients with idiopathic HES and EGPA) should be offered the possibility of increasing the dose before concluding that treatment is ineffective or not justified economically. This concept is implemented in omalizumab-treated asthmatic patients whose dose is adapted to serum IgE levels. Unfortunately, there are as of yet no validated biomarkers reflecting the level of endogenous IL-5 production that could be used to guide the choice of dosing of anti-IL-5 in patients with marked hypereosinophilia, although serum TARC levels appear promising in this regard (79). Changes in approved dosing regimens should be evaluated in the setting of carefully monitored clinical studies. Post-marketing authorization data is awaited for mepolizumab in the phase 4 "multinational single-arm observational study to evaluate the real-world effectiveness and pattern of use of mepolizumab in patients with severe eosinophilic asthma" (100). Clinicians and patients will likely be tempted to increase between-dose intervals when disease is stabilized, and such modifications require close follow-up as they may result in disease worsening, due to decreases in the antibody:antigen ratio and/or release of biologically active IL-5.

Special attention should also be paid to the consequences of tapering background therapy in patients with chronic eosinophilic disorders whose condition improves significantly after initiation of treatment with anti-IL-5. Indeed, variable degrees of adrenal insufficiency develop over time after prolonged systemic and even topical CS use (117), and its under-estimation may result in serious complications during CS tapering. In a recent open-label extension study with mepolizumab in asthmatic patients, the maintenance dose of OCS was higher when tapering was left to the clinicians' discretion, than when done in the setting of placebo-controlled clinical trial constraints (i.e., following a predefined algorithm to reach the lowest possible dose), indicating that clinicians naturally take the risks and discomforts associated with OCS withdrawal into account (101). Furthermore, a subset of asthmatic patients successfully treated with low-dose (100 mg) anti-IL-5 and tapered off OCS may progress to EGPA, as previously reported for other agents like leukotriene antagonists and omalizumab (115). All of these eventualities mandate close monitoring.

Finally, with the increasing availability of compounds targeting other mediators and cells involved in chronic inflammatory diseases associated with eosinophilia, optimal treatment strategies may involve combination therapy. Identification and increased availability of reliable biomarkers predicting treatment response will help design efficacious and minimally toxic tailored treatment regimens for patients with these complex disorders in great need of well-tolerated therapeutic options.

AUTHOR CONTRIBUTIONS

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REVIEW
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Anti-IgE Treatment for Disorders Other Than Asthma

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Immunoglobulin E (IgE) plays a key role in the pathogenesis of many allergic diseases. Thus, IgE-mediated immunologic pathways are an attractive target for intervention in allergic diseases. Omalizumab is a recombinant humanized monoclonal antibody that binds IgE and has been used treat allergic asthma for over a decade. Currently, omalizumab is approved for the treatment of both allergic asthma and chronic spontaneous urticaria. Since IgE plays a critical role in other allergic diseases, anti-IgE therapy has been evaluated in other allergic diseases in small clinical trials and case reports. Omalizumab has demonstrated efficacy in treating allergic rhinitis, atopic dermatitis, physical urticarias, mast cell disorders, food allergy, and other allergic diseases. In addition, the use of omalizumab with conventional allergen immunotherapy improves both safety and effectiveness.

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IMMUNOGLOBULIN E (IgE) BACKGROUND

Immunoglobulin E is responsible for the pathogenesis of many allergic diseases including asthma. The primary role of IgE is defense against parasitic disease such as helminthes and protozoa (1). IgE, like all immunoglobulins, is composed of two light chains and two identical heavy chains (2). The heavy chain for IgE is epsilon. IgE is a monomer and consists of four constant regions. The constant region, C-epsilon-3, binds to both the low- and high-affinity IgE receptor. Production of IgE requires two signals between T cells and B cells to override the default production of IgM by plasma cells (3). The first signal involves interleukin-4 (IL-4) and interleukin-13 (IL-13) release by T helper type 2 (Th2) cells, mast cells, and basophils interacting with their respective receptors on the B cell. The second signal is the interface between the B cell CD40 and the T cell CD40 ligand (CD154).

Immunoglobulin E functions *via* its high- and low-affinity receptors on mast cells, basophils, and other cells (4). The high-affinity receptor for IgE is Fc-epsilon-RI expressed on mast cells and basophils. When bound with IgE subsequent cross-linking leads to activation of the cell and release of preformed mediators and the production of other inflammatory cytokines. The inflammatory mediators released by mast cells and basophils include histamine, tryptases, tumor necrosis factor-alpha, leukotrienes, and prostaglandins. In addition, the production of the Th2 cytokines IL-4, IL-5, and IL-13 further initiate late-phase inflammation and promotes more IgE production. The low-affinity receptor Fc-epsilon-RII (CD23) in its inducible form is present on B cells, T cells, dendritic cells, monocytes, macrophages, neutrophils, eosinophils, intestinal epithelial cells, and platelets (5, 6). The low-affinity receptor helps regulates IgE synthesis and has a role in antigen presentation (5, 6).

ANTI-IgE ANTIBODY (OMALIZUMAB)

Omalizumab is a recombinant humanized immunoglobulin (IgG1) monoclonal antibody that binds IgE with high affinity developed for the treatment of allergic diseases (7). Omalizumab binds to

the same C-epsilon-3 region that interacts with the IgE receptors forming complexes with free IgE preventing its interaction with these receptors (8). The omalizumab-IgE complexes are subsequently cleared by the hepatic reticuloendothelial system. Omalizumab is specific to IgE and does not bind to IgG or IgA. An important property of omalizumab is that it cannot bind to the IgE receptors or to IgE already attached to Fc-epsilon-RI, and therefore does not interact with cell-bound IgE or activate mast cells or basophils. Administration of omalizumab results in a rapid and significant decrease in free IgE levels. Due to this dramatic decrease in circulating IgE omalizuamb subsequently decreased the expression of the high affinity FcεRI receptor on the surface of both mast cells and basophils (9).

OMALIZUMAB'S EFFECT ON EOSINOPHILS

In a pooled analysis of over 2,200 patients, omalizumab treatment reduced blood eosinophil counts which correlated with the reduction seen in free IgE (10). In asthmatic patients, two studies evaluated the effect of omalizumab on sputum eosinophils and bronchial biopsies. The first study by Djukanović et al. examined induced sputum and bronchial biopsies on 45 moderate to severe asthma patients with baseline sputum eosinophils ≥2% (11). Omalizumab treatment for 16 weeks reduced mean sputum eosinophils from 6.6 to 1.7%, while the reduction in the placebo group was only 8.5 to 7.0%. In the submucosal bronchial biopsies median eosinophil counts decreased from 8.0 to 1.5 cells/mm^2 with omalizumab treatment while the counts were 6.3 to 6.4 cells/mm^2 in the placebo group. There was a weak correlation with the reduction in submucosal eosinophils and reduction in cells producing IL-4. Van Rensen et al. studied the effects of omalizumab on allergen challenge with 25 atopic asthmatics (12). In their study, omalizumab decreased sputum eosinophils from 4 to 0.5% and bronchial biopsy eosinophil count from 15 to 2 cells/0.1 mm^2.

One proposed mechanism for the reduction of eosinophils is by inducing eosinophil apoptosis. Nineteen patients with allergic asthma were treated 3 months of omalizumab (13). A marker of eosinophil apoptosis (annexin V) was increased in those patients treated with omalizamab and annexin-positive eosinophils were increased compared to baseline. In addition, cellular production of GM-CSF, used for eosinophil growth and survival, was decreased.

ASTHMA

In the United States and worldwide, omalizumab is approved for use in patients 6 years of age and older with moderate-to-severe persistent perennial asthma (14). In patients with moderate-to-severe asthma, multiple studies have demonstrated that treatment with omalizumab (compared with placebo) decreases the incidence of exacerbations and significantly reduces the dose of inhaled or oral glucocorticoids required to control symptoms. In two studies of pooled data omalizumab reduced emergency room, asthma-related outpatient visits, and hospitalizations (15, 16). Several studies have examined omalizumab therapy in children. These studies demonstrated reduced exacerbations, asthma symptoms, inhaled corticosteroid (ICS) doses, daily systemic corticosteroid dose, and hospitalizations with omalizumab therapy (17–19). In 2014, a meta-analysis of 25 randomized trials of patients with moderate or severe asthma requiring inhaled glucocorticoids, omalizumab reduced the risk of experiencing an exacerbation from 26 to 16% over 16 to 60 weeks of treatment (20). In addition, omalizumab reduced the risk of hospitalization for asthma from 3 to 0.5% over 28 to 60 weeks as well as decreased the amount of ICSs needed for asthma control. In this analysis, subjects receiving omalizumab were more likely to be able to completely withdraw inhaled glucocorticoids compared with those receiving placebo (40 versus 21%). However, omalizumab did not appear to increase the likelihood that subjects could discontinue oral glucocorticoids or consistently improve lung function in this meta-analysis.

PREDICTORS OF RESPONSE TO OMALIZUMAB IN ASTHMA PATIENTS

Asthma is a heterogenous disease with several different phenotypes. When based on inflammatory markers the Th_2 pattern is the most common. The EXTRA omalizumab study evaluated 850 patients with severe perennial allergic asthma divided into low- and high-biomarker groups (F_{ENO}, blood eosinophils, and serum periostin) (21). Patients in the high eosinophil group (≥260/μL) had a greater reduction in exacerbations with omalizumab treatment than those in the low group (<260/μL). In a pediatric trial of omalizumab to prevent fall asthma exacerbations during the run in period patients those who had an exacerbation had a higher serum eosinophil count (350 cells/μL) compared to those without an exacerbation (280 cells/μL) (22). A 24-week, multicenter, parallel group, double-blind, randomized, placebo controlled trial on patients with symptomatic asthma despite ICSs evaluated exacerbation rates with 6 months of anti-IgE therapy (23). In a subgroup analysis, patients with a baseline eosinophil count of ≥300/mL treated with omalizumab had a reduction in protocol-defined exacerbations by nearly 60% compared to placebo. When using the exacerbations defined by ATS/ERS criteria, the rate was reduced by 45% with omalizumab treatment. In those patients with the low baseline eosinophil counts, no improvement was noted in protocol defined exacerbation. There are limited characteristics that predict a positive response to omalizumab therapy for asthma but findings suggest that patients with a higher baseline serum eosinophil count may have a better clinical outcome.

CHRONIC URTICARIA

Urticaria and/or angioedema that occurs daily or near daily for more than 6 weeks has been termed chronic idiopathic urticaria (CU) or chronic spontaneous urticaria (CSU). First-line therapy, oral H1 antihistamines are effective for 50–60% of these patients (24). The initial proof-of-concept study involved 90 patients with antihistamine refractory CSU treated with a single administration of three different doses of omalizumab, 75, 300, or 600 mg versus placebo (25). Only the 300 and 600 mg doses demonstrated

improvement in the urticaria scores 4 weeks after treatment and there was not a significant difference in efficacy between these doses. This led to three large, phase III, randomized, double-blind, placebo controlled studies, Asteria I, Asteria II, and Glacial (26–28). These studies evaluated patients aged 12–75 with CSU that was refractory to oral H1-antihistamines.

In the Asteria I trial, 318 patients were randomized to one of three different doses of omalizumab (75, 150, and 300 mg) or placebo every 4 weeks for 24 weeks after failing licensed doses of H1-antihistamine therapy (26). Within the first week, the 300 mg dose improved urticaria compared to placebo. At 12 weeks, all three omalizumb doses significantly reduced patient's symptoms at 12 weeks compared to placebo. By week 12, 52% of the high-dose omalizumab patients were well controlled and 36% completely controlled. In addition, the 300 mg dose improved associated angioedema symptoms.

The Asteria II trial was similar in design with the same doses of omalizumab or placebo and enrolled 323 patients with CSU who remained symptomatic despite H1-antihistamine therapy (27). In this study, patients were treated for only 12 weeks compared to 24 in the Asteria I. At the end of the study, the patients on 150 and 300 mg doses of omalizumab demonstrated significant improvements in symptom scores and number of hives compared to placebo with 53% of the group receiving 300 mg of omalizumab becoming hive free and 44% free from both hives and itching.

The Glacial trial had a significant difference compared to the two Asteria trials. These patients all failed H1-antihistamines up to four times licensed doses plus patients were allowed to have been on an H2-blocker and/or leukotriene antagonist (28). In this study, 335 patients were randomized to either 300 mg of omalizumab or placebo monthlym for 24 weeks of treatment with 16-week follow-up. This study demonstrated the effectiveness of 300 mg omalizumab monthly in reducing urticarial lesions and symptoms after 12 weeks of therapy which was sustained for the 24 weeks of therapy.

In a review of over 900 patients with CSU symptomatic despite conventional therapy omalizumab improved symptoms in 65% with complete resolution in 40%. The improvement was noted in a just a few days in a subset of these patients (29). Omalizumab has also improved symptoms in patients with different types of physical urticarias, including solar, cold, localized heat, cholinergic, dermatographic, and pressure (30–37). In successfully treated patients with CSU or with physical uriticaria discontinuing therapy may lead to, relapse within a few weeks. Retreatment with omalizumab was effective resolving those symptoms (38).

Several associations such as the American Academy of Allergy, Asthma & Immunology, American College of Allergy, Asthma & Immunology, European Academy of Allergy and Clinical Immunology, Global Allergy and Asthma European Network, European Dermatology Forum, and the World Allergy Organization have recommended the use of omalizumab for CU in their urticaria guidelines (39, 40). In March of 2014, the US Food and Drug Administration approved the use of omalizumab in chronic urticaria patients 12 years and older who remain symptomatic despite H1 antihistamines.

The exact mechanisms of how omalizumab works in CU are unclear. In a subset of patients, IgG autoantibodies against FcεRI, IgE, or both may exist (41). Since omalizumab decreases the free IgE available with subsequent down-regulation of the FcεRI receptor, it was natural to postulate that omalizumab's effects might be due to decreasing the targets for these autoantibodies. However, no differences in effectiveness have been found in patients with or without the autoantibodies. Furthermore, analysis of previous data suggests that it takes time to significantly decrease the expression of the high affinity IgE receptors on either basophils (2 weeks) or mast cells (10 weeks); whereas a therapeutic effect within 1 week is noted in some patients (42, 43). Another possible mechanism is that IgE antibodies against autoallergens are present, and omalizumab reduces the level of these autoantibodies. Similar autoantibodies have been noted in patients with systemic lupus erythematosus contributing to the pathogenesis of that disease and similar autoimmune disorders (44).

ATOPIC DERMATITIS

Atopic dermatitis is another potential allergic target for anti-IgE therapy. Atopic dermatitis has been treated with omalizumab in several case series of both adult and pediatric patients, but results have been mixed. In a small series of seven pediatric patients with severe atopic dermatitis clinical improvement was noted 3 to 6 months after starting therapy and all patients had improvement after 12 months of therapy (45). Heil et al. performed a randomized, placebo-controlled, double-blind pilot study on 20 atopic dermatitis patients (46). Patients were randomized to omalizumab (13) or placebo (7) treatments for 16 weeks. At the end of the study, omalizumab reduced free IgE, surface IgE, and FceRI expression. Despite these changes, no significant improvement was noted with omalizumab therapy on clinical symptoms of atopic dermatitis. Iyengar et al. performed another randomized, double-blind, placebo-controlled study of eight patients (four omalizumab, four placebo) with severe refractory atopic dermatitis (47). After 24 weeks of therapy, omalizumab decreased levels of allergic inflammatory mediators but clinical symptoms were comparable between the omalizumab and placebo groups. A recent meta-analysis included the previously mentioned randomized studies as well as 13 case series evaluating the effectiveness of omalizumab in treating atopic dermatitis (48). There was no concrete evidence that omalizumab was effective in treating atopic dermatitis. Despite that conclusion, it was noted that 43% of patients improved clinically with omalizumab. There may be specific types of patients who are more responsive than others based on multiple variables, age, baseline IgE level, atopic status, or presence of filaggrin mutation (49).

ALLERGIC RHINITIS

Allergic rhinitis is another potential target for anti-IgE therapy. An early study in the United States evaluated 536 ragweed allergic patients at 25 different sites (50). This randomized, double-blind, placebo-controlled study evaluated several doses of omalizumab (50, 150, and 300 mg) and placebo administered every 3–4 weeks just prior to and during ragweed season.

Patients treated with 300 mg of Omalizumab had significantly lower rhinitis symptoms. Those treated with the 300 mg dose of omalizumab had better quality of life scores than the other groups and did not decline during the peak ragweed season. A follow-up open-labeled study of 300 mg of omalizumab therapy every 3–4 weeks demonstrated that the therapy is well tolerated without any significant immunologic reactions (51). Further studies demonstrated omalizumab's effectiveness in reducing symptoms and rescue medication usage in patients with allergic rhinitis to ragweed, birch, cedar, and perennial allergens (52–58).

A meta-analysis published in 2014 retrieved 352 citations with 78 articles eligible for review (59). Of those studies, 11 qualified for evaluation with a total of 2,870 patients treated for seasonal or perennial allergic rhinitis. Omalizumab significantly reduced both daily nasal symptoms and daily nasal rescue medication usage. No significant adverse events were reported.

ALLERGEN IMMUNOTHERAPY

Allergen immunotherapy has been used for 100 years for the management of allergic disorders and is the only antigen-specific immunomodulatory treatment. The addition of omalizumab to standard maintenance-dose immunotherapy was evaluated in 221 pediatric patients sensitized to birch and grass pollen (60). During birch season, the addition of omalizumab reduced symptoms by 48% compared to birch SCIT alone. Similar results were seen in grass season, with a 57% decrease in symptoms with the addition of omalizumab to grass immunotherapy compared to grass immunotherapy alone. When these findings were further analyzed for the grass pollen–allergic children, the groups treated with omalizumab plus immunotherapy had significantly diminished rescue medication use and number of symptomatic days compared to omalizumab or immunotherapy alone (61). Casale et al. evaluated omalizumab starting 9 weeks before rush immunotherapy followed by 12 weeks of therapy (62). Overall, in ragweed-allergic patients the combination of omalizumab and immunotherapy showed a significant improvement in severity scores during the ragweed season compared with those receiving immunotherapy alone after rush immunotherapy buildup. Overall, these findings demonstrate that combined treatment with omalizumab and immunotherapy is more effective than omalizumab or immunotherapy alone.

Immunotherapy for allergens carries a risk of anaphylaxis with each administration. In those patients treated with rush ragweed immunotherapy, omalizumab added to immunotherapy had fewer adverse events than those receiving immunotherapy alone. The addition of omalizumab to SCIT resulted in a decrease in risk of anaphylaxis caused by immunotherapy by fivefold (62). In asthma patients, the use of omalizumab in conjunction with SCIT resulted in fewer systemic reactions (13.5 versus 26.2% for placebo) (63). The use of omalizumab in conjunction with venom immunotherapy in a few patients demonstrated conflicting results in preventing systemic reactions caused by immunotherapy (64, 65). Recently, the use of oral immunotherapy for food allergy noted benefit in patients with milk, egg, and peanut allergy. The data suggest that omalizumab may facilitate oral desensitization to peanut and milk (66, 67). The addition of omalizumab has allowed some children to successfully receive oral immunotherapy to multiple foods simultaneously, including milk, egg, peanut, wheat, soy, and tree nuts (66–69). The addition of omalizumab to oral immunotherapy for milk not only improved safety but also decreased basophil activation (70).

FOOD ALLERGY

Food allergies affect about 6% of children younger than 3 years of age and 2% of adults, with 1.5 million suffering from peanut allergy in the United States. An early double-blind, placebo-controlled, randomized trial in 84 peanut allergic patients evaluated three doses of another humanized mAb against IgE, TNX-901 (71). All groups, including the placebo group, had a greater threshold of peanut tolerability, but only the high-dose TNX-901 group significantly improved from tolerating about 1/2 peanut to more than 8 peanuts at the end of therapy. Despite this 25% of the high-dose group had no improvement with therapy. A more recent attempt at a phase II, multicenter, randomized, double-blind, placebo-controlled, parallel-group trial was designed to assess the efficacy of omalizumab in peanut allergic patients (72). Due to concern of severe anaphylactic reactions during the qualifying food challenges before therapy, only 14 patients (9 omalizumab and 4 placebo) completed treatment. Of these, four (44.4%) omalizumab-treated subjects compared to one (20%) placebo-treated subjects could tolerate >1,000 mg peanut flour after 24 weeks, but this difference was not significant. Both of these studies suggest that omalizumab may be beneficial for food allergy, but the findings are not conclusive.

EOSINOPHILIC ESOPHAGITIS

Eosinophilic esophagitis is another potential target for anti-IgE therapy. In two case studies of patients with eosinophilic esophagitis and multiple food allergies, the addition of omalizumab to the patient's standard therapy reduced symptoms of eosinophilic esophagitis but did not improve endoscopic and histologic changes (73). A prospective randomized, double-blind, placebo-controlled trial of omalizumab therapy monthly for 16 weeks in 30 eosinophilic esophagitis patients who were either refractory to or relapsed after topical corticosteroids found no improvements in either esophageal eosinophil counts or symptom scores (74). In an open label study, omalizumab was administered for 12 weeks to 15 subjects with long standing EoE, only 5 of the subjects had histological and clinical improvement after 3 months of treatment (75). Omalizumab induced remission of EoE was limited to those subjects with low baseline peripheral blood absolute eosinophil counts (<450 cells/μl). Foroughi et al. examined the effect of omalizumab on assorted eosinophilic gastrointestinal disorders (76). Of the nine subjects, eight had multiple areas of eosinophilic disease with seven having at least the esophagus involved. Patients were treated with omalizumab every 2 weeks for 16 weeks. There was a non-significant decrease in eosinophils present in the duodenum and stomach while the esophageal eosinophils were unchanged. Despite this lack of histological

changes, symptom scores were significantly decreased by 70% at the end of the study. No effect on T-cell function was noted in those patients treated with omalizumab (77). Overall, these studies suggest that for select patients with eosinophilic-based GI diseases anti-IgE therapy may be effective, especially those with low blood eosinophil counts.

ALLERGIC BRONCHOPULMONARY ASPERGILLOSIS (ABPA)

Allergic bronchopulmonary aspergillosis is associated with pediatric cystic fibrosis and adult asthma. One review on children with cystic fibrosis and ABPA analyzed 8 cases in 13 children (78). Treatment with omalizumab improved lung functions and reduced respiratory symptoms and systemic corticosteroid use. A Cochrane meta-analysis attempted to evaluate the effects of omalizumab for ABPA in patients with cystic fibrosis, but concluded that there were no studies that met inclusion criteria (79). A retrospective analysis of four adult stage IV ABPA patients (corticosteroid dependent) treated with omalizumab 375 mg every 2 weeks for 12 months found it was steroid sparing and reduced inflammatory markers and symptom scores, even with elevated IgE levels (80).

NASAL POLYPS

Nasal polyps are frequently associated with eosinophilic inflammation and local production of IgE. In a randomized, double-blind, placebo-controlled study, patients with nasal polyps and comorbid asthma were treated with either omalizumab (16) or placebo (8) for 16 weeks (81). In the omalizumab-treated group, nasal polyp size decreased by both endoscopy and CT scan assessment, regardless of allergic status. Only the omalizumab-treated patients had a significant improvement in their nasal and asthma symptom scores. A similar study of patients with nasal polyps who were continued on their medical regimen (nasal corticosteroids, leukotriene modifiers, and as needed courses of prednisone and antibiotics) found that the addition of omalizumab decreased nasal polyp size but had no significant effect on symptoms compared to the placebo group (82). A retrospective analysis of eight subjects demonstrated that omalizumab after polypectomey may reduce the severity of nasal polyp recurrence (83).

TABLE 1 | Clinical benefit with omalizumab therapy.

Strong evidence	Allergic asthma
	Chronic urticaria
Good evidence	Allergic rhinitis
	Allergen immunotherapy (inhalants)
Fair evidence	Atopic dermatitis
	Food allergy
	Oral immunotherapy (foods)
	Mast cell disorders
Weak evidence	Eosinophilic gastrointestinal diseases
	Allergic bronchopulmonary aspergillosis
	Nasal polyps

OTHER DISEASES

A recent review on the use of omalizumab in mast cell disorders noted case studies of patients with mastocytosis, cutaneous mastocytosis, venom anaphylaxis, and mast cell activation syndrome showed improvement with omalizumab therapy (84). Case reports and small studies have noted the benefit of omalizumab treatment in Churg–Strauss Syndrome, bullous pemphigoid, Kimura's disease, aspirin-exacerbated respiratory disease, recurrent anaphylaxis, laryngeal angioedema, chronic eosinophilic pneumonia, drug allergy, and vernal keratoconjunctivitis (85–93).

CONCLUSION

Omalizumab is the first immune modifier to be approved for the treatment of allergic diseases (**Table 1**). The excellent strength of evidence for the effectiveness of omalizumab in allergic asthma and chronic urticaria have resulted in the FDA approval for use in those diseases. While evidence points to omalizumab's effectiveness in allergic rhinitis, and as an adjunct to allergen immunotherapy, due to costs or dosing limitations omalizumab will unlikely be widely used for those instances. Large clinical trials are needed for omalizumab and other anti-IgE strategies to treat the other allergic diseases were the evidence is not as strong.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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Targeted Treatment Options in Mastocytosis

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Mastocytosis refers to a heterogeneous group of disorders resulting from the clonal proliferation of abnormal mast cells and their accumulation in the skin (cutaneous mastocytosis when only in the skin, CM) or in various organs (systemic mastocytosis, SM). This leads to a wide variety of clinical manifestations resulting from excessive mediator release in CM and benign forms of SM (indolent SM, ISM) and from tissue mast cell infiltration causing multiorgan dysfunction and failure in more aggressive subtypes (aggressive SM, ASM, or mast cell leukemia). In addition, SM may be associated with hematological neoplasms (AHN). While treatment of ISM primarily aims at symptom management with anti-mediator therapies, cytoreductive and targeted therapies are needed to control the expansion of neoplastic mast cells in advanced forms of SM, in order to improve overall survival. Mast cell accumulation results from a gain-of-function mutation (mostly the D816V mutation) within the KIT tyrosine kinase domain expressed by mast cells and additional genetic and epigenetic mutations may further determine the features of the disease (ASM and AHN). Consequently, tyrosine kinase inhibitors and targeted therapies directed against the oncogenic signaling machinery downstream of KIT are attractive therapeutic approaches. A better understanding of the relative contribution of these genetic and epigenetic events to the molecular pathogenesis of mastocytosis is of particular interest for the development of targeted therapies and therefore to better choose patient subgroups that would best benefit from a given therapeutic strategy.

Keywords: systemic mastocytosis, mast cell, KIT, targeted treatment, tyrosine kinase inhibitor

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INTRODUCTION

Mastocytosis refers to a heterogeneous group of disorders characterized by the pathologic accumulation of mast cells in different tissues or organs, predominantly skin, bone marrow, and visceral organs (1).

This rare disorder with its high variety of subtle and non-specific clinical manifestations is a real diagnostic challenge. Its exact incidence and prevalence is unknown, but a recent European retrospective population-based study gives an estimate of 1 case per 10,000 persons (2).

In the 2016 WHO classification, mastocytosis is no longer considered a subcategory of myeloproliferative neoplasms (MPN), but a separate entity in myeloid neoplasms with its distinctive clinical and pathologic features (3). Two major forms of mastocytosis are described: cutaneous

mastocytosis (CM) and systemic mastocytosis (SM). CM is the most frequent presentation in children, and in most cases, regresses spontaneously at the puberty onset (4). In contrast, SM more often develops in adults and may persist throughout life (5). SM implies an extracutaneous site involvement, most commonly the bone marrow and the gastrointestinal tract, but lymph nodes, spleen, and liver can also be affected (1). Skin involvement is frequent in the benign form of SM, namely, indolent SM (ISM), whereas rarely present in the life-threatening SM subtypes, aggressive SM (ASM), and mast cell leukemia (MCL) (6). SM can also be associated with a non-mast cell clonal hematological neoplasm (SM-AHN), more often myeloproliferative disorders or myelodysplastic syndromes (7).

PATHOGENESIS

Recent advances have been made in the understanding of mastocytosis pathogenesis, paving the way for the development of different targeted treatments.

Mast cells derive from hematopoietic progenitors and express on their surface high levels of tyrosine kinase receptor KIT (CD117) that binds the stem cell factor (SCF), a growth factor essential for their survival, maturation, proliferation, migration, and activation (8). KIT is expressed widely on hematopoietic stem cells and on multipotent progenitor cells, but is then downregulated in all mature lineages, except in the mast cell one. Acquired activating KIT mutations lead to SCF-independent receptor activation and signaling, survival, clonal expansion, and uncontrolled activation in mast cells.

In adults, the most common mutation occurs in the codon 816 and consists of a valine-to-aspartate substitution (9). This D816V mutation is located in the phosphotransferase domain of the receptor and causes conformational change in its juxtamembrane region leading to its dimerization and consequently its constitutive activation.

KIT D816V is detected in >80% of all SM cases (9). More than 20 other KIT mutations have been identified such as V560G, D815K, D816Y, VI816_816, D816F, D816H, and D820G (10–16). Disease phenotype and prognosis is apparently not dependent on the type of mutation encountered (12, 17, 18) but is rather correlated to the KIT D816V allele burden. Indeed, a strong correlation exists between the allele burden of KIT mutant determined by highly sensitive techniques such as allele specific quantitative PCR and neoplastic mast cell load, survival, and prognosis (19, 20).

The effect of KIT mutation on mastocytosis phenotype may also be influenced by the development stage of the mutated cell. Indeed, KIT mutations present in multiple lineages (mast cells, myeloid, and lymphoid lineages) have been associated with more aggressive forms of SM (21). In contrast, mutations affecting committed mast cell progenitors or mature mast cells result in milder forms of the disease (22).

In advanced systemic mastocytosis (ASM, MCL, SM-AHN), epigenetic alterations are believed to play a role in the molecular pathogenesis of SM and are of particular interest as potential therapeutic targets. The next-generation sequencing of 70 patients revealed that the most frequently affected genes were TET2 (47%), SRSF2 (43%), ASXL1 (29%), RUNX1 (23%), JAK2 (16%), N/KRAS (14%), CBL (13%), and EZH2 (10%) (23). These mutations are not specific of SM, as they were also identified in other myeloid neoplasms including MPN/myelodysplasic syndrome (MDS) or MPN. These mutations seem to develop before KIT mutations, in almost all patients (24). Such additional lesions may be co-expressed with KIT D816V in the same cells or subclones but may also be detectable in other myeloid lineages, especially in SM-AHN. The prognostic impact of these mutations has been recently studied. Overall survival was adversely affected by mutation in SRSF2, ASXL1, and RUNX1, and the clinical course was also worsening with the number of mutations in the SRSF2/ASXL1/RUNX1 panel (23). SRSF2 has the worse prognosis, and remains, with ASXL1, an independent poor prognostic factor in multivariate analysis (23). Mutations in the tumor suppressor gene TET2 act in synergy with KIT D816V mutation, enhance its oncogenic potency, and induce aggressiveness of the mastocytosis (25), but in contrast to other mutations have not been associated with decreased overall survival (26–28).

Downstream of the phosphotransferase domain mutated KIT, the Jak/Stat5 pathway, and to a less extent the PI3K–AKT signaling cascade are essential for neoplastic mast cells development and proliferation (29) and offers a panel of targeted treatment possibilities, as will be discussed further below.

In childhood, CM is also associated with germline or acquired activating KIT mutations, signing a clonal disease. Available data suggest that approximately 40 percent of children with CM have exon 17 mutation, with another 40 percent carrying KIT mutations outside of exon 17 (17, 30). Some familial mutations of KIT also have been identified, in rare cases of familial mastocytosis (31–33). In contrast to somatic KIT mutations in mastocytosis that were mainly found in exon 17, germline KIT mutations are located in exons 8, 9, 10, 13, and 17 (34). *In vitro,* all these mutations are oncogenic (in contrast of those of the exon 17) and are inducing mainly the PI3-AKT and MAP kinase pathways.

CLINICAL MANIFESTATIONS, DIAGNOSIS, AND CLASSIFICATION

Clinical manifestations depend on the subtype of mastocytosis and can be divided in three non-exclusive categories.

Cutaneous Lesions due to Skin Involvement

Three major variants of CM have been defined by the 2016 WHO classification (see **Table 1**), the most frequent one being maculopapular mastocytosis (also named urticaria pigmentosa, UP) (35, 36). UP consists in reddish-brown macules or slightly raised papules, classically affecting the upper and lower extremities, sometimes the thorax and the abdomen, but rarely the face or other sun exposed areas. The pathognomonic Darier's sign refers to swelling, itchiness, and redness appearing after scratching an UP lesion and is due to localized release of mast cell mediators (35). Pruritus and flushing can also be triggered by temperature changes, hot showers, emotional stress, spicy food, fever, exercise, friction, and certain drugs (5).

TABLE 1 | WHO 2016 mastocytosis classification.

1. Cutaneous mastocytosis
   a. Urticaria pigmentosa or maculopapular cutaneous mastocytosis
   b. Diffuse cutaneous mastocytosis
   c. Solitary mastocytoma of skin
2. Systemic mastocytosis
   a. Indolent systemic mastocytosis
   b. Smoldering systemic mastocytosis
   c. Systemic mastocytosis with an associated hematological neoplasm
   d. Aggressive systemic mastocytosis
   e. Mast cell leukemia
3. Mast cell sarcoma

Symptoms Associated With Mast Cell Mediator Release

Mediator-related symptoms are a constellation of non-specific signs making the clinical diagnosis of mastocytosis very challenging. These include fatigue, nausea, vomiting, abdominal pain, diarrhea, anaphylaxis, hypotension, diffuse musculoskeletal pain, osteopenia, and osteoporosis (37, 38). Abnormal mast cell degranulation may also occur in the central nervous system leading to psychiatric symptoms such as depression, anxiety, and cognitive impairment (39). More specifically, patients with mastocytosis are more prone to anaphylaxis during allergic reactions, particularly in response to hymenoptera stings (40).

Importantly, in these indolent forms, osteopenia and osteoporosis may occur leading to bone fractures (41, 42). All these symptoms are prominent in smoldering SM (SSM) and ISM while seldom present in ASM.

Symptoms Related to Organ Infiltration (Only Present in SM)

In advanced SM (ASM, SM associated with another hematological neoplasm and mast cell leukemia), organ damage or dysfunction due to organ infiltration with neoplastic mast cells can include cytopenias, hepatosplenomegaly, portal hypertension, lymphadenopathy, impairment of liver function, ascites, hypersplenism, malabsorption, weight loss, and pathological lytic bone fractures (which must be differentiated from those associated with osteoporosis) (1, 43). The classification of these symptoms in B findings (for "Borderline Benign," that reflects the disease burden), or C findings (for "require Cytoreductive therapy"), reflecting organ dysfunction and disease aggressiveness, helps to define the subcategory and severity of SM (44) (see **Table 2**).

Systemic mastocytosis diagnosis, according to the WHO classification, requires the presence of both the major criterion and one minor, or at least three minor criteria (1). The major criterion is defined as the presence of multifocal, dense infiltrates of mast cells (>15 mast cells in aggregates) detected in bone marrow and/or other extracutaneous organs. Minor criteria are the following: (1) >25% of mast cells in infiltrates with atypical morphology; (2) detection of an activating point mutation at codon 816 of KIT in bone marrow, blood, or an extracutaneous organ; (3) mast cells abnormal expression of CD2 and/or CD25; and (4) serum total tryptase level >20 ng/ml.

TABLE 2 | B and C findings.

"B" findings	"C" findings
1. BM biopsy showing >30% infiltration by MC (focal, dense aggregates) and/or serum total tryptase level >200 ng/mL	1. Bone marrow dysfunction manifested by one or more cytopenia(s) (ANC < 1.0×10^9/L, Hb < 10 g/dL, or platelets < 100×10^9/L), but no obvious non-mast cell hematopoietic malignancy.
2. Signs of dysplasia or myeloproliferation, in non-MC lineage(s), but insufficient criteria for definitive diagnosis of a hematopoietic neoplasm (AHN), with normal or slightly abnormal blood counts.	2. Palpable hepatomegaly with impairment of liver function, ascites, and/or portal hypertension.
3. Hepatomegaly without impairment of liver function, and/or palpable splenomegaly without hypersplenism, and/or lymphadenopathy on palpation or imaging	3. Skeletal involvement with large osteolytic lesions and/or pathological fractures.
	4. Palpable splenomegaly with hypersplenism.
	5. Malabsorption with weight loss due to gastrointestinal mast cell infiltrates.

BM, bone marrow; MC, mast cell; ANC, absolute neutrophil count; Hb, hemoglobin.

The 2016 WHO classification recognizes further five subtypes of SM in order to stratify mast cell disorders according to their aggressiveness (3):

- ISM, displaying no evidence of extracutaneous organ dysfunction;
- SSM, defined by the presence of two or more B findings (see **Table 2**);
- Aggressive systemic mastocytosis (ASM), defined by the presence of one or more C findings (see **Table 2**);
- SM associated with another hematological neoplasm (SM-AHN);
- Mast cell leukemia (MCL), defined by >20% mast cells on bone marrow smear or >10% in peripheral blood.

Systemic mastocytosis prognosis differs according to the disease subtype and will subsequently guide treatment strategy. A study of 342 patients with SM of the Mayo clinic showed that ISM survival is comparable to age-and sex-matched control population, whereas advanced SM patients clearly have a significantly inferior survival with a median of 41 months for ASM, 24 months for SM-AHN, and 2 months for MCL (45). Recently, a form of chronic MCL with a low index of proliferation (Ki67) has been described with a better prognosis (46).

TREATMENT OF ADVANCED SM

A proposed treatment algorithm is presented in **Figure 1**. Various cytoreductive treatments have been used for advanced SM, including 2-Chlorodeoxyadenosine (2-CDA), interferon-alpha (IFN-α), classical chemotherapy agents (such as cytarabine or fludarabine), but all with modest and disappointing response rates, highlighting the need for innovative therapies (47, 48).

Cytoreductive Therapies

For many years, IFN-α has been considered as the first line treatment for patients with advanced mastocytosis, but the efficacy

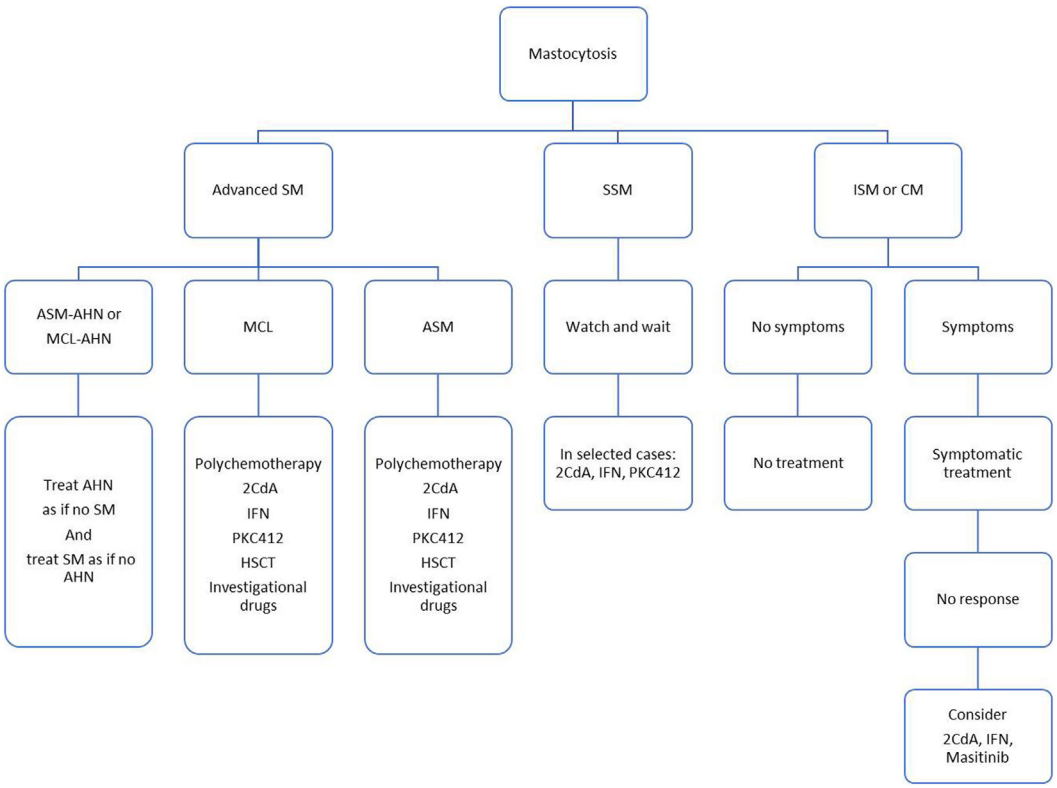

FIGURE 1 | Proposed treatment algorithm.

was variable, and the exact posology and treatment duration remained unknown. In the Mayo Clinic study published in 2009, the overall response rate (ORR) in 40 IFN-α-treated patients was 53% with only 1 complete response (CR), 6 major responses (MR), and 14 partial responses (PR), and a median duration of response of 12 months (range 1–67 months) (49). Major toxicities were observed, including fatigue, depression, and thrombocytopenia. Interestingly, IFN-α has also a role in treating skeletal symptoms because of its ability to increase bone density. The median weekly dose was 15 million units/week, ranging from 0.5 to 10 MU three times a week (49).

Cladribine (or 2-CdA) has shown therapeutic activity in all SM subtypes, including MCL. In the Mayo Clinic study, the ORR in 22 cladribine-treated patients was 55% (CR 5%, MR 32%, and PR 18%), with a mean duration of response of 11 months (range 3–74). Major toxicities included myelosuppression and infections (49). Improved response rates were observed in a recent French study on 68 patients (36 with ISM and 32 with advanced SM) treated with 2-CdA with 72% ORR, 92% in ISM (by reducing symptoms and skin involvement), and 50% in advanced SM and a median duration of response of 3.7 and 2.47 years for ISM and advanced SM, respectively. The administered dose was 0.14 mg/kg intravenous or subcutaneously for 5 days, repeated at 4–12 weeks, with a median number of cycles of 3.7 (range 1–9) (50). As expected, major toxicities were leukopenia and opportunistic infections.

Tyrosine Kinase Inhibitors

Tyrosine kinase inhibitors (TKIs) are an attractive therapeutic approach, given the pathogenesis of SM and the involvement of KITD816V mutation in more than 80% of patients, and other KIT mutations that map to the TK juxtamembrane domain or transmembrane domain in sporadic cases of SM (48).

Imatinib

Imatinib is an efficient inhibitor of wild-type KIT, PDGFR, and BCR-ABL, but has no activity against the KITD816V mutation (51). Indeed, this mutation induces structural alterations at the KIT binding site, resulting in a decreased affinity for type I TKIs, such as imatinib, that recognize the active conformation of the kinase (52). Consequently, imatinib failed to demonstrate any response in KIT D816V mutated SM (53). However, imatinib may be an appropriate candidate in the rare SM cases that display an imatinib-sensitive KIT mutation (F522C, K5091, V560G, V559G, and del419) (54, 55), or those without the KITD816V mutation (56, 57). For this reason, the US Food and Drug Administration approved imatinib only for ASM patients not harboring the KITD816V mutation or ASM patients with an unknown mutational status.

The difficult distinction between certain forms of hypereosinophilic syndrome (HES) and SM has contributed to the recommendation of imatinib as therapy for mast cell disease with hypereosinophilia in earlier reports. Indeed, mast cells and

eosinophils may be found in both disorders; however, when present, genetic mutations (KIT mutations and FIP1L1-PDGFRa rearrangement) are the diseases distinctive signature (58). Only a few patients carry both KITD816V and FIP1L1-PDGFRa rearrangement (59). The delineation between FIP1L1-PDGFRa HES and KIT D816V advanced SM with eosinophilia has important clinical implications, as those with FIP1L1-PDGFRa rearrangement respond to imatinib and not the others (60).

Dasatinib

Dasatinib is a multikinase inhibitor active against BCR-ABL1, KIT, and PDGFRa and has shown promising *in vitro* activity against various KIT mutants, including D816V (61, 62), but its very short half-life *in vivo* may be responsible for the disappointing clinical response. In the largest phase 2 study of dasatinib in SM (33 patients, 15 with advanced SM), 2 (6%) of the 33 patients achieved CR and 9 (27%) achieved a symptomatic improvement. ORR was 33% but 58% experienced grade 3 toxicities, mainly pleural effusions and thrombocytopenia (63). In view of these elements, dasatinib is nowadays not recommended in the treatment of advanced SM patients.

Nilotinib

Nilotinib has been investigated in a phase 2 trial with 61 patients (including 37 with advanced SM), at the dose of 400 mg twice daily. The ORR was 21.6% overall and 21% in advanced SM (64). Regarding to its modest activity, nilotinib has currently no place in the treatment of SM.

Bosutinib

Bosutinib is a dual SRC/ABL kinase inhibitor, with minimal anti-KIT activity. *In vitro*, bosutinib is able to decrease neoplastic mast cell growth by inhibiting LYN and BTK activity (65). However, no clinical response has been shown in a patient with ASM treated with bosutinib (66).

Ponatinib

Ponatinib, another multikinase inhibitor, has shown activity on KITV560G and, less effectively, on KITD816V in the human mast cell leukemia cell line human mast cell line-1 (HMC-1) (67, 68). Ponatinib also synergizes with midostaurin to obtain growth inhibition against neoplastic mast cells harboring the KITD816V mutant (67). However, clinical trials are needed to assess the *in vivo* efficacy of ponatinib, alone or in combination.

Masitinib (AB1010)

Masitinib (AB1010) is a KIT inhibitor with activity against KIT and LYN kinases, but with no activity on KITD816V mutants (69). Few anecdotal cases with aggressive forms bearing KIT mutations outside exon 17 or no KIT mutation have responded durably (70). Its emerging role in the treatment of indolent mastocytosis will be discussed further below.

Midostaurin (PKC412)

Midostaurin (PKC412) is an oral potent multikinase inhibitor with activity against protein kinase C (PKC), FMS-related tyrosine kinase 3 (FLT3), PDGFRA/B, vascular endothelial growth factor receptor 2, and KIT (71). Interestingly, midostaurin shows clinical activity and efficacy regardless of the KIT mutation status. In the recently published phase 2 multicenter international study, 116 patients with advanced SM received 100 mg of midostaurin twice daily until progression or unacceptable toxicity (72). Eighty-nine patients were evaluable for efficacy, including 16 with ASM, 57 with SM-AHN, and 16 with MCL. After a median follow-up of 26 months (range 12–54 months), the ORR was 60% with 45% of MR and 15% of PR and the median OS was 28.7 months (72). Responses occurred in multiple organ systems, including resolution of pleural effusions, hypoalbuminemia, reversion of weight loss, improvement in liver function, and increase in hemoglobin and platelet counts. In responding patients, durable responses were observed, with a median duration of response of 24.1 months and a median OS of 44.4 months. Results were similar no matter the KIT mutational status and were similar in different subtypes of advanced SM. In the 16 patients with the highly aggressive MCL subtype, the ORR was 50%, 7 patients experienced MR (44%). Their median OS was 9.4 months overall, but median OS in responders has not been reached. A significant (>50%) decrease in bone marrow MC burden and tryptase levels has also been observed. Toxicities included mainly grade 1–2 gastrointestinal adverse events (AEs), and grade 3–4 anemia, neutropenia and thrombocytopenia was observed in 41, 24, and 29%. respectively, mainly in patients with preexisting cytopenias. Sixty-five patients (56%) needed dose reduction, mainly because of AEs, with possible reescalation to the initial dose in 21 of the 65 patients (32%). Midostaurin has therefore a favorable efficacy and safety profile. It can induce durable responses in patients with advanced SM, even in MCL patients, and should be considered as a part of the first-line treatment in advanced SM.

BLU-285

BLU-285, a potent and selective KITD816V inhibitor, has shown encouraging results in preclinical studies (73) but also in an ongoing phase I trial. So far, 12 patients with advanced SM have been treated with BLU-285 at three dose levels (30, 60, or 100 mg once daily) (74). Eleven of the 12 patients harbored the KIT D816V mutation. BLU-285 appeared to be well tolerated at all doses since no patients discontinued treatment due to AEs, and no grade ≥ 4 AEs were reported. The majority of the AEs were grade 1 or 2 and included fatigue, dizziness, headache, rash, shingles, anemia, and thrombocytopenia ($n = 1$ for each). Objective decreases in mast cell burden were observed in six out of eight evaluable patients, including decline in peripheral blood and BM KIT D816V DNA levels. Serum tryptase levels declined in 10 out of 12 patients (83%), and half of the patients experienced a decrease in BM infiltrate. Symptomatic improvement was also reported with less allergy symptoms, improved UP and increased albumin, and weight gain (74).

Antibody-Mediated Targeted Therapy

Normal and neoplastic mast cells express on their surface a number of cell surface antigens that might be considered as potential targeted therapies in advanced SM, some of them being already

available and used in other hematological diseases. These antigens include CD13, CD25, CD30, CD33, CD44, CD52, CD87, CD117, and CD123 (75).

In contrast to normal mast cells, neoplastic mast cells abundantly express the cell-membrane protein CD30 on their surface but also in their cytoplasm (76, 77). As assessed by flow cytometry, CD30 was found on neoplastic mast cells in 12% of patients with ISM and 57% of patients with ASM or MCL, making it an attractive target for advanced SM (78). Brentuximab vedotin (a CD30-targeted antibody conjugated with the antimitotic agent auristatin E) is already an established treatment for Hodgkin lymphoma and anaplastic large cell lymphoma. In patients with CD30(+) SM, brentuximab vedotin induces apoptosis of neoplastic MCs, downregulates IgE-mediated histamine release in CD30(+) MCs, and synergizes with midostaurin to inhibit neoplastic MC growth (78). In a small case series of four patients with ASM or ISM, brentuximab vedotin led to a reduction in the disease burden in half of them, of which one experienced a durable response for more than 3 years (79). Besides, treatment with brentuximab vedotin was well tolerated with toxicities manageable by dose reduction only. Together, CD30 appears to be a promising new drug target for patients with CD30(+) advanced SM, with a favorable toxicity profile. Further studies are needed to determine its efficacy and potential combination with midostaurin.

CD52 is another potential target widely expressed on the surface of neoplastic MCs mainly in advanced SM (80). The CD52-targeted antibody alemtuzumab has been shown to induce neoplastic mast cell death *in vitro* but also *in vivo*, in xenotransplanted mice with HMC-1. So far, no clinical studies have been performed.

In a similar way, gemtuzumab ozogamicin (Mylotarg®), an anti-CD33 monoclonal antibody conjugated with a cytostatic agent, can also induce cell death in neoplastic MCs and their progenitors *in vitro* (81). We have treated a patient with MCL, who was refractory to all previous treatment (cladribine, midostaurin, chemotherapy), with a MR allowing bone marrow transplantation (O. Hermine, personal observation).

CD123, the α-subunit of the interleukin-3 receptor, represents also a potential therapeutic target as it is aberrantly expressed on neoplastic MCs and absent on normal MCs (82, 83). Clinical trials are ongoing to evaluate its efficacy in patients with SM.

More recently, it has been shown that mast cells in mastocytosis may express PD-L1, suggesting that effects of checkpoint inhibitor antibodies should be tested in clinical trials in this disease (84).

Targets Related to Signaling or Apoptosis

Several studies have reported quantitative and qualitative defects of signal transduction in SM. These altered pathways play a role in the pathogenesis of SM and targeted drugs may provide therapeutic options by selective inhibition of some of these critical pathways.

Neoplastic MCs development seems to be essentially governed by the STAT5–PI3K–AKT–mTOR signaling cascade downstream of the mutated KIT (85, 86). PI3K (phosphoinositide 3-kinase), a lipid kinase, is important for the function of intracellular signaling molecules, like BTK, AKT and PDK1. Mutated KIT constitutively activates PI3K, which in turn phosphorylates AKT and subsequently mTOR, promoting abnormal mast cell development *in vivo* and *in vitro* (86). Activated KIT also recruits the JAK/STAT signaling pathway and STAT5 especially (87). Small inhibitor molecules targeting STAT5 or AKT might therefore be of particular interest in treating patients with SM. Unfortunately to date, AKT inhibitors have shown efficacy only *in vitro*, and STAT5 targeting drugs can be effective only at high concentrations *in vivo* (48).

mTOR, a conserved Serine/Threonine kinase, exists in two distinct multimolecular complexes: mTOR complex1 and mTOR complex2. Expression and activation of mTORC1 and mTORC2 is increased in neoplastic human MC lines and in immature normal MCs, compared with mature normal MCs (88). Rapamycin has shown to specifically block mTORC1 in normal MCs and to inhibit cell survival of tumor mast cells bearing the C-KIT D816V mutation (89). In contrast, everolimus, another mTOR inhibitor, was found ineffective in patients with SM (90). We have treated a patient with an ASM refractory to cladribine and midostaurin, who responded to the combination of temsirolimus and high-dose aracytine, with a MR and who is now cured 2 years after an allogeneous stem cell transplantation. BEZ235, a dual PI3K/mTOR blocker, produces growth-inhibitory effects in immature neoplastic MC and inhibits IgE-dependent activation of mature basophils and MCs (91). Whether these potentially beneficial drug effects have clinical implications is currently under investigation.

Bim, a proapoptotic Bcl-2 family member, is downregulated by KITD816V and has been identified as a tumor suppressor in neoplastic mast cells (92). Midostaurin and the proteasome-inhibitor Bortezomib enhance the expression of Bim in MC leukemia cell lines HMC-1.1 (D816V negative) and HMC-1.2 (D816V positive), decreasing their survival. Furthermore, midostaurin was found to cooperate with bortezomib and with the pan-Bcl2 family inhibitor obatoclax in reducing proliferation and survival in both HMC-1 subclones (92). Targeting Bcl-2 family members by drugs promoting Bim (re)-expression, or by BH3-mimetics such as obatoclax, may be an attractive therapy concept in SM.

MCL-1, a BCL-2 family member with antiapoptotic properties, is expressed in neoplastic MCs (93). MCL-1 inhibition with antisense oligonucleotides increased apoptosis in these cell lines, and increased responsiveness to TKI such as midostaurin, suggesting a novel interesting target that could help overcome resistance to TKI.

NF-kB and NFAT (nuclear factor of activated T cells), two transcription factors of the REL family, have been found to be constitutively activated in KIT mutated cells and could also represent interesting targets (94, 95). Indeed, by inhibiting NF-kB activity with IMD-0354, HMC-1 cells spontaneous proliferation was completely repressed (94). Similarly, in *in vitro* assays on KIT mutated mast cell lines, combining a KIT inhibitor with a NFAT-regulator such as a calcineurin phosphatase inhibitor, leads to synergistic increase in cell apoptosis (95).

More recently, the epigenetic reader bromodomain-containing protein-4 (BRD4) has been identified as a novel potential target, as neoplastic MCs express substantial amounts of BRD4 in ASM

and MCL (96). The BRD4-targeting drug JQ1 (a drug blocking the specific interactions between BRD4 and acetylated histones) induces dose-dependent growth inhibition and apoptosis in primary neoplastic cells obtained from patients with advanced SM as well as in HMC-1 and ROSA cells (96). Interestingly, drug effects could be potentiated by addition of PKC412 or ATRA (all trans retinoic acids). Whether these targeted drugs are effective *in vivo* has yet to be determined.

Histone deacetylase inhibitors (HDACi) may also be of clinical interest for treatment of SM. In particular, suberoyl anilide hydroxamic acid (SAHA), also known as vorinostat, have been shown to induce apoptotic cell death in mast cell lines as well as in MCs from patients with SM, through a specific epigenetic downregulation of KIT, whereas healthy bone marrow MCs are less sensitive (97). The HDACi AR-42 has also been described to downregulate constitutively active KIT in malignant murine and canine MCs (98).

Allogeneic Hematopoietic Stem Cell Transplantation (AlloHSCT)

As currently available treatment options fail to achieve durable remissions, alloHSCT remains the only potentially curative treatment for patients with advanced SM and has to be considered in those patients. In the largest published case series, 57 patients received stem cell transplant, mostly from HLA-identical ($n = 34$) or unrelated donors ($n = 17$), and with myeloablative conditioning ($n = 36$) or reduced-intensity conditioning ($n = 21$) (99). Overall survival was 57% at 3 years for all patients, 74% for patients with SM-AHNMD, 43 and 17% for those with ASM and MCL, respectively. The strongest risk factor for poor OS was a diagnosis of MCL (99). Consensus opinion on HSCT in advanced SM and consensus criteria of treatment response were recently published in order to help standardize assessment of treatment response and optimal management in this rare, heterogenous, and severe disease (100, 101).

Treatment for SM Associated With Another Hematological Neoplasm (SM-AHN)

In any variant of SM, another associated hematological neoplasm may be diagnosed as a concomitant disease. As for SM, the AHN component has to be determined by WHO criteria. In most of the patients with AHN, a myeloid neoplasm is diagnosed: chronic myelomonocytic leukemia is commonly detected, but also acute myeloid leukemia (AML), JAK2-mutated myeloproliferative neoplasms (MPN), or MDS overlap disorders. Lymphoproliferative disorders (myelomas and lymphomas) have also been reported, however, more rarely. Coexistence of SM with Philadelphia positive chronic myeloid leukemia is an extremely rare condition. The SM component in SM-AHNMD often presents as ASM and less frequently as MCL. The standard recommendation is to treat the SM component of the disease as if no AHN was diagnosed and to treat the AHN component as if no SM was found, with special attention to potential drug interactions and side effects (44). Patients with ASM-MDS for example could be successfully treated with hypomethylated agents like azacitidine in combination with midostaurin (102). In a similar way, ASM or MCL with associated AML should be treated with midostaurin combined to high-dose chemotherapy, with consideration for allogeneic stem cell transplantation if a certain degree of response is obtained. Moreover, recent data showed that high-risk hematologic neoplasms such as FLT3-positive AML can be managed effectively with midostaurin in combination with chemotherapy (103). Interestingly, in AML associated with ASM, even patients not in CR could be transplanted with a favorable outcome. Finally, patients with an associated myeloproliferative neoplasm exhibiting JAK2 mutations or a JAK2 fusion gene product can be responsive to JAK2-targeting drugs, such as ruxolitinib.

TREATMENT FOR ISM AND SSM

Treatment for ISM is based on mediator-related symptom management, as they are likely to have a normal life expectancy. The keystone of the treatment is to recognize and avoid triggers of MC degranulation. These often are food, stress, excessive heat or cold, hymenoptera stings, alcohol (red wine), and medications as non-steroidal anti-inflammatory drugs, aspirin, or opioids (104).

Most patients with ISM respond to a combination of H1- and H2-histamine receptor antagonists, the standard therapy for pruritus and flushing, and abdominal pain, cramping, and diarrhea, respectively. In patients with persistent gastrointestinal symptoms, adding a proton pump inhibitor may be beneficial in combination with anti-H2 drugs. Cromolyn sodium, a MC stabilizer, can also be useful if gastrointestinal symptoms control is insufficient (105). Adding leukotriene antagonists may be useful, particularly in recalcitrant skin symptoms (106). When conservative measures are unsatisfactory, short courses of corticosteroids may be required to curb refractory symptoms (48, 104). Finally, some patients refractory to optimal conventional therapy will require mast cell cytoreductive treatments, mainly 2-CdA or IFNα (104). In those cases, a careful evaluation of the handicap linked to the symptoms is critical to weigh the beneficial/risk ratio of cytoreductive treatment in ISM.

In patients with SM, special attention has to been made for osteoporosis. Indeed, a cohort study of 75 patients with SM revealed that osteoporosis was present in 31% of patients (42). Osteoporosis should be screened actively and treated with bisphosphonates if indicated. In case of resistant osteoporosis or intolerance to bisphosphonate, alternative drugs may be considered, including low-dose IFN-α or RANKL inhibitors such as denosumab.

Prognosis and natural clinical course of patients with SSM has not been clearly defined, but risk of disease progression and leukemic transformation may be higher and survival shorter than in ISM (107). However, according to general recommendations, patients with SSM who have no symptoms or signs of progression do not require any specific therapy (47, 104). In case of mediator-related symptoms, treatment is identical to that of ISM. In SSM patients with severe anaphylaxis or signs of progression, 2-CdA or IFN is often recommended and is usually effective in reducing the MC burden.

Masitinib (AB1010) is a KIT inhibitor with activity against wild-type KIT, PDGFR, and Lyn, but with no activity on KITD816V

mutants (69). Nevertheless, in a phase 2 study in 25 patients with CM or ISM harboring symptoms refractory to conventional therapy, masitinib showed a significant improvement in the frequency of flushing (62%), pruritus score (36%), and Hamilton rating for depression (43%) (108). The overall clinical response, defined as >50% improvement in baseline symptom, was 56% and maintained at 60 weeks. Toxicity profile was acceptable, with mostly nausea and vomiting (52%), edema (44%), muscle spasms (28%), and rash (28%); however, one patient experienced reversible agranulocytosis (108). In a recently published phase 3 randomized trial, 135 patients were randomized to receive either masitinib (6 mg/kg/day over 24 weeks with possible extension) or placebo (109). By 24 weeks, masitinib was associated with a cumulative response (i.e., >75% improvement from baseline within weeks 8–24) of 18.7% compared with 7.4% for placebo for the following symptoms: pruritus, flushes, depression according to Hamilton scale, and severe fatigue (109). The most frequent severe AEs included diarrhea (11%), rash (6%), and asthenia (6%). Surprisingly, with time, mast cell burden decreases with tryptase level reduction and improvement of skin lesions. These studies indicate that masitinib provides symptomatic improvement in ISM or SSM with severe symptoms refractory to conventional treatment, with an acceptable toxicity profile. Its effects might be due to the inhibition of WT C-KIT, FYN, and LYN, which participates to mast cell activation.

Management of Allergy in Mastocytosis

Prevalence of allergy and atopic disorders in patients with mastocytosis is identical to that of the general population (110–112), but incidence of anaphylaxis is significantly higher, and ranges from 20 to 49% (112–114). Anaphylaxis in mastocytosis may be IgE-mediated or IgE-independent without any identified triggers and is more likely to manifest itself with hypotension as well as life-threatening circulatory collapse. Therefore, some authors recommend the prescription of an epinephrine pen for patients with coexisting allergies, in case of the acute onset of severe symptoms of anaphylaxis (47, 104). Major triggers are Hymenoptera stings, foods, and medications, although in approximately 40%, the elicitor is not known (115). Besides, patients with detectable IgE against bee or wasp venom should undergo life-long hymenoptera venom immunotherapy (116).

In some patients with refractory symptoms of allergy, with high risk of life-threatening anaphylaxis, antibody-mediated depletion of IgE with omalizumab may be useful (117). This humanized IgG kappa monoclonal antibody against IgE has shown a reduction in the frequency of anaphylaxis in limited case studies (117–121). It can also be used in patients with daily symptoms whose disease has been unresponsive to classical treatment, even if the underlying mechanism is not completely understood. Omalizumab inhibits IgE binding to the surface of mast cells and basophils by forming complexes with free IgE in the serum. This triggers the downregulation of the high-affinity IgE receptor (FcepsilonRI) expression on mast cells and basophils and subsequently the reduction of mast cell activation and reactivity (122) However, omalizumab does not seem to decrease mast cell burden, as reflected by stable serum tryptase levels during the treatment (118).

CONCLUSION

During the last two decades, major discoveries have been made for better diagnosis, identification of the clinical and biological abnormalities, and for a better classification of the different forms of mast cell disease, allowing now a better prognosis stratification. In addition, the knowledge of molecular pathways involved in the pathophysiology of mastocytosis has led to the emergence of new symptomatic and cytoreductive drugs that have dramatically improved the quality of life and survival of patients with mastocytosis. However, progress is still needed particularly for controlling psychiatric and neurological symptoms in ISM and to decipher molecular pathways involved in ASM, MCL, and sarcoma in the hope to find new targeted drugs or to use new combination.

AUTHOR CONTRIBUTIONS

MV, FB, and OH: design of the review. MV: manuscript writing. FB and OH: critical revision and final approval.

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REVIEW
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New Insights into Drug Reaction with Eosinophilia and Systemic Symptoms Pathophysiology

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Drug reaction with eosinophilia and systemic symptoms (DRESS), also known as drug-induced hypersensitivity syndrome, is a severe type of cutaneous drug-induced eruption. DRESS may be a difficult disease to diagnose since the symptoms mimic those of cutaneous and systemic infectious pathologies and can appear up to 3 months after the initial culprit drug exposure. The symptoms of DRESS syndrome include rash development after a minimum of 3 weeks after the onset of a new medication, associated with facial edema, lymphadenopathy, and fever. Biological findings include liver abnormalities, leukocytosis, eosinophilia, atypical lymphocytosis, and reactivation of certain human herpes viruses. In DRESS, liver, kidneys, and lungs are frequently involved in disease evolution. Patients with serious systemic involvement are treated with oral corticosteroids, and full recovery is achieved in the majority of cases. DRESS is a rare disease, and little is known about factors that predict its occurrence. The key features of this reaction are eosinophil involvement, the role of the culprit drug, and virus reactivation that trigger an inappropriate systemic immune response in DRESS patients. Interestingly, it was evidenced that at-risk individuals within a genetically restricted population shared a particular HLA loci. In this respect, a limited number of well-known drugs were able to induce DRESS. This review describes the up-to-date advances in our understanding of the pathogenesis of DRESS.

Keywords: eosinophil, drug-induced eruption, virus replication, cytokines, side effects

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INTRODUCTION

Drug reaction with eosinophilia and systemic symptoms (DRESS) is a severe cutaneous drug-induced eruption (DIE) characterized by a virus like clinical presentation. Typically, the patient presents fever, lymphadenopathy, facial edema, and a maculopapular rash. Systemic involvement includes hepatitis and interstitial pneumonia. Severe renal and cardiac [eosinophilic myocarditis (EM)] involvement may be also found. Since DRESS is triggered by long-term drug exposure, it is essential to seek and identify the culprit drugs in the months prior to eruption. Other severe DIEs include Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), both characterized by skin detachment. They occur after a short drug exposure and do not present systemic involvement (1). Non-severe DIE characterized by a benign maculopapular eruption does not present systemic signs or skin detachment. On the one hand, the sequence of immunological and biological events at the onset of DIE that play a key role in the pathogenesis may be shared between the three different forms of severe DIE; on the other hand, specific clinical manifestations may be influenced

TABLE 1 | DRESS inducers.

Anticonvulsant
Carbamazepine
Lamotrigine
Oxcarbamazepine
Phenobarbital
Phenytoin
Antibiotic
Minocycline
Sulfalazine
Sulfamethoxazole
Vancomycin
Others
Abacavir
Allopurinol
Dapsone
Mexilletine
Nevirapine
Salazosulfapyridine
Strontium ranelate

Adapted from Cacoub et al. (4).

by patient-intrinsic genetic factors or external factors such as viral infection or reactivation that are not yet clearly identified and deciphered. DRESS may be difficult to diagnose and identify because symptoms evidenced could mimic several other diseases including infectious diseases and can appear a long time after initial culprit drug exposure. The RegiSCAR criteria were created to better evidence DRESS in drug-treated patients presenting a DIE (2). RegiSCAR is based on seven independent parameters and three of them are required (fever > 38°C, acute skin rash, lymphadenopathy, internal organ involvement, blood count abnormalities including atypical lymphocytes and eosinophilia) for the diagnosis of DRESS. Other criteria were developed in Japan: the Japanese consensus group diagnostic criteria for drug-induced hypersensitivity syndrome (3). These new diagnostic criteria require that a minimum of seven of nine symptoms be found to diagnose DRESS [skin eruption a minimum of 3 weeks after starting medication, symptoms not stopped when the drug is discontinued, fever, liver biological abnormalities, circulating leukocyte abnormalities including leukocytosis, atypical lymphocytosis, eosinophilia, lymphadenopathy, and reactivation of human herpesvirus 6 (HHV-6)] (Table S1 in Supplementary Material). In DRESS, the organs frequently involved are liver, kidneys, and lungs, and usual blood abnormalities include eosinophilia, atypical lymphocytes, and lymphocytopenia. Interestingly, a limited number of well-known drugs mainly including anticonvulsants are able to induce DRESS (**Table 1**). Patients with DRESS are usually treated with immunosuppressive drugs including mainly systemic corticosteroids, whereas usage of intravenous immunoglobulin is controversial. Full recovery is achieved in 90% of patients (4).

Drug-specific T-cells have been identified in patients and are supposed to be the primary effectors of the pathology in DIE patients (5). However, T-cells derived from healthy donors can also be activated with drugs without previous drug exposure (6–8). These interesting data could predict a higher occurrence of DIE in patients taking drugs than observed in real life. All the factors that may identify "at-risk" individuals in patients exposed to drugs are not yet determined. Interestingly, some genetic risk factors of DIE are associated with different HLA loci (9–14), these findings of primary importance cannot account alone for DIE occurrence, because HLA risk alleles are considered neither fully necessary nor fully sufficient for disease development (10). Interestingly, a relationship was also clearly evidenced between DIE and virus infection or reactivation. In this respect, endogenous herpes virus (HSV) can be reactivated and presented to the immune system in DRESS patients (15). However, there is no evidence that reactivation of HSVs can also occur in other DIE such as SJS and TEN, despite some isolated clinical cases, whereas some virus and mycoplasma induced eruptions may mimic SJS and TEN (16). Recurrence of DRESS with unrelated drugs can be observed in 25% of cases, whereas very little or no recurrence is found with TEN and SJS patients (17). There may also be factors related to the nature of the culprit drug, severe systemic involvement has been associated with allopurinol and minocycline, and prolonged evolution with non-Caucasian ethnicity and minocycline (18, 19).

PATHOPHYSIOLOGY

The Hapten Theory and p-i Concept

A hapten is a small non-immunogenic molecule that becomes antigenic when it is bound to a carrier protein [reviewed in Ref. (20)]. By contrast, pro-hapten molecules require metabolization to become immunogenic and to be able to bind to proteins. Since detoxification enzymes are expressed by all patients, it has been proposed that detoxification enzyme polymorphisms could be responsible for the development of DIE and DRESS in only a subgroup of patients. However, no such polymorphism has been identified yet in patients with DIE (20). Indeed, the majority of small drug molecules can be recognized by the human immune system including T-cells despite lacking hapten structure (21). The direct binding of drugs and their metabolites to HLA that trigger T-cell responses has been called "p-i" concept (pharmacological interaction of drugs with immune receptor) (22). T-cells isolated from healthy donors and patients present the capacity to be stimulated by certain drugs indicating that some individual susceptibility factors are required to mount a pathological immune response (20).

Drug Interactions with HLA Type

Very interesting results have been obtained showing that specific HLA variants are responsible for very high increased risk of DRESS or hypersensitivity occurrence. The first study to show a clear relationship between DIE and HLA subtype was performed in 2002 (11), the authors identified a very strong link between HLA-B*5701 in HIV-positive caucasians and the development of hypersensitivity to abacavir ($p < 0.0001$). The specific mechanism of T-cell activation by abacavir in the HLA groove was then identified in 2012 (23). The results were largely confirmed by other teams (24). Abacavir was found able to bind noncovalently and specifically to the peptide-binding groove of HLA-B*5701 molecule. The presence of the abacavir molecule in the groove induces a change in the repertoire of peptide presentation and

as a consequence a T-cell response against a HLA/self-peptide complex. Those modifications of the immune presentation of endogenous and self proteins induce an important inflammatory response that triggers systemic clinical and biological signs. Interestingly, they also evidenced that the non-covalent abacavir binding to the HLA groove modified the self-peptide repertoire presented in the groove and represented a possible immunological mechanism of autoimmunity that can appear after DIE (24, 25). In this respect, some cases of autoimmune diseases including diabetes and thyroiditis were evidenced in patients after occurrence of a DRESS (26, 27).

HLA links were also found for specific DRESS inducers. Carbamazepine is an anticonvulsant drug considered as a major DIE inducer. Susceptibility to carbamazepine reactions has been evidenced in patients with HLA-B*1502 variant (14). The mechanism of T-cell activation induced by carbamazepine is supposed to be the same as that described for abacavir (23). HLA-B*1502 is commonly found and exclusive to South East Asia populations. In contrast, carbamazepine immune response in European populations is associated with the presence of HLA-B*3101 (9). Allopurinol induces reactions in HLA-B*5801 patients (12). All these HLA associations probably share a similar T-cell immune activation mechanism that depends on culprit drug/HLA interaction and HLA/peptide repertoire presentation. The identification of DIE risk-associated HLA variants opens new avenues for physicians by using patient stratification for DIE risk using HLA typing. In a prospective study, carbamazepine was not used in Taiwanese patients carrying the HLA-B*1502 variant (28). By determining HLA phenotype before drug introduction, the incidence of DIE was dramatically reduced, since none of the 4,120 HLA-B*1502 negative included patients developed SJS, TEN, or DRESS compared to an estimation of 10 expected SJS and TEN cases. Mild rash was found in 6% of the non-HLA-B*1502 patients. These results highlight the major role of the major histocompatibility complex in DIE, whereas additional risk factors for benign eruption may play a role in 6% of the population. In conclusion, for certain drugs and particular populations, screening patients HLA haplotype before drug introduction could be used to reduce DIE occurrence (29).

Antiviral Responses

It is now largely accepted that DRESS can be associated with reactivation of inactive viruses, especially in individuals infected with members of the human herpes viridae family including mainly HHV-6, EBV, and CMV (30–35). As a consequence, we proposed that viruses may play a key role in DRESS pathogenesis. Interestingly, HHV-6 and EBV both induce a disease associated with fever and skin rash. HHV-6 is able to infect T-cells (36) and to dysregulate $CD8^+$ lymphocytes by inducing abnormal expression of CD4 that may increase T-cell activation and antiviral response (37). Picard et al. evidenced a massive anti-HSV T-cell response in the blood and involved organs of DRESS patients (15). In this study, 40 cases of DRESS were analyzed; Picard and colleagues showed that circulating EBV-specific $CD8^+$ T-cells were expanded within the T-cell population, representating up to 21% of the total cytotoxic T-cell population in DRESS patients compared with <0.1% in control patients. Activated T lymphocytes produced large amounts of TNFα, IL-2, and IFNγ, considered as key mediators of the cytokine release that induces the symptoms found in DRESS patients. Interestingly, EBV-specific T lymphocytes were detected in affected organs in DRESS patients including liver, skin and lungs. Moreover the authors demonstrated that the culprit drug is able to induce *in vitro* viral reactivation (15). In this respect, reactivation of the HSV that triggers uncontrolled antiviral T lymphocyte response leads to systemic inflammation associated with organ failure. These immunological events may represent a specific feature of DRESS compared to other DIE (**Figure 1**). In addition, IL-10 secretion by B cells and inflammation may promote viral reactivation. The inflammation induced by the virus and the systemic inflammation found in DRESS may represent a loop that induces a long lasting inflammation process. In this respect, multiple HHV family member reactivations were identified in DRESS patients (15, 35, 38, 39). By contrast, HSV reactivation or infection in SJS and TEN patients is not proven (40, 41). Further investigation is now required to decipher the mechanisms and roles of the culprit drug-specific T lymphocytes response in DRESS patients, in order to better understand the role of culprit drugs on the onset and the amplification of anti-HSV immune responses. Interestingly, expansion of regulatory T-cell populations (T-reg) can be found in DRESS patients (42). This phenomenon could also play a role in infection or reactivation of HHV- 6 (43). Altered function of T-reg may also plays a role in the occurrence of autoimmune disease evidenced in DRESS patients after initial DIE.

Eosinophilia

Cutaneous DIEs are usually associated with eosinophilia, and cutaneous eosinophil infiltration plays a key role in cutaneous eruption. Interestingly, cutaneous eosinophil infiltration is more pronounced in DRESS (44). For DRESS, eosinophilia is a diagnosis criterion and is found in 80% of patients (4). The number of eosinophils is increased in blood and in skin and involved organs, whereas in physiologic conditions eosinophils are not present in skin, liver, and lungs. Eosinophils are circulating granulocytes involved in the host defense against parasites, bacteria, viruses and in allergic reactions. Eosinophils are also involved in diverse inflammatory responses and can regulate innate and adaptive immunity. Eosinophils are derived from bone marrow precursor and differentiate mainly in response to IL-5. After, they enter the peripheral blood and circulate. Finally, eosinophils enter and home into tissues following an eotaxin gradient. Within the tissue, they can develop an extracellular trap formation. IL-5 again is a key cytokine for eosinophil survival, proliferation, and activation (45). Both $CD4^+$ and $CD8^+$ T-cells are thought to be involved in IL-5 production prior to eosinophil recruitment. The main factors in DRESS that activate and recruit eosinophil are IL-5 and eotaxin. In synergy with IL-5, eotaxin-1 has been identified to be a very selective and potent recruiter for eosinophil (46, 47). Eotaxin-1 is a CC chemokine, also known as cysteine cysteine ligand 11 (CCL11). Under basal conditions or during allergy and inflammation, eotaxin *via* interaction with its receptor CCR3 acts in synergy with IL-5 to recruit eosinophils into tissues. Interestingly, an increase in serum eotaxin level has been highlighted during

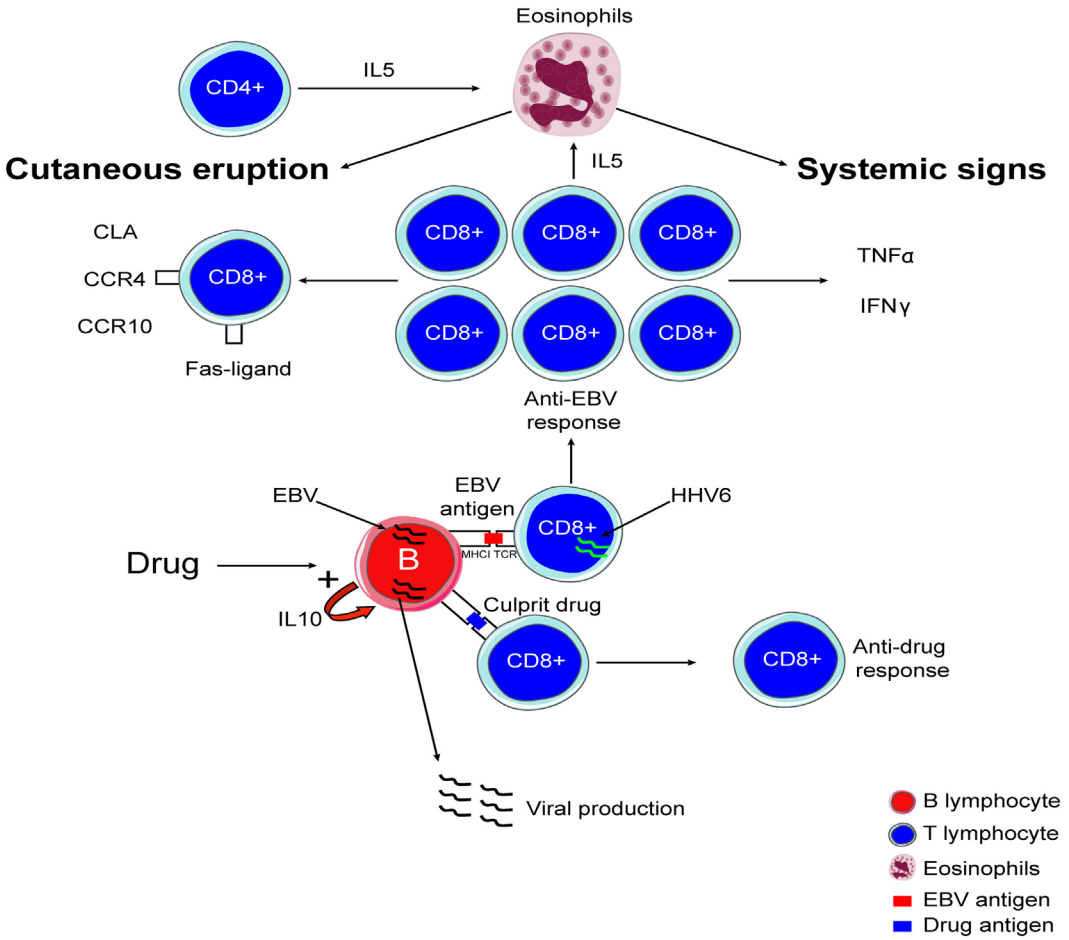

FIGURE 1 | Immunological mechanism involved in drug reaction with eosinophilia and systemic symptoms.

the course of DRESS syndrome and eotaxin, in synergy with IL-5 has been identified as a key player in activating and recruiting eosinophils in drug-induced cutaneous eruption (47).

In addition to IL-5 and eotaxin, eosinophil migration from circulation can also be controlled by thymus activation-regulated chemokine (TARC/CCL17) (48). TARC is a member of the CC chemokine family that is constitutively expressed in the thymus. It is the ligand of CCR4 that is expressed mainly by Th2 lymphocytes, basophils, and natural killers and is also produced by endothelial cells, bronchial epithelial cells, fibroblasts, keratinocytes, and dendritic cells (48). The pathogenic role of TARC has been highlighted in skin diseases such as atopic dermatitis, and bullous pemphigoid. TARC is known to be present in cutaneous lesions massively infiltrated by eosinophils; moreover, serum TARC levels reflect disease activity (48). In addition to the activity of attraction of Th2 lymphocytes, studies have shown that TARC is a potent eosinophil chemoattractant and has been associated with eosinophilic pustular folliculitis, highlighting a correlation between serum TARC levels and peripheral blood eosinophil number (48). Interestingly in drug eruption, a strong correlation between serum TARC levels and blood eosinophil count has been highlighted. Serum TARC levels during the acute phase were higher in DRESS patients compared with SJS/TEN patients or in cases of benign maculopapular eruption. TARC levels were correlated with the occurrence of skin eruptions, serum IL-5 levels and eosinophil counts (49). It has been demonstrated that the $CD11c^+$ dermal dendritic cells in DRESS patients may be the main source of TARC. Interestingly, due to 100% sensitivity and 92.3% specificity in diagnosing DRESS and elevated levels observed in the serum especially at the early stage of DRESS, serum TARC measurement could even be a potent diagnostic value for DRESS among patients with various types of drug eruptions (49) (**Figure 2**).

Interestingly, we found in DRESS patients an over expression of IL-17 including IL-17E (IL-25) that play a key role in eosinophil blood increase (15). IL-17E over expression may increase circulating eosinophils, IL-4, IL-5, eotaxin, and IgE. As a consequence, IL-17E may play a key role in the control and amplification of the eosinophilic immune responses found in DRESS patients (50).

Damage Induced by Eosinophilia

In multiple target tissues, eosinophils specifically eliminate antibody bound parasites through the release of cytotoxic granule proteins (45). Therefore, cytotoxic release produces organ

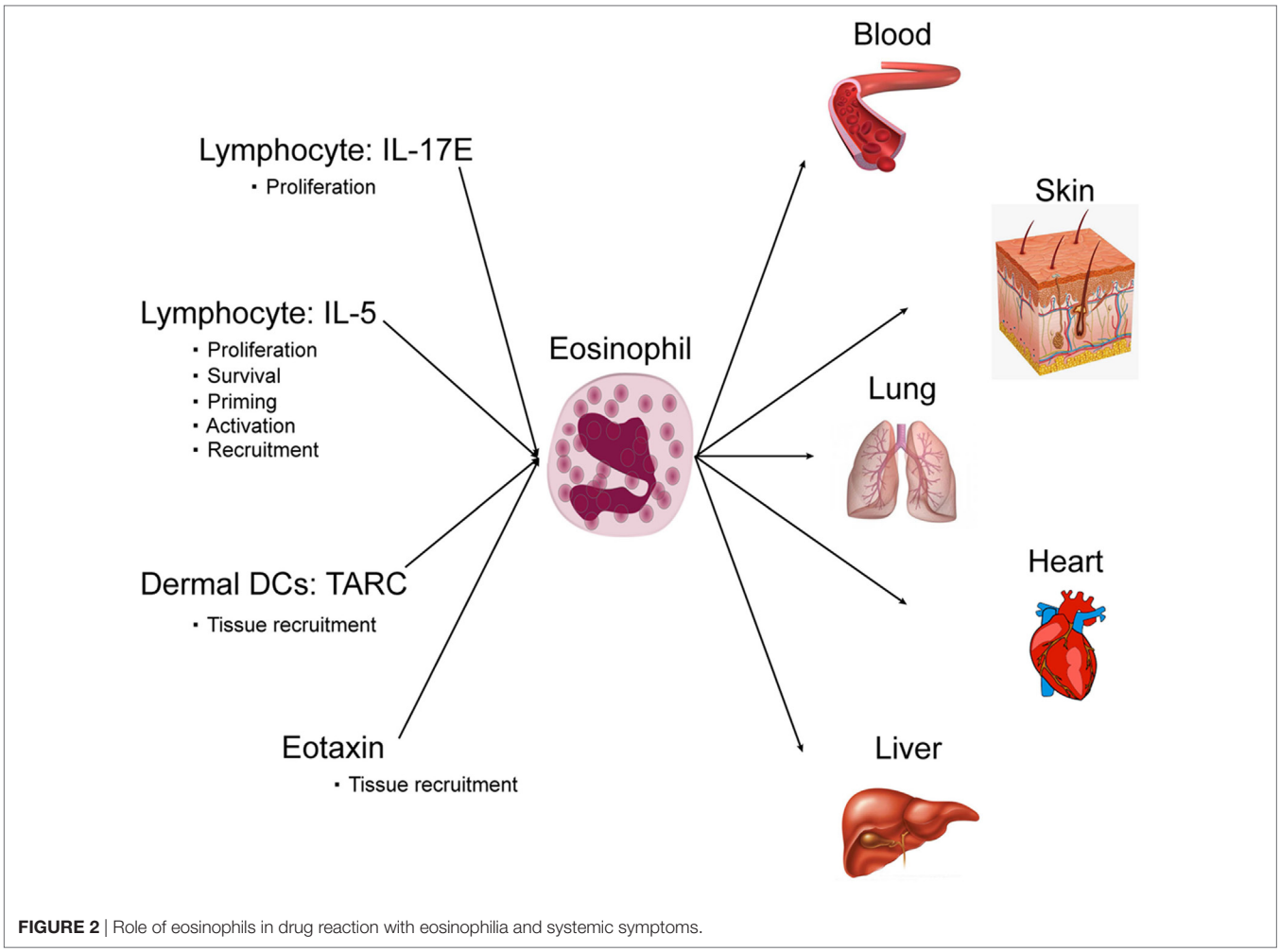

FIGURE 2 | Role of eosinophils in drug reaction with eosinophilia and systemic symptoms.

damage. The most dangerous involvement in DRESS patients is caused by heart eosinophil damage.

Eosinophilic myocarditis is a rare and potentially fatal condition if left untreated. EM can have a delayed presentation and can appear even after a long delay. Delayed corticosteroid treatment can result in heart failure and death. Cardiac involvement must be detected early by echocardiography, and elevated serum troponin since ECG signs may not be present and may be evidenced too late. Eosinophil toxicity may also involve lung causing interstitial pneumonitis detected by early chest radiography. Interstitial pneumonitis requires systemic steroid treatment. Hepatitis, detected in blood by hepatic enzyme increase, is a diagnostic criterion of DRESS. In rare cases, hepatic involvement may lead to fulminant hepatitis with dramatic consequences including severe hepatic failure. In a limited number of cases of severe hepatic failure, hepatic transplantation may be required. In severe hepatic failure, steroid usage is debated. In diverse organs including digestive tract, thyroid, and central nervous system, nerves may also be infrequently involved (**Figure 2**).

Finally, eosinophil activation and multiplication is related to antiviral and culprit drug immunological response leading to organ eosinophil infiltration. Granule release represents a key factor of tissue damage in DRESS patients.

In conclusion, DRESS is a systemic drug reaction wherein eosinophil activation and multiplication is driven by an immunological response directed against viral reactivation and a culprit drug. Eosinophils infiltrate organs in response to chemokines including eotaxin-1 and TARC, in synergy with IL-5, and granule release represents a key factor of tissue damage.

AUTHOR CONTRIBUTIONS

BJ and PM wrote the manuscript and made the figures.

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SUPPLEMENTARY MATERIAL

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