SEVERE EOSINOPHILIC DISORDERS: MECHANISMS AND CLINICAL MANAGEMENT

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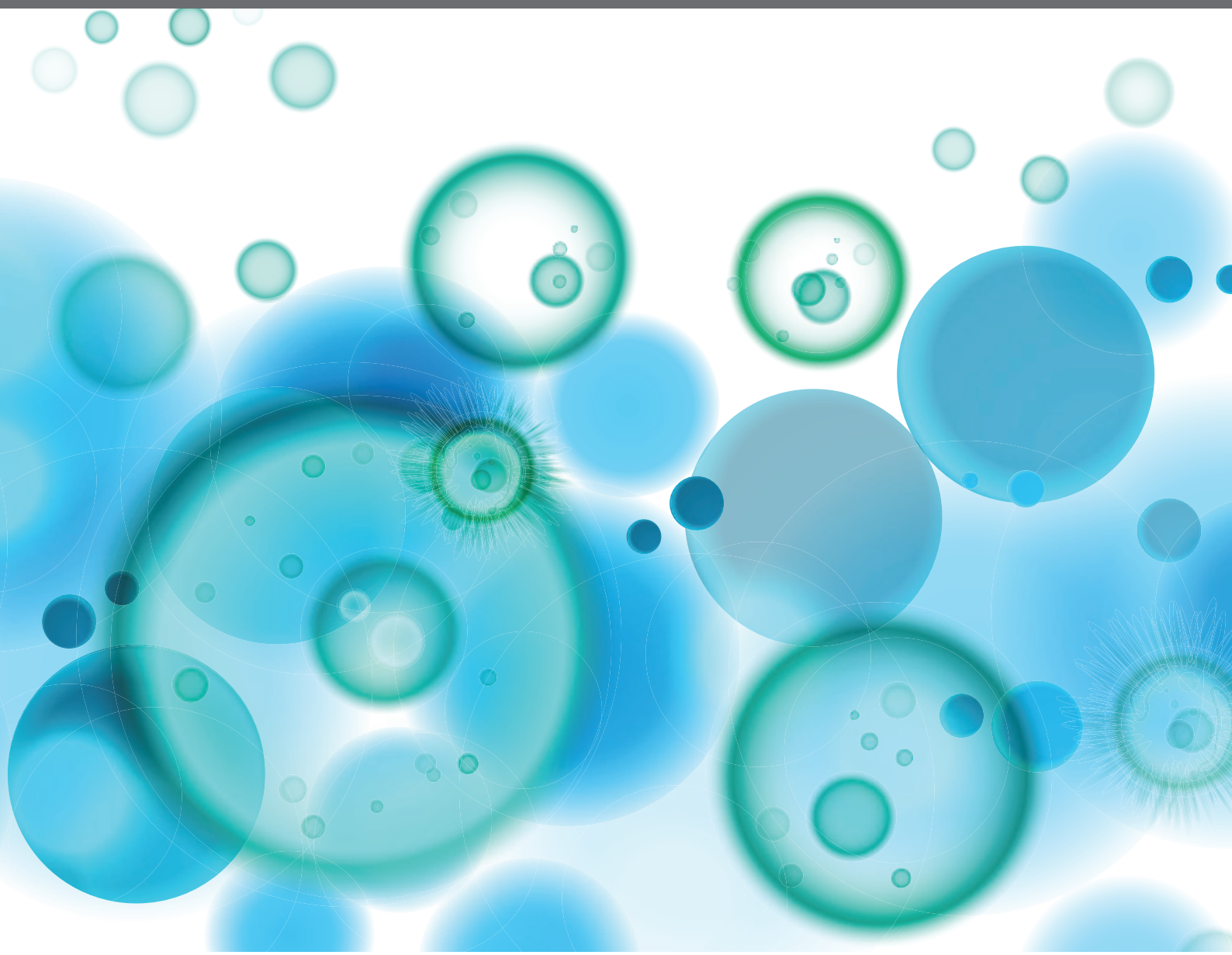

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SEVERE EOSINOPHILIC DISORDERS: MECHANISMS AND CLINICAL MANAGEMENT

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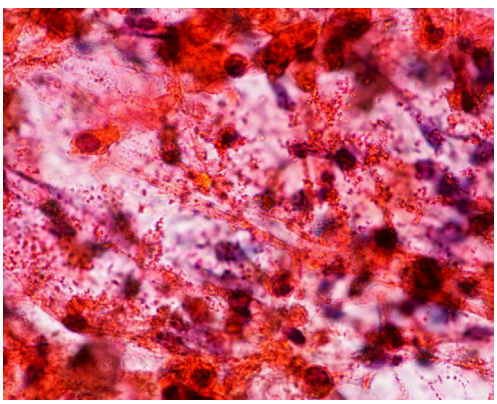

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EDITORIAL
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Editorial: Severe Eosinophilic Disorders: Mechanisms and Clinical Management

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Keywords: asthma, eosinophils, hypereosinophilia, IL-5, degranulation, rhinosinusitis

Editorial on the Research Topic

Severe Eosinophilic Disorders: Mechanisms and Clinical Management

Severe eosinophilic disorders present a significant clinical challenge. This is due to several factors, including the varied etiologies, a high tendency to recur, and a frequent need to treat with long-term systemic steroids, accompanied by a host of short- and long-term side effects. Eosinophilic disorders can in certain circumstances be life threatening. Lessons from the recent emergence of anti-IL-5 monoclonal antibody therapies have highlighted the clinical benefits of eosinophil depletion. To promote improved understanding of the eosinophil, a pleiotropic multifunctional leukocyte, and associated eosinophilic diseases, potentially leading to advances in clinical management, we sought with this Research Topic both original research and review articles that were truly bench to bedside in their consideration of severe eosinophilic disease, encompassing studies of eosinophil cell biology and immunology, murine models of eosinophilic inflammation, and translational/clinical investigations.

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EOSINOPHIL EXTRACELLULAR TRAPS

Mukherjee et al. review a growing body of literature that delineates the ability of eosinophils to release DNA extracellular traps. These are similar to neutrophil extracellular traps, in that they may have important functions in defense against extracellular pathogens. An important consideration in this article is contextualizing the process of "ETosis" alongside the other canonical pathways for eosinophil degranulation.

Conversely, Ueki et al. (co-editor for this Research Topic) extend the discussion of the potential pathogenic role of eosinophil extracellular traps in the specific case of allergic bronchopulmonary aspergillosis (ABPA). In this entity, the accumulation of eosinophils and formation of eosinophil extracellular traps in response to fungal elements may thicken pathologic mucous and further perpetuate airway inflammation, representing a novel perspective into this eosinophilic lung disease.

PIECEMEAL DEGRANULATION

One of the other now canonical pathways for eosinophil degranulation is known as piecemeal degranulation (PMD). Dias et al. demonstrate in a murine model of *Schistosoma mansoni* infection that the eosinophils recovered from the liver were predominantly undergoing PMD. Their data also supported the release of the granule protein MBP by PMD, suggestive of well-coordinated, selective release of granule proteins by eosinophils during parasitic infection.

EX VIVO STUDIES OF MURINE AND HUMAN EOSINOPHILS

Amorim et al. reported on novel effects of leptin stimulation of human and murine eosinophils, extending knowledge on the emerging role of leptin in immune regulation. In their study, leptin was shown to activate leukotriene C_4 synthesis with an increase in lipid body/lipid droplet formation through the sequential release of CCL5 and prostaglandin D_2.

Comprehensive metabolic profiling by extracellular flux analysis from Porter et al. provides valuable insights into the metabolic flexibility of human eosinophils. The study indicates that while eosinophils have similar capacity for glycolysis in comparison to neutrophils, they have a greater ability to utilize glucose for oxidative metabolism. These findings have important implications in contextualizing the diverse immunoregulatory capacities of eosinophils.

MURINE MODELS OF ALLERGIC AIRWAYS INFLAMMATION

Gubernatorova et al. report the results of conditional gene targeting of IL-6 in a house dust mite model of allergic asthma. IL-6 has been noted in human disease to be an important biomarker of severity by the Severe Asthma Research program [Peters et al. (1)]. The authors of the study in this Research Topic found that dendritic cells and macrophages were the sources of IL-6 and that ablation of IL-6 reduced eosinophilic inflammation, as did specific macrophage IL-6 ablation.

Two manuscripts in the Research Topic involved the role of non-apoptotic Fas signaling in resolution of allergic airways inflammation. In the first, Williams et al. show that non-apoptotic Fas signaling is key to the resolution of eosinophilic inflammation and that disruption of non-apoptotic Fas signaling results in prolonged eosinophilic airways inflammation. In the second manuscript, Ferreira et al. utilize T cell-specific Fas conditional knockout mice to show that antigen exposure during homeostatic T cell proliferation after non-lethal radiation in these animals was sufficient to induce prolonged eosinophilic airways inflammation. Together, these manuscripts highlight the likely importance of Fas signaling in resolution of Th2 inflammation.

CLINICAL CONSIDERATIONS IN ASTHMA AND RHINOSINUSITIS

In the era of anti-IL5 therapies for asthma, it has been well-established that eosinophil-targeting strategies reduce asthma exacerbations. In their mini-review, Nakagome and Nagata discuss the potential roles of eosinophils in promoting asthma exacerbation, with a section of particular interest that details the ability of viral infection to augment eosinophil-mediated pathology.

Kobayashi et al. report a blinded, placebo controlled study testing a novel delivery of inhaled steroid to the upper airway in patients with chronic rhinosinusitis in the setting of asthma whose upper airway disease was refractory to the usual delivery method of intranasal steroid via the inhalation through the nares. In this study, inhaling beclomethasone through for delivery to the lower airways followed by exhalation through the nose for deposition to the upper airways led to radiographic and symptomatic improvements in the upper airway, suggesting that this alternative delivery strategy may be a viable therapeutic option.

Eotaxin-3 (CCL26) has been noted as an important epithelial-derived chemokine associated with an array of eosinophilic disease. Yamada et al. extend this growing body of work to establish circulating eotaxin-3 as a biomarker that correlates with eosinophil infiltration in tissue samples from chronic rhinosinusitis independent of blood eosinophil counts.

HYPEREOSINOPHILIA AND HYPEREOSINOPHILIC SYNDROMES

The evaluation of hypereosinophilia is challenging in pediatric patients and important distinctions compared to the evaluation of hypereosinophilia in adults exist. Schwartz and Fulkerson present their differential diagnosis and approach to the child presenting with hypereosinophilia.

There are common cytogenic abnormalities associated with myelodysplastic syndromes (MDS), but there are less well-characterized abnormalities that have been described. Rai et al. report on two cases of MDS with hypereosinophilia that led to fatal end organ damage, with both cases associated with the poorly characterized cytogenetic abnormality der(1;7)(q10;p10).

CONCLUSIONS

Eosinophilic disorders represent a wide range of conditions that share the commonality of the eosinophil itself, allowing for studies of eosinophil biology to inform multiple disease entities and, in turn, for studies of one eosinophilic disorder to inform understand of others. The editors of this Research Topic believe that continuing to explore eosinophil biology and commonalities between eosinophilic diseases will continue be a fruitful endeavor.

AUTHOR CONTRIBUTIONS

PA drafted the manuscript. JN and SU have made a substantial, direct and intellectual contribution to the work. All authors provided approval for publication of the content.

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REVIEW
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Eosinophil Extracellular Traps and Inflammatory Pathologies—Untangling the Web!

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Eosinophils are an enigmatic white blood cell, whose immune functions are still under intense investigation. Classically, the eosinophil was considered to fulfill a protective role against parasitic infections, primarily large multicellular helminths. Although eosinophils are predominantly associated with parasite infections, evidence of a role for eosinophils in mediating immunity against bacterial, viral, and fungal infections has been recently reported. Among the mechanisms by which eosinophils are proposed to exert their protective effects is the production of DNA-based extracellular traps (ETs). Remarkably, DNA serves a role that extends beyond its biochemical function in encoding RNA and protein sequences; it is also a highly effective substance for entrapment of bacteria and other extracellular pathogens, and serves as valuable scaffolding for antimicrobial mediators such as granule proteins from immune cells. Extracellular trap formation from eosinophils appears to fulfill an important immune response against extracellular pathogens, although overproduction of traps is evident in pathologies. Here, we discuss the discovery and characterization of eosinophil extracellular traps (EETs) in response to a variety of stimuli, and suggest a role for these structures in the pathogenesis of disease as well as the establishment of autoimmunity in chronic, unresolved inflammation.

Keywords: eosinophils, degranulation, extracellular traps, ETosis, cytolysis, airways, sputum

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INTRODUCTION

Eosinophils have intrigued physicians and scientists alike since the time of their first report in 1879. While lower numbers in systemic circulation ($<0.5 \times 10^9$/L) are generally considered healthy (1), increased peripheral numbers and, more importantly, tissue accumulation is associated with disease (2). Essentially, eosinophilia is best known in host responses to helminth infections, followed by the recognized pathological role of eosinophils as end-stage effector cells in allergic diseases such as asthma, atopic dermatitis, rhinitis, eczema, and related conditions. Eosinophils have also been implicated in non-allergic disease pathologies, such as Crohn's disease, chronic obstructive pulmonary disease (COPD), and more recently, non-atopic asthma. The common denominator underlying all these pathologies is the ability of eosinophils to secrete potent immunomodulatory factors stored as pre-formed mediators within their granules (cytokines, chemokines, growth factors), as well as *de novo* synthesized lipid mediators and oxidative metabolites (3, 4). Moreover, the presence of clusters of free intact, membrane-bound eosinophilic granules (FEGs) and cationic granule proteins [major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived

neurotoxin, and eosinophil peroxidase (EPX)] have been identified in inflammatory foci in eosinophilic diseases (5, 6). Indeed, presence of FEGs and cationic granule proteins is a better marker of disease activity than intact eosinophils as reported for asthma (5, 7). Despite their association with diseased states, granule proteins have been naturally (evolutionarily) selected for host defense against viruses, bacteria, fungi, and helminths [reviewed elsewhere in (8)]. The mechanisms of mediator release and tissue dispersal of eosinophil granules have therefore remained a topic of intense research.

EOSINOPHILS: DEGRANULATION, PRIMARY LYSIS, AND GRANULE RELEASE

In response to receptor stimulation, eosinophils typically release their granules (intact) or products of granules through degranulation. Degranulation is an umbrella term used to define processes where there is release of granule proteins from viable cells, or the release of intact/ruptured granules from "dying" cells, without reference to the specific underlying regulatory mechanism (6, 9). Four modes of degranulation have been reported for eosinophils using transmission electron, confocal, and super resolution microscopy studies, namely: (i) *classical exocytosis* (ii) *compound exocytosis* (10); (iii) *piecemeal degranulation* (PMD); and (iv) *cytolysis* [reviewed extensively, (9)].

The fourth physiologic form of degranulation is *cytolysis* which involves chromatolysis (disintegration of the chromatin of cell nuclei), followed by rupture of the cell's plasma membrane, leading to release of FEGs (11). Research in the past decade has suggested that eosinophil cytolysis leading to the production of FEGs may be a major *modus operandi* of eosinophils *in vivo*, and is not a crush artifact of *in vitro* experimentation [release of cell granules due to mechanical damage to cells or inadequate tissue handling (12)]. Indeed, it is the second most commonly observed eosinophil degranulation mode after PMD in allergic tissues (13), ranging from 10 to 33% of all degranulation modes (13–15). Cytolysis has been observed as a dominant secretory mechanism in other diseases such as eosinophilic esophagitis (16). Cytolysis is also referred to as necrosis, where ruptured eosinophils release FEGs into the surrounding milieu (9). Whether cytolysis is synonymous to necrosis has been debatable ever since the early observations of FEG clusters in diseased inflammatory tissue. Persson and Erjefelt termed the phenomenon as "primary lysis" and concluded such events to be the fate of highly activated eosinophils (12). It is now considered that eosinophil cytolysis includes spilling of cellular contents, including nuclear materials such as histones and DNA, in addition to granular proteins onto the extracellular matrix, and can be from either live or lytic cells (17). The current review article focuses on recent observations of extracellular DNA traps associated with granule release from both live (18) and lytic eosinophils (19), largely defined as eosinophil "cytolysis," and their role in host defense and potential contribution to disease pathology.

Abbreviations: ANA, anti-nuclear antibodies; DAMPs, danger associated molecular patterns; EET, eosinophil extracellular traps; ECP, eosinophil cationic protein; EPX, eosinophil peroxidase; IF, immunofluorescence; MBP, major basic protein; SEM, scanning electron microscopy; LPS, lipopolysaccharide.

EVOLUTION OF EXTRACELLULAR TRAPS: ETosis

The defining feature of our immune system is host defense involving recognition and elimination of pathogens that endanger our health. Typically, innate and adaptive immunity work in a continuum for achieving immune homeostasis. In the innate circle, polymorphonuclear neutrophils (PMNs) have been well-studied as the first line of defense against invading pathogens, while eosinophils are classically known for their proliferation and recruitment in response to helminthic parasites. Phagocytosis of microbial pathogens by innate cells (neutrophils and macrophages) followed by intracellular lysosomal degradation is the best-known innate immune mechanism for host defense against these microorganisms.

While anti-microbial mechanisms associated with phagocytosis have been very well-characterized, an unexpected observation was recently made demonstrating that neutrophils were capable of releasing nuclear DNA onto extracellular pathogens to control their growth and proliferation. These extracellular DNA deposits were termed neutrophil extracellular traps (NETs), and were first proposed in 2004 as a phagocytosis-independent anti-microbial pathway (20). Neutrophil extracellular traps (NETs) were found studded with high local concentrations of anti-microbial agents (peptides, proteases, reactive oxygen species), released into extracellular matrix to ensnare or degrade virulence factors and aid in the killing of bacteria (20, 21).

Similar extracellular traps (ETs) have subsequently been observed from other cells of the innate family members, for example, mast cells (22), monocytes (23), tissue macrophages (24), and eosinophils (18, 25–27). Recently, even lymphocytes, key effector cells of the adaptive immune system, were demonstrated to eject ETs *in vitro* when incubated with serum obtained from systemic erythematous lupus (SLE) patients (28). Based on such elegant observations, the release of ETs from immune cells that was associated with rupture of the cell membrane, was considered to be a novel cell death pathway (distinct from necrosis and apoptosis), and referred collectively as ETosis [extensively reviewed in (29)].

ETosis is proposed to be an immune-protective host defense mechanism, particularly at barrier sites, that has been conserved through evolution. Indeed, ET-like structures containing DNA have been discovered in plant root-tips that protect against fungal infections (30). Regulated release of chromatin ETs that ensnares microorganisms has also been demonstrated in several invertebrates (remarkably, an acoelomate), and essentially strengthens the notion that DNA traps are evolutionarily conserved as a defense weapon (31).

Notably, eosinophil-derived ETs have been observed in higher vertebrate forms. For example, both neutrophils and eosinophils undergo ETosis to trap and kill *Haemonchus contorta*, a gastrointestinal nematode in ruminant animals. Being trapped

in ETs, the migration of the larvae to sites of infection is limited (32). In the case of the bovine respiratory pathogen *Manheimia haemolytica*, ETosis from innate immune cells including macrophages was shown to limit the spread of this bacterium (33).

EOSINOPHIL EXTRACELLULAR TRAPS: ARE EOSINOPHILS DEAD OR ALIVE?

Upon appropriate stimulation, eosinophils release intracellular DNA to form web-like ETs, embedded with granular proteins (refer to **Figure 1**). The deposition of extracellular DNA by immune cells is considered to be a double-edged sword, i.e., implicated both as a host defense machinery and in the development of certain pathologies. Compared to NETosis, coined by Steinberg and Grinstein, which is now accepted as a novel cell death pathway (21), the concept of extracellular trap formation with respect to eosinophils is conflicted. At present, there are two schools of thought regarding the formation of eosinophil extracellular traps (EETs) and the fate of cells releasing them (34, 35). In 2008, DNA net-like structures, similar to those reported for neutrophils, were observed for the first time by Yousefi et al. to be catapulted out of "live" eosinophils (18). Interestingly, the DNA present in these EETs was reported to be of *mitochondrial* origin, and DNA strands were embedded with granule proteins such as MBP and ECP, evident from co-localization studies both *in vitro* (18) and *in vivo* (27) (refer to **Figure 1**). Extracellular traps (ET) formation was independent of cellular cytoskeletal remodeling, since there was no inhibitory effect by cytochalasin D on EET release (18). The mitochondrial origin of EETs is yet to be confirmed in other reports (discussed in the next section).

In contrast to the above report, Ueki et al. (19) elegantly demonstrated the release of similar web-like structures from lytic eosinophils upon multiple triggers (demonstrated previously to cause eosinophil cytolysis), such as immobilized IgG, IgA (1 mg/mL, 120 min) (38), and the calcium ionophore A23187 (1 μM, 60 min) (39) (refer to **Figure 1**). These findings were obtained using transmission electron microscope and confocal (immunofluorescence) microscopy. Immunostaining with anti-histone antibody on non-permeabilized cells post-treatment confirmed that the DNA was *nuclear* in origin (histone-coated). To ascertain the difference between ETotic, apoptotic and necrotic eosinophils, anti-Fas activated eosinophils (undergoing apoptosis with high annexin V staining) and heat-inactivated eosinophils (with characteristic necrotic blebs) were compared with those undergoing ETosis. In ETotic eosinophils, surface annexin V staining was weak (indicative of low/no phosphatidylserine on the outer surface of the cell membrane, and suggesting these are not apoptotic) compared to Fas-activated cells. In addition, necrotic blebs were not observed. The casting of filamentous ETs was preceded by chromatolysis, involving rupture of both nuclear and cell membranes [events noted in primary lysis (11)].

Concurrently with EETs, intact membrane-bound granules were also observed to be released from cells. Since EET formation occurred in parallel with eosinophil lysis, the term EETosis was coined, and cells were presumed to undergo necrotic death (19, 35). Later, this was disputed, since EET formation does not always accompany cell death (40). Eosinophil extracellular traps (EETs) were shown to be released from live eosinophils as depicted by Yousefi et al. (18) and Gevaert et al. (41), and it is interesting that EETosis was dependent on NADPH (nicotinamide adenine dinucleotide phosphate) oxidase activity. In this study, EETs were studded with granule proteins, suggesting degranulation; properties that resemble EETotic eosinophils observed by Ueki et al. (19). It is also perhaps compelling to emphasize that the number of EET^+ live eosinophils reported by Gevaert et al. (41) in nasal polyps (∼8.8% of the total eosinophils) was similar to the percentage of lytic eosinophils with FEG clusters (9.9%) reported by Erjefält and co-workers in nasal tissue (13).

In recent years, there have been reports of EETs accompanied with features of cell lysis. Charcot-Leyden crystals, the classical feature of eosinophil "activity," were shown to closely associate with EETosis both *in vivo* and *in vitro* (42). EETosis was demonstrated *in vivo* in induced sputum plugs from COPD (chronic obstructive pulmonary disease) patients. Increased eosinophil free granules and cellular debris were found associated with EETs, and MBP was shown to co-localize with observed DNA-based ETs (25). Again, immobilized IgG (50 μg/mL) and immunoprecipitated immunoglobulins from the sputum of severe eosinophilic asthmatics with increased anti-EPX IgG titers were shown to trigger extensive EETs *in vitro* from isolated peripheral eosinophils within 3–12 h of incubation (36). Sputum immunoglobulins with autoantibodies were more potent than affinity-purified IgGs from pooled healthy donors in inducing extensive histone-coated EETs in a time-dependent manner. Increased EETs over time was accompanied with an increase in extracellular lactose dehydrogenase (LDH) levels, indicative of membrane rupture and cell lysis (36).

According to a recent comment by Rosenberg and Foster (17), it is now realized that all EETs are not created equal, and some may be released from live cells while some emerge from lytic (necrotic) cells. However, the process of EET formation is morphologically and functionally distinct from any forms of programmed cell death. Looking at the current literature and ongoing discussion, it may be prudent to remark that the fate of eosinophils (whether dead or alive) during EET formation may be dependent on the stimuli either *in vitro* or *in vivo*. Specifically, *in vitro* experimental conditions such as priming, the nature of stimuli, the time-span involved, and the experimental technique used for assessment may have very distinct outcomes for EET formation from either live or dead cells. The incidence of live or dead eosinophils may also be dependent on the state of eosinophil activation itself at inflamed tissues/foci. For example, the same stimuli, such as *Staphylococcus aureus*, on isolated eosinophils *in vitro* caused EETs to catapult out of live cells within 15 min or less (37), while longer incubations with bacteria or other stimuli for >60 min allowed EET formation with concomitant FEG release (indicative of primary cytolysis) and reduced numbers of viable intact eosinophils (37). In contrast, LPS stimulation of IL-5/IFNγ-primed eosinophils with time-lapse automated confocal imaging confirmed that EETs from live eosinophils were released

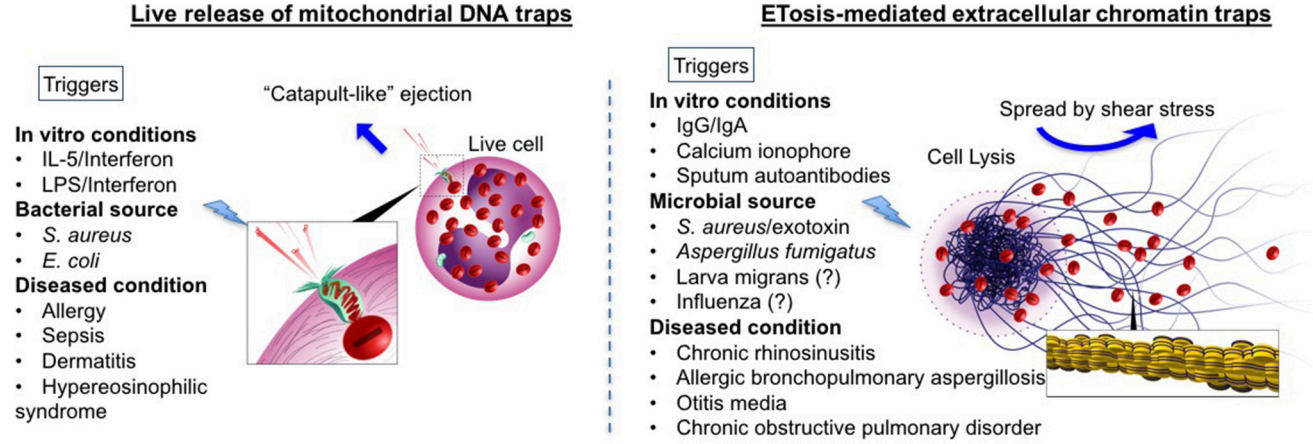

FIGURE 1 | Comprehensive schematic of two theories of eosinophil extracellular trap formations: Comparison between mitochondrial DNA (mtDNA) traps **(Left)** and ETosis mediated extracellular traps **(Right)** are given. Blue arrows indicate how traps are spread extracellularly. In mtDNA theory, live eosinophil rapidly ejects mtDNA, which are loaded with specific eosinophil granule protein. MtDNAs are launched and spread into the extracellular space with a speed of at least 10–20 micrometer s^{-1}, by unknown mechanism [suggested to be the stored elastic energy like plants catapulting pollen in the air (18)]. In contrast, ETosis-generated extracellular traps are mediated through active cell death program. Nuclear and plasma member disintegration during an event of cytolysis, allow for the release of chromatin-based web-like extracellular traps (as depicted in the enlarged image in square). Extracellular traps spread by a passive process. Shear stress (for example, cough, respiration, fluid flow including Brownian motion) enable to distribute free granules (red) and extracellular traps. Other triggers such as immunoglobulins and microbes have also been shown to release histone-coated EETs with evidence of cell death (19, 36, 37). The different triggers known (or reported in literature) to release EETs via both mechanisms have been listed (please note that this is not an exclusive list). For those with inconclusive evidence (or with evidence only from mouse models) have been indicated with a question mark.

within 1–11 s, with the kinetics of DNA spread estimated at 10–20 $\mu m\ s^{-1}$ (18). A later independent study demonstrated that EETs were released from activated eosinophils co-cultured with *S. aureus*/exotoxins (43), but the mechanism of EET release was not described.

SOURCE OF EOSINOPHIL EXTRACELLULAR DNA TRAPS: NUCLEAR OR MITOCHONDRIAL?

There are several possible reasons that could explain the discrepancy in findings regarding the source of EETs being either mitochondrial or nuclear in origin. In the former study suggesting a mitochondrial source for EETs (18), the concern is that the ejection of small particles of mitochondrial DNA may be physically challenging due to high fluid resistance ("drag") within the cytoplasm. The amount of energy required to eject mitochondria through the cytoplasm is predicted to be very high, and is akin to throwing strings from underwater so that they fly up into the air. In contrast, the formation of "DNA clouds" and "DNA traps" as reported elsewhere were typically observed as deriving from the nucleus of cells (19). Perhaps most importantly, human eosinophils contain very few mitochondria (24–36/cell compared with ∼1,300/cell for hepatocytes), suggesting that very little mitochondrial DNA can be released (44). Each mitochondrion contains ∼2–10 copies of mitochondrial DNA, which is highly susceptible to reactive oxygen species-mediated damage because it lacks protective histones and has limited DNA repair mechanisms. Finally, the lack of nuclear DNA in eosinophil supernatants may be explained by the highly adhesive properties of these DNA strands, which are likely to clump and adhere to cells and plate surfaces, particularly after shaking during incubation. This would precipitate the majority of nuclear DNA from supernatants while mitochondrial DNA, being smaller in its size, will remain soluble in the supernatant and more readily detected by PCR. In contrast, using sensitive molecular methods, increased release of dsDNA in the supernatant in an event of induced-EETs *in vitro* has been demonstrated (36). As described above, recent findings by Ueki et al. (19) suggest that most EETs consist of histone-bound DNA that is nuclear in its origins (26). Therefore, the ability of mitochondrial DNA to generate extensive and large DNA traps from eosinophils observed *in vitro* and *in vivo* is physically very challenging and conceptually difficult to apply. These discrepancies and subsequent speculations demand further investigation.

EETs AND HOST DEFENSE

As discussed in section Evolution of Extracellular Traps: ETosis, ET formation by innate cells is an evolutionarily conserved mechanism. Extracellular traps (ET) formation and function was first described in neutrophils, and these are well-known for their microbicidal properties both *in vitro* and *in vivo*, including mouse models of sepsis (45). In recent years, compelling evidence has accumulated suggesting similar roles for EETs and their possible role in host defense.

EETs and Parasites

Eosinophils are primarily implicated in helminthic parasite infections. ETosis has been proposed to be a novel extracellular

killing mechanism for pathogens that are too large to allow phagocytosis. There is limited evidence regarding EETs and parasites. Although a recent veterinary report showed that both neutrophils and eosinophils release ETs to trap *H. contorta* larva, a known pathogenic nematode in ruminant animals (32), the role of EETs is doubtful due to the low purity of isolated eosinophils (30%) using Percoll gradients in this study. EET^+ eosinophils have been reported in the deep dermis of skin biopsies from patients with parasitic infections (larva migrans, ectoparasitosis) (46) however, *in vitro* evidence of parasite-induced EETs has not yet been obtained.

EETs and Bacterial Infection

As mentioned briefly in section Eosinophil Extracellular Traps: Are Eosinophils Dead or Alive?, primed eosinophils were demonstrated to cast EETs in response to a bacterial product trigger (LPS). Yousefi et al. (18) demonstrated that priming of eosinophils with IL-5 and/or IFNγ, or eotaxin, allowed catapult-like EET formation within 1–15 s on subsequent bacterial stimulation by *E.coli* (18) or *S. aureus* (41). Further, they demonstrated that activated eosinophils cultured with opsonized *E. coli* could kill approximately 90% of inoculated bacteria within 45 min by a phagocytosis-independent mechanism (18). The authors also demonstrated that in a model of post-caecal ligation and puncture, IL-5-transgenic and not wild-type mice showed demonstrable intestinal eosinophil infiltration and extracellular DNA deposition (indicative of EETs), which allowed protection against microbial sepsis (18). Prince et al. later demonstrated that EET release by lytic eosinophils, when co-cultured with *S. aureus*, was mediated by bacterial virulence factors (37). The first evidence of EETs embedded with eosinophilic granule proteins (ECP and MBP) *in vivo* was obtained in bacteria-infected caecum (*Spirochetosis*) (18). Caecal tissues in this model were infiltrated by EET^+ eosinophils, rather than neutrophils (confirmed by the absence of a $CD16^+$ population). It has also been demonstrated that EETs provide a larger adhesive surface that allows for encapsulation of fungi and bacteria (26).

EETs and Fungal Infection

Eosinophils are prominent cells in allergic bronchopulmonary mycosis and fungal-associated asthma (47), but their molecular interactions and consequent immunopathological roles in fungal infections are yet to be conclusively defined. The first evidence of EETs forming in fungal disease was reported in a recent clinical case (48), where a patient diagnosed with ABPA (allergic bronchopulmonary aspergillosis) showed CT evidence of bronchiectasis and mucus plugging. Bronchial secretions from this patient exhibited an intense accumulation of eosinophils in the mucus and chromatolytic nuclei aggregated to form filamentous structures that co-stained with anti-human histone 1 antibody and Hoechst 33342 DNA stain. Clusters of free eosinophil granules attached to DNA traps were also detected (48), in agreement with their previous report that EETosis releases intact membrane-bound free eosinophil granules (19). In addition, using scanning electron microscope, the same group demonstrated the capture of *Candida albicans* by EETs *in vitro* (19). Recent evidence from Muniz et al. (49) revealed that *Aspergillus fumigatus* can induce EETs from isolated eosinophils *in vitro* in a ROS-independent manner, but occurred via CD11b binding (a receptor for fungal antigens) and activation of the Syk tyrosine kinase pathway (49). However, EETs were incapable of fungicidal activity. This is similar to findings relating to ETs released from neutrophils in response to *A. fumigatus*, where two independent studies failed to show any NET-related fungicidal activity (50), while there was some evidence that NETs could inhibit germination of fungal spores (51).

EETs and Virus Infections

The role of eosinophil-induced ETs in limiting virus infections still remains to be elucidated. Interestingly, extracellular histones coated on EETs have recently been implicated in influenza-induced lung pathogenesis. Ashar et al. demonstrated a high accumulation of extracellular histones in mice infected with influenza virus that was associated with widespread pulmonary microvascular thrombosis (52). Increased accumulation of extracellular histones was also evident in nasal lavage from influenza-infected patients; however, this study did not investigate whether histones were generated due to EET formation. Neutrophil extracellular traps (NETs) were also observed to be induced by human respiratory syncytial virus *in vitro* (53), but there is no evidence of virus-induced EETs despite evidence of an anti-viral role for eosinophils (54). In fact, it is interesting that eosinophil recruitment to the lungs in response to *A. fumigatus* in a mouse model leads to active eosinophil degranulation and consequent protection from lethal respiratory virus infection (55).

EXTRACELLULAR TRAPS IN DISEASE PATHOLOGY

Despite their demonstrated and important role in host defense, ETs have also been associated with host damage, appearing in association with several pathologies including sepsis, diabetes, and autoimmunity (56). Most of our current knowledge about ETs is based on investigations of neutrophils and the phenomenon of NETosis [reviewed extensively (56)]. Neutrophil extracellular traps (NETs) are studded with self-antigens such as histones, dsDNA, and myeloperoxidase, all of which are implicated in a host of autoimmune diseases, and their role in autoimmune pathogenesis is well-defined (56–59). The association of free FEGs and eosinophil granule proteins with disease severity (5, 7, 15, 60), coupled with recent *ex vivo* evidence of EETs in diseased tissues (tabulated chronologically in **Table 1**) have led researchers to investigate and speculate possible contribution of EETs to disease etiology, sustenance, and progression in continuing studies of cell function, animal models, and clinical cases.

Delayed Resolution of Inflammation, Inefficient Clearance, and Autoimmunity

The presence of EETs, as shown in **Table 1**, has been reported in several inflammatory tissues obtained by biopsy—from bronchial, nasal, skin, esophageal, and intestinal sources, as well

TABLE 1 | *In vivo* evidence of eosinophil extracellular traps in human disease.

Year	Disease	Sample	Methodology used	References
2008	Crohn's disease	Paraffin-embedded intestinal sections	Confocal laser scanning microscope: anti-MBP polyclonal antibody, Sytox orange, and Mito tracker	(18)
2011	Asthma and allergic airways	Endobronchial biopsies	IF with propidium iodide and anti-MBP antibody	(27)
2011	Atopic dermatitis, allergic contact dermatitis, urticaria, bullous pemphigoid, hypereosinophilic syndrome, dermatitis herpetiformis	Skin biopsies	Confocal laser scanning microscopy with propidium iodide and anti-human ECP antibody	(46)
2013	Allergic sinusitis and hypereosinophilic syndrome	Tissue biopsies	Transmission electron microscopy	(19)
2015	Eosinophilic esophagitis	Esophageal biopsy	IF with propidium iodide and anti-human EPX antibody	(61)
2015	Eosinophilic cellulitis or Well's syndrome	Skin biopsies (flame figures)	IF with propidium iodide and anti-histone H2 antibody	(62)
2016	Eosinophilic otitis media and Chronic rhinosinusitis	Sinus and ear exudative secretions	Confocal IF with anti-human histone H1 mAb and Hoeschst 33342 DNA staining; SYTOX green	(26)
2017	Chronic obstructive pulmonary disease	Induced sputum plugs	DAPI staining colocalized with anti-MBP antibody and electron microscopy	(25)
2018	Eosinophilic otitis media (case studies)	Bronchial lavage fluid, bronchial secretions and mucus	Confocal IF with anti-human histone H1 mAb and Hoeschst 33342 DNA staining	(48) (63)
2018	Allergic bronchopulmonary aspergillosis	Bronchoscopic mucus plugs	Confocal IF with anti-citrullinated histone H3 mAb and Hoeschst 33342 DNA staining and SEM	(49)

NB, only in vivo/tissue evidence. in vitro evidence is not given.

as sinus and airway secretions. Against the backdrop of host defense as discussed in the previous section, a plausible outcome of EETs in inflammatory pathology may involve exaggerated responses to microbial stimuli coupled with a reduced capacity in clearance of EET products, such as histones, dsDNA, and peroxidases. Eosinophil extracellular trap (EET) products are all known DAMPs (damage-associated molecular patterns) capable of activating both the innate and adaptive immune systems. Damage-associated molecular patterns (DAMPs) are capable of activating plasmacytoid dendritic cells that present these as self-antigens to cognate lymphocytes, triggering self/autoreactivity (64). For instance, EPX was shown to activate and mobilize dendritic cells to lymph nodes (65). Furthermore, inefficient degradation of DNA-EPX EET products or clearance of EPX released upon ETosis could allow accumulation and subsequent activation of the adaptive system to trigger autoimmune responses. The presence of EETs in tissues with pre-existing autoimmune responses could aggregate immune complexes between autoantibodies and their cognate antigens embedded on ETs. Clearance of such immune complexes by macrophages and dendritic cells could then potentially lead to proinflammatory cytokine secretion, which sustains inflammation (64), interferes with clearance, and prevents resolution, thereby contributing to a vicious cycle of inflammation.

An increased presence of EPX, and FEGs in the airways (sputa) of severe eosinophilic asthmatics were found to be positive predictors of sputum autoantibodies (anti-EPX IgG and anti-nuclear antibodies including anti-dsDNA, anti-histones) (36). Eosinophil extracellular traps (EETs) staining positive for DNA and MBP has been observed in bronchial biopsies obtained from allergic asthmatics that further correlated with infiltrating eosinophils (27). Peripheral blood eosinophils isolated from severe eosinophilic asthmatics compared to non-severe demonstrated higher potential of EET formations *in vitro* when stimulated with LPS and IL-5. The percentage of EET^+ eosinophils negatively correlated with the lung function. Eosinophil extracellular traps (EETs) were shown to have autocrine effect on inducing further eosinophil degranulation and demonstrated a capability of activating epithelial cells to release pro-inflammatory cytokines (IL-6 and IL-8) (66). In a separate study, EETs were demonstrated to be released on stimulation with thymic stromal lymphopoietin (TSLP), a known Th2 alarmin (61). Thus, in a Th2 environment, EETs could be a potential source of self-antigens. Interestingly, *in vitro* experiments revealed that pharmacologically relevant concentrations of dexamethasone were incapable of reducing EET formation from autoantibody-activated eosinophils (36), hinting at a mechanism for a sustained inflammatory environment in steroid-resistant asthmatics. Furthermore, because of this sustained inflammatory environment, a delayed resolution of EET-induced inflammation may potentially trigger self-reactivity and initiate the production of autoantibodies against ET products such as DNA, histones, and granule proteins, as recently reported in severe asthmatic airways (36).

The concomitant presence of EETs and NETs in COPD sputum was reported recently (25). The authors concluded

that EETs may contribute to the severity of COPD. The accumulation of EET-related debris and subsequent phagocytosis by neutrophils may also serve to activate and trigger NETosis, an event that was extensively evident in the high exacerbation severe COPD group (25). It is important to note that in an earlier study, sputum derived from severe COPD patients had detectable anti-nuclear antibodies (ANAs), and an autoimmune pathology for COPD has been proposed (67). However, a direct role of EETs or NETs in the pathogenesis of COPD and any possible underlying autoinflammation has not yet been confirmed. Anti-nuclear antibodies (ANAs) were also reported in nasal exudative secretions from patients with sinus disease and Samster's triad (68), along with evidence of extensive histone-coated EETs in the nasal tissue (26).

Autoantibodies are potent triggers of EETs *in vitro* (36). Sputum with detectable titers of anti-neutrophil cytoplasmic antibody (ANCA), derived from patients diagnosed with eosinophilic granulomatosis and polyangiitis triggered extensive NETs and EETs *in vitro* (69). NETosis and pathogenesis of ANCA is well-known (58), but an avenue has now been opened to decipher the role of EETs in vasculitis and driving pulmonary complications in patients with eosinophilic granulomatosis and polyangiitis. EETs have also been demonstrated in skin biopsies from systemic autoimmune disorders such as Wegner's granulomatosis and bullous pemphigoid (46), as well as intestinal tissue from autoimmune disease such as Crohn's (18) (**Table 1**).

Heightened EET Response in Infection and Associated Inflammatory Pathology

Airway epithelial damage by exaggerated EET anti-bacterial response and collateral tissue damage could possibly explain epithelial (barrier) dysfunction and inflammatory pathology in diseases such as eosinophilic chronic rhinosinusitis (CRS) (70), atopic dermatitis (71), and asthma (72). With respect to intestinal inflammatory diseases, Yousefi et al. (18) demonstrated that increased numbers of eosinophils infiltrated the intestinal lining in *IL-5* transgenic mice after caecal ligation and puncture compared to wild type animals. Increased tissue eosinophilia was associated with an increased level of detectable EETs deposited in the tissue, possibly as a consequence of an exaggerated response to intestinal bacteria. Moreover, the authors showed the presence of EETs in inflamed intestinal tissue from patients with Crohn's disease (18). Again, within eosinophilic asthmatics, there is a subset of patients who suffer from recurrent infective bronchitis (with mixed granulocytic sputum) and low lung function that exhibit autoantibodies in their sputum samples (73). Interestingly, ANAs and anti-EPX IgGs in the sputa of these patients also has the ability to induce EETs *in vitro* (36). It is possible that in addition to NETs, EETs in response to bacterial infection (discussed earlier in section EETs and Bacterial Infection) could be associated with a breakdown of local tolerance as well as a source of potential self-antigens.

Mucus Plugging and Sticky Secretions

Neutrophil extracellular traps (NETs) have been known to contribute to the viscosity of airway secretions in cystic fibrosis patients (74), and DNase treatment was shown to have clinically relevant improvement (75). Dornase alfa [recombinant human deoxyribonuclease I (rhDNase)] is used therapeutically to cleave the DNA in mucus plugs which improves mucocilliary clearance, and leads to improved pulmonary function. Recent evidence suggests that EETs add substantially to the viscosity of eosinophil-rich exudates of patients with CRS, eosinophilic otitis media and ABPA [reviewed in (35)]. Muniz et al. (49) and Ueki et al. (19) demonstrated the presence of abundant nuclear histone-bearing EETs in mucus secretions obtained from the airways of ABPA patients and nasal secretions of CRS patients, respectively. Compared to NETs, EETs assembled to form more stable aggregates that entrapped fungi and bacteria through hydrophobic interactions, demonstrated *ex vivo* in nasal exudates from CRS patients. When compared to NETs, the EETs exhibited thicker fibers coated by higher number of histone molecules and were less susceptible to proteolytic degradation (19).

Cunha et al. showed that ovalbumin sensitization and challenge in mice led to the accumulation of EET^+ EPX^+ live eosinophils in lung tissues and induced increased extracellular DNA in bronchoalveolar lavage (76). In subsequent years, the same group showed that recombinant DNase treatment significantly decreased airway resistance, concomitantly with goblet cell hyperplasia and reduced EET formation (77). Eosinophil extracellular trap (EET) secretions also increased mucin/airway secretions in these mice post-allergen challenge. Recent observations of Dunican et al. (78) showed that mucus plugs in asthmatics correlated with increased airway eosinophilia and EPX content, which occurred in 58% of asthmatics compared to 4.5% of healthy volunteers, and was also associated with reduced lung function. It may be speculated that in asthmatics with increased eosinophilic activity (indicated by increased EPX), there may be a loss of immune tolerance, increasing presence of local autoantibodies, and generation of EETs that contribute to goblet cell hyperplasia and IL-13-related mucin overproduction, both of which lead to the production of highly viscous airway-blocking mucus plugs that are unique to eosinophilic disorders for their exceptionally thick peanut butter-like consistency.

CONCLUSIONS

We now have incontrovertible evidence that EETs form in response to many different stimuli deriving from microbes and their products, as well as non-infectious stimuli such as DAMPs, complement proteins, and immunoglobulins. These traps are important in protection of the host in response to invasive pathogens such as bacteria, and potentially, viruses, fungi, and parasites. However, in the case of chronic, unresolved inflammation, it is apparent that EETs can cause health complications by promoting the production of highly viscous mucus secretions, and potentially setting the stage for the development of autoantibodies. The localized presence of autoantibodies in the lungs (79) is proposed to further exacerbate EET formation, setting up a vicious cycle of inflammation that is not readily ameliorated by glucocorticosteroid treatment.

Further studies are anticipated in this area to understand more about how EETs may be prevented in patients with severe inflammatory conditions including steroid-dependent asthma and sinus disease.

AUTHOR CONTRIBUTIONS

MM prepared the first draft. SU and PL contributed to the development of the manuscript. All authors have read and agreed to the submitted manuscript.

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Allergic Bronchopulmonary Aspergillosis–A Luminal Hypereosinophilic Disease With Extracellular Trap Cell Death

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Allergic bronchopulmonary aspergillosis (ABPA) is characterized by an early allergic response and late-phase lung injury in response to repeated exposure to *Aspergillus* antigens, as a consequence of persistent fungal colonization of the airways. Here, we summarize the clinical and pathological features of ABPA, focusing on thick mucus plugging, a key observation in ABPA. Recent findings have indicated that luminal eosinophils undergo cytolytic extracellular trap cell death (ETosis) and release filamentous chromatin fibers (extracellular traps, ETs) by direct interaction with *Aspergillus fumigatus*. Production of ETs is considered to be an innate immune response against non-phagocytable pathogens using a "trap and kill" mechanism, although eosinophil ETs do not promote *A. fumigatus* damage or killing. Compared with neutrophils, eosinophil ETs are composed of stable and condensed chromatin fibers and thus might contribute to the higher viscosity of eosinophilic mucus. The major fate of massively accumulated eosinophils in the airways is ETosis, which potentially induces the release of toxic granule proteins and damage-associated molecular patterns, epithelial damage, and further decreases mucus clearance. This new perspective on ABPA as a luminal hypereosinophilic disease with ETosis/ETs could provide a better understanding of airway mucus plugging and contribute to future therapeutic strategies for this challenging disease.

Keywords: eosinophil, extracellular traps, extracellular trap cell death, allergic bronchopulmonary aspergillosis, inflammation, mucus plugs, ETosis, NETosis

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INTRODUCTION

Allergic bronchopulmonary aspergillosis (ABPA) is a disease entity first proposed by Hinson and colleagues as bronchopulmonary aspergillosis in 1952 (1). ABPA develops mainly in adolescent and adult patients with asthma or cystic fibrosis. It has been estimated that 2.5% (0.7–3.5%) of adult patients with asthma suffer from ABPA (2). Clinically, it is characterized by peripheral blood eosinophilia, increased levels of serum IgE, an immediate skin reaction and/or specific IgE/IgG antibodies to *Aspergillus fumigatus* due to type I and III hypersensitivity reactions, and

radiographic findings including pulmonary opacities, central bronchiectasis, and mucus plugs (3–5). Systemic corticosteroids and/or anti-fungal drugs are effective, although approximately half of ABPA patients experience relapse (6, 7).

Besides blood eosinophilia, a massive accumulation of eosinophils and clustering of these immune cells in the bronchial lumen, resulting in bronchial impaction, are hallmarks of ABPA. Clinically, this has been defined by different terms depending on the context, such as "allergic mucin," "high attenuation mucus," or "allergic mucus plugs." Considerable evidence has indicated the close association between the sputum eosinophil count and/or the eosinophil granule protein concentration and asthma severity (8, 9), although much less attention has been paid to luminal eosinophils (and their lytic components) in ABPA. This may be simply because of the lack of an explicit relationship and/or the difficulties in measuring protein concentrations due to the inspissated bronchial secretions. In this review, we discuss the clinical features of ABPA, focusing on new insights into the fate of eosinophils and their "cell debris" in the airways.

FUNGUS IN THE AIRWAYS

Germination and saprophytic growth of fungi in the mucus are interesting and unique features of ABPA. *Aspergillus fumigatus* is the major causative fungus of ABPA, but other *Aspergillus* spp., such as *A. flavus*, *A. niger*, and *A. oryzae*, can also cause ABPA, although less frequently. *Schizophyllum commune*, a filamentous basidiomycete commonly found on the rotten wood of trees, can cause similar pathology and a condition known as allergic bronchopulmonary mycosis (ABPM) (7, 10–12). Because fungal hyphae are immunologically active expressing and releasing various proteases and pathogen-associated molecular patterns (13–15), repeated exposures to airborne fragments of fungal hyphae, either dead or alive, can cause asthma and hypersensitivity pneumonitis. For the development of ABPM, by contrast, inhalation of viable fungi as conidia, not their hyphae fragments, and their germination in the lower airways is essential. *A. fumigatus* has advantages for the development of ABPA/ABPM over other fungi because of the small size of its conidia (3–6 μm) and thermophilicity (16). The conidia of *S. commune* are also small (3–4 μm), and prefer a relatively high temperature (30–35°C) to germinate (17). *A. fumigatus* conidia are also known for their high dispersibility due to their remarkable hydrophobicity (18).

Unlike fungal infections, germinated hyphae cannot penetrate the lung tissues in the presence of a normal immune system and bronchial structure. Therefore, *A. fumigatus* remains in the mucus plugs of the bronchi. Compared with other fungi, *A. fumigatus* has another advantage in this process in that it induces the *Muc5ac* gene, one of the mucin genes, and mucus production in bronchial epithelial cells (19). Induction of *Muc5ac* gene expression is dependent on the high serine protease activity of *A. fumigatus*, which activates membrane-bound TNFα-converting enzyme and TGF-α, stimulating epidermal growth factor receptors. Compared with *A. fumigatus*, other fungi, such as *Penicillium* and *Alternaria*, and other *Aspergillus* spp., such as *A. flavus* and *A. niger* have lower serine protease activity (19, 20).

RADIOGRAPHIC FEATURES OF ABPA

In the first description of ABPA, Hinson and colleagues advocated that the features of ABPA included (i) wheezing and blood eosinophilia, (ii) repetitive infiltrations visible on chest X-ray, and (iii) "allergic" (eosinophilic) mucus plugs with fungal hyphae (AMwF) (1). In 1967, Scadding (21) reported the presence of central bronchiectasis by bronchography, with the initial mucus-filled bronchi being less apparent but bronchial ectasis remaining. The diagnostic criteria for ABPA in the pre-computerized tomography era, proposed by Rosenberg and Patterson (22), included pulmonary opacities and central bronchiectasis.

Currently, the radiographic features of ABPA include bronchiectasis, mucoid impaction, pulmonary opacities, mosaic attenuation, centrilobular nodules, tree-in-bud opacities, and pleuropulmonary fibrosis (3). Pulmonary opacities, which are usually transient, are frequently observed during the course of the disease, with 89% of ABPA cases with bronchiectasis demonstrating pulmonary opacities/ground grass attenuation in a nationwide survey in Japan (7); however, this is not classed as a specific feature of this disease. Central bronchiectasis with peripheral tapering of bronchi has been considered as a relatively specific finding for ABPA, but bronchiectasis can extend to the peripheral bronchi in 33–63% of cases (23, 24). Mucus plugs in the bronchi are common in ABPA, and may present as high attenuation mucus (HAM) with a CT density higher than the values of paraspinal skeletal muscle (25) or 70 Hounsfield units (26). Magnetic resonance imaging of HAM showed hypodense lesions in T1- and T2-weighed images, suggesting that the mucus is desiccated or inspissated (27). HAM can be observed in more than half of the cases with mucus plugs due to ABPA (7, 23), and is more specific for this disease. The presence of HAM also correlates with a higher number of eosinophils in the peripheral blood and greater susceptibility to disease relapse (6, 28).

CLINICOPATHOLOGICAL FEATURES OF ABPA

Regarding the pathological features of ABPA, Katzenstein and colleagues emphasized the presence of AMwF, bronchocentric granulomatosis with tissue eosinophilia (BrCG-eo), eosinophilic bronchiolitis, and eosinophilic pneumonia (29). In 1988, Bosken and coworkers indicated, based on the investigation of surgically resected specimens, that mucoid impaction of bronchi with allergic mucin or BrCG-eo, together with fungal hyphae detected in the lesion, were sufficient for the diagnosis of ABPA/ABPM (30). However, the diagnostic criteria proposed by Rosenberg and

Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; ABPM, allergic bronchopulmonary mycosis; AMwF, allergic mucus plugs with fungal hyphae; BrCG-eo, bronchocentric granulomatosis with tissue eosinophilia; ET, extracellular trap; EET, eosinophil extracellular trap; ETosis, extracellular trap cell death; EETosis, eosinophil extracellular trap cell death; HAM, high attenuation mucus; NET, neutrophil extracellular trap; NETosis, neutrophil extracellular trap cell death; ROS, reactive oxygen species

Patterson (22) excluded pathological findings; instead, clinical, radiological, and laboratory findings, especially allergy tests, were included.

AMwF was detected in the biopsy samples by bronchoscopy, expectorated airway secretions, and the resected lungs. AMwF has been described by pathologists as follows (31, 32). "*AMwF could be found in any bronchi of any lobes, including lobar and segmental bronchi and 3rd—5th bifurcated small bronchi. AMwF was typically white-yellowish, dark-yellowish, or yellow-greenish in color, and sticky with elastic soft or elastic hard in consistency. The shape ranged from branching and clubbing to lumpy. The H&E stained section of the AMwF demonstrated concentric multiple layers consisting of eosinophils, Charcot-Leyden crystals, and fibrin (32, 33). The eosinophils might be viable, necrobiotic (with pyknotic nuclei and nuclear dusts) or necrotic. The latter two types formed inspissated eosinophil aggregates with indistinct cell margins and occasionally exhibited many fissures likely due to dehydration inside of AMwF, demonstrating 'fir-tree structure' (33). Fungi were usually found in the clusters of eosinophils as fragmented hyphae or branching septate hyphae, and occasionally in the mucus surrounding the eosinophil clusters. While fungi floats in the air as unicellular spores, they reside in the AMwF as multicellular hyphae, suggesting the proliferation of fungi.*"

Central bronchiectasis and peripheral lung lesions in ABPM were suggested as secondary pathological changes after the formation of AMwF. BrCG-eo, eosinophilic bronchiolitis, and eosinophilic pneumonia were found to be in the airways peripheral to the AMwF plugs, previously described as follows (32). "*The investigation of the resected lungs of ABPM patients revealed the airway walls plugged by AMwF were invaded with eosinophils and small round cells including plasma cells and were ulcerated, resulting in eosinophilic infiltration into the allergic mucin. The intense inflammation in the bronchial walls destructed and dissipated airway structures i.e., elastic fibers and smooth muscles, and even bronchial cartilages. Central bronchiectasis could be caused by the vulnerability of diseased bronchial walls and by expanding AMwF. In the exudate from BrCG-eo and eosinophilic bronchiolitis, inspissated eosinophil aggregates, fir-tree structures and fungi were detected, similar to the AMwF. However, the inspissated eosinophil aggregates and fir-tree structures were fragmented and the number of fungi was smaller than that in the central lesions of AMwF (30–33). These findings suggested that the peripheral lung lesions might be formed by the bronchogenic spread of AMwF to the peripheral lung.*" Thus, careful morphological analysis has indicated the AMwF is an initial and crucial step in the development of ABPA.

EOSINOPHIL EXTRACELLULAR TRAP CELL DEATH (ETOSIS) AND EXTRACELLULAR TRAPS

Eosinophils are bone marrow-derived, short-lived, non-dividing granulocytes that have been implicated as integral components of allergic airway inflammation. Eosinophils contain ∼200 granules per cell (34), which are preformed stores of the specific granule proteins (35). The highly cationic granule proteins possess cytotoxicity by disrupting the integrity of lipid bilayers, exerting neurotoxic properties, RNase activities, and participating in the generation of reactive oxidants and radical species (36, 37). Since eosinophil degranulation does not occur in the circulation (38), it is critical to understand the actual mode of degranulation and cell fate in the airways. Our recent studies indicated eosinophil cell-death mediated degranulation, i.e., eosinophil ETosis, which might play an important role in the generation of mucus plugs (39, 40).

In 2004, Brinkmman and colleagues provided the first evidence that neutrophils release filamentous chromatin structures, i.e., neutrophil extracellular traps (NETs) (41). A subsequent study revealed that the novel cell death process mediates the release of NETs and was designated NETosis (42). NETosis has unique features that are different from apoptosis and necrosis. Unlike apoptosis, nuclear DNA fragmentation is spared during the process of NETosis. Histone citrullination induces chromatin decondensation and granules are intracellularly disrupted before plasma membrane disintegration, thereby NETs are associated with various antimicrobial molecules (including histones, elastase, myeloperoxidase, cathepsin, and lactoferrin). Once NETs are released, they immobilize and potentially kill pathogens (41, 43, 44). Based on this, roles for these structures in preventing microbial spread and in creating an antimicrobial environment have been postulated.

Since chromatin traps are cytolytically released by other leukocytes, such as macrophages (45) and mast cells (46, 47), "ETosis" has been proposed as a similar cell death pathway (48). Extracellular trap (ET) formation is now considered to be a common mechanism of the innate immune system in vertebrates (49). A recent report showed that even invertebrate cells are capable of ETosis, indicating an ancient and evolutionary conserved mechanism (50). Eosinophil ETosis (EETosis) was first reported as a mechanism of "cytolysis" (also called lytic degranulation, necrosis, primary lysis, and eosinophil lysis) that has been observed (but often overlooked) in eosinophilic inflammatory diseases (35). This finding indicates that eosinophil cytolysis does not represent a process of passive/accidental necrosis, rather eosinophils are actively selecting their death program at the inflammatory site. The process of EETosis is akin to that of neutrophils but notably differs in that intact granules are released extracellularly (35, 51–53).

It is well-established that the process of leukocyte activation and ET release plays a role in health and disease, including innate immunity, autoimmunity, metabolic disorders, malignancies, and coagulation (44, 46, 54–57). Excess production of ETs could be pathogenic (49); for instance, large amounts of NETs contribute to the thickness of airway fluids from patients with cystic fibrosis (58). Inhalation of recombinant human DNase improved lung function in cystic fibrosis patients, indicating the pathogenicity of NETs (58–60). Similar to NETs, EETs provide a sticky scaffold for secretions, a fact confirmed by the decrease in viscosity following disruption of EET polymers with DNase (61). Recently, we have shown abundant EETs associated with clusters of cell-free extracellular granules in the bronchial mucus plugs of ABPA patients (39, 40). We also observed a drastic decrease in EETs in the bronchoscopically-obtained secretions from an

ABPA patient after corticosteroid treatment, which correlated with clinical improvements (40).

It is noteworthy that NETs and EETs have different attributes in terms of stability and structure. The protein content of ETs plays an important role in its mechanical properties (62). Compared with neutrophils, eosinophils contain far less proteases, and thus eosinophil chromatin is spared from endogenous protease digestion (61, 63). Neutrophil elastase promotes the chromatin decondensation of NETs by proteolytic processing of histones (64). *In vitro*, NETs deteriorated within 24 h, whereas EETs were stable for at least 7 days (61). Electron microscopy showed that released NETs consisted of 5–10-nm smooth stretches (composed of stacked cylindrical nucleosomes) and 25–50-nm globular domains (41, 65), although EETs consisted mostly of chromatin fibers with diameters of 25–35 nm in conjunction with larger fibers (35). In terms of innate immune responses, staunch fibers might offer an advantage by immobilizing and hampering the progression of large parasites (66), and may also pathologically contribute to the highly viscous nature of eosinophil-dominant airway secretions, as clinically observed in ABPA, but also in other diseases, such as eosinophil chronic rhinosinusitis (51, 61), eosinophilic otitis media (63, 67), and severe asthma (40, 68).

There is evidence *in vitro* that human eosinophils rapidly activate in response to various stimuli by releasing mitochondrial DNA via a non-cytolytic mechanism (69, 70). The mitochondrial DNA would be present in mucus plugs since EETosis liberates total cellular contents through plasma membrane disintegration. However, mitochondrial DNA, lacking the histone and nucleosome structure, is only 16 kbp in size, constituting <1% of total cellular DNA (e.g., genomic DNA has 3.2 billion bp, which equates to 2 m in length/cell) (71). In addition, human eosinophils contain a small number of mitochondria (∼30/cell) (72). Therefore, nuclear-derived chromatin fibers are a major component contributing to viscosity (35), as evidenced by positive staining of airway EETs with specific antibodies against histones/citrullinated histones (39, 61).

A. FUMIGATUS INDUCED EOSINOPHIL ETOSIS

The immunopathological mechanisms that underlie the molecular interactions between *A. fumigatus* and eosinophils are also of interest (see review (73)). Our *in vitro* studies have shown that ETs are released by lytic human eosinophils in direct response to *A. fumigatus* (39). When human eosinophils were co-cultured with *A. fumigatus*, EETs were released in a time and ratio (fungus: cell) dependent manner (39).

Unlike necrosis, ETosis is an active form of cell death and is frequently accompanied by the production of reactive oxygen species (ROS) (35, 41, 43, 52, 69, 70, 74–76); however, we observed that human eosinophils release EETs *in vitro* in response to *A. fumigatus* in a ROS-independent manner (39). Regarding *Aspergillus* specifically, neutrophils are described to respond to different morphotypes of this fungus by releasing NETs in a ROS-dependent process (77–79). However, the ROS-independent release of NETs was observed when stimulated by bacteria (80), parasites (81), fungi (*Candida* and *Paracoccidioides brasiliensis* conidia) (82, 83), and other stimuli (84–86). The participation of ROS in the process of ETosis might be essentially dependent on the stimuli, time point, or microorganisms examined.

Mechanistically, *A. fumigatus* induced EETosis *in vitro* is a process dependent on the Syk tyrosine kinase pathway (known to mediate cell signaling via different classes of receptors involved in fungus recognition) and adhesion molecule CD11b β-integrin (which recognizes β-glucan, a molecular pattern commonly found in the cell wall of fungi) (39). Consistent with these findings, CD11b has been described to play an important role in eosinophil interactions with the fungus *Alternaria alternata* (87). Previous studies with eosinophils and *A. alternata* suggest a critical role for the CD11b I domain in eosinophil activation and degranulation, but not for the lectin domain, which recognizes the β-glucan (87). In neutrophils, both the C-type lectin receptor dectin-1 and the β-integrin CD11b/CD18 (CR3) have been implicated in the recognition of β-glucans on the cell surface of *A. fumigatus* (79, 88, 89). However, we did not detect the expression of dectin-1 protein in human blood eosinophils (39).

Airway eosinophils display enhanced responses to a variety of ligands and become further activated to degranulate (90, 91). Human eosinophils undergo ETosis in response to immobilized IgG or IgA (used as *in vitro* defined models of immunoglobulin-coated pathogens including parasites), calcium ionophore, IL-5/GM-CSF with platelet activating factor, or phorbol myristate acetate to liberate ETs (35, 68, 92). In addition to the direct response to *A. fumigatus*, it is possible that locally produced immunoglobulins, cytokines, and other mediators could activate eosinophils to induce EETosis. Indeed, a recent study indicated that IgG-type autoantibodies were present in the airways of patients with severe eosinophilic asthma, potentially induced by the release of EETs (68). It is also noteworthy that biological molecules associated with the fungal cell wall, such as chitin and β-glucan from *Aspergillus* species, induce eosinophilic Th2 inflammation in mouse lungs (93–95).

LUMINAL EOSINOPHILS AND ETOSIS MATTER

EETs exhibited intimate contact with *A. fumigatus* conidia and with cell-free extracellular granules, indicating that EETs provide an adhesive surface for organelle and microorganism entrapment (39). The highly hydrophobic surface of *A. fumigatus* might be easily entrapped by EETs, since EETs ensnare microorganisms mainly via hydrophobic interactions (39, 61). It has been reported that NETs may efficiently kill microbial pathogens (96–99); however, several fungi may resist the antimicrobial effect of NETs (79, 83, 100, 101).

Interestingly, our study revealed that human EETs did not promote the damage or killing of *A. fumigatus* (39). Eosinophils exhibit killing activity against different pathogens both *in vitro* and in experimental models (reviewed in (102)). *In vitro*, Yoon

and colleagues demonstrated that human eosinophils release their cytotoxic granule proteins in response to *A. alternata* and kill the fungus in a contact-dependent manner (87). Mouse bone marrow-derived eosinophils exhibited killing activity against *A. fumigatus in vitro*, which did not require cell contact (103). By contrast, using a Th2-dominant murine model of chronic invasive aspergilosis, eosinophil-deficient mice showed decreased morbidity and improved clearance of *A. fumigatus* (94). Intriguingly, studies based on mouse experimental models revealed that Th2-type immunity to chronic fungi exposition is generally accompanied by detrimental allergic inflammation, including tissue eosinophilia, goblet cell hyperplasia, and airway remodeling, but no signs of hyphae or invasive fungal growth were reported at any time after conidia challenge (104). Thus, the development of an animal model showing fungal colonization of the airways similar to that observed in ABPA pathology is still lacking. It remains unclear whether these discrepancies are due to the differences between mouse and human eosinophils (105) and/or experimental settings. Nevertheless, *A. fumigatus* resistance to EET killing activity might be an important feature of ABPA pathogenesis, possibly explaining the previously suggested "innate immunological defect" in this disease (3).

The rheological properties of mucus and the mucociliary transport system function as a self-cleaning mechanism for the respiratory tract (106). However, difficult-to-remove eosinophilic airway secretions are associated with disease severity (107, 108). As described above, the lower protease content and stable chromatin traps of eosinophils contribute to airway mucus viscosity. Most recently, Dunican and coworkers indicated that oxidants generated by eosinophil granule protein oxidize cysteine thiol groups to stiffen airway mucus (109).

We propose that EETosis contributes significantly to the fate of luminal eosinophils and plays a critical role in the pathogenesis of ABPA (**Figure 1**). Under Th2 conditions, blood eosinophils actively accumulate in the airway tissue. Eosinophils are usually eliminated by migration into the airway lumen followed by mucociliary clearance (110). Unlike apoptosis that produces fragmented DNA, luminal eosinophils undergo ETosis by direct interaction with fungi and/or local stimuli to release a sticky chromatin structure. EETs contribute to the increased viscosity of mucus rather than the direct elimination of fungi. Eosinophil cytolysis also releases intact granules and a wide range of nuclear and cytosolic damage-associated molecular patterns (92). Free granules may act as a reservoir of toxic granule proteins and secretion-competent extracellular organelles (35, 111). Perpetuating epithelial damage may inhibit the effectiveness of ciliary beating, thereby decreasing mucus transport and also resulting in the release of alamins and further eosinophilic inflammation. Thus, EETosis might promote a perpetuating cycle of thickening secretions (51). However, this new perspective requires further evaluation.

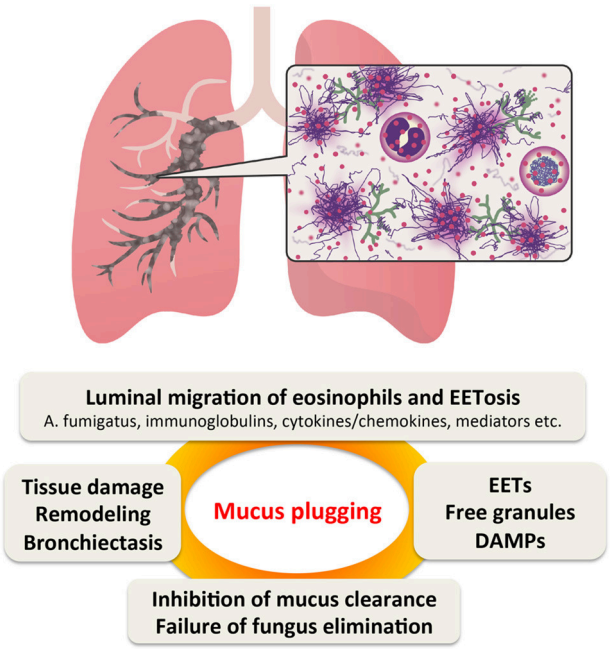

FIGURE 1 | Proposed perpetuating cycle of mucus plugging caused by eosinophil ETosis (EETosis). In Th2-type airway inflammation, eosinophils are the major effector cells. They are supplied from the blood and massively accumulate in the bronchial lumen, where they are highly activated by fungi and other stimulants. EETosis-mediated cytolysis releases their total cellular contents including eosinophil extracellular traps (EETs), which contributes to the higher viscosity of eosinophilic mucus. Persistent fungal colonization of the airways and inhibition of "cytotoxic" mucus clearance further contributes to tissue damage, resulting in bronchiectasis. DMAPs, damage-associated molecular patterns.

CONCLUDING REMARKS

As reviewed by Persson and Uller (112), cytolysis has often been overlooked by researchers, although several clinical studies illustrated that cytolysis accounted for a major proportion of bronchial eosinophils in severe and lethal asthma (112). Because apoptosis is a rare event among tissue-migrated eosinophils, ETosis-mediated cytolysis and luminal entry are likely to be the major fates of these cells. Indeed, Charcot–Leyden crystals, often observed in mucus plugs, were generated in association with the EETosis process (53). Considering the clinical features of ABPA, it is conceivable that increased eosinophil turnover, rather than prolonged lifespan, might be a major cause of luminal eosinophilia. It should be recognized that EETosis-mediated cytolysis and EETs are potentially important therapeutic targets in ABPA, as well as in other eosinophilic inflammatory diseases.

AUTHOR CONTRIBUTIONS

SU wrote and finalize the manuscript. AH and MK contributed pathological part; KA contributed clinical part. JN wrote cell biology part.

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Identification of Piecemeal Degranulation and Vesicular Transport of MBP-1 in Liver-Infiltrating Mouse Eosinophils During Acute Experimental *Schistosoma mansoni* Infection

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Eosinophils have been long associated with helminthic infections, although their functions in these diseases remain unclear. During schistosomiasis caused by the trematode *Schistosoma mansoni*, eosinophils are specifically recruited and migrate to sites of granulomatous responses where they degranulate. However, little is known about the mechanisms of eosinophil secretion during this disease. Here, we investigated the degranulation patterns, including the cellular mechanisms of major basic protein-1 (MBP-1) release, from inflammatory eosinophils in a mouse model of *S. mansoni* infection (acute phase). Fragments of the liver, a major target organ of this disease, were processed for histologic analyses (whole slide imaging), conventional transmission electron microscopy (TEM), and immunonanogold EM using a pre-embedding approach for precise localization of major basic protein 1 (MBP-1), a typical cationic protein stored pre-synthesized in eosinophil secretory (specific) granules. A well-characterized granulomatous inflammatory response with a high number of infiltrating eosinophils surrounding *S. mansoni* eggs was observed in the livers of infected mice. Moreover, significant elevations in the levels of plasma Th2 cytokines (IL-4, IL-13, and IL-10) and serum enzymes (alanine aminotransferase and aspartate aminotransferase) reflecting altered liver function were detected in response to the infection. TEM quantitative analyses revealed that while 19.1% of eosinophils were intact, most of them showed distinct degranulation processes: cytolysis (13.0%), classical and/or compound exocytosis identified by granule fusions (1.5%), and mainly piecemeal degranulation (PMD) (66.4%), which is mediated by vesicular trafficking. Immunonanogold EM showed

a consistent labeling for MBP-1 associated with secretory granules. Most MBP-1-positive granules had PMD features (79.0 ± 4.8%). MBP-1 was also present extracellularly and on vesicles distributed in the cytoplasm and attached to/surrounding the surface of emptying granules. Our data demonstrated that liver-infiltrating mouse eosinophils are able to degranulate through different secretory processes during acute experimental *S. mansoni* infections with PMD being the predominant mechanism of eosinophil secretion. This means that a selective secretion of MBP-1 is occurring. Moreover, our study demonstrates, for the first time, a vesicular trafficking of MBP-1 within mouse eosinophils elicited by a helminth infection. Vesicle-mediated secretion of MBP-1 may be relevant for the rapid release of small concentrations of MBP-1 under cell activation.

Keywords: schistosomiasis, eosinophil degranulation, major basic protein-1, granuloma, inflammation, liver, immunonanogold electron microscopy, piecemeal degranulation

INTRODUCTION

Eosinophils are innate immune cells with a broad distribution in tissues and notably associated with allergic and helminth parasitic diseases [reviewed in (1–3)]. Increase in the numbers of eosinophils has long been reported during the acute phase of schistosomiasis, a neglected tropical disease of great clinical and socioeconomic relevance (4–7). The etiological agents of human schistosomiasis are trematode worms of the genus *Schistosoma* with most species, including *Schistosoma mansoni*, the only one that occurs in the Americas, affecting mainly the liver and the intestines (8). Human infection with this parasite causes significant chronic morbidity with the development of a granulomatous reaction and severe tissue inflammation, which can lead to life-threatening hepatosplenomegaly [reviewed in (9)].

There is considerable debate on the role of eosinophils during schistosomiasis. It remains uncertain if eosinophils act as major effector cells against the parasite; as immunomodulators of the immune response; as participants in tissue homeostasis and metabolism, which could favor establishment and maintenance of parasitic worms in their hosts, or merely as operators in remodeling and clearance of debris following injury (7, 10–14).

Eosinophil responses to inflammatory and/or immunoregulatory situations are underlined by the ability of these cells to release granule-stored products, that is, to degranulate (15). Direct attention to events of eosinophil degranulation during schistosomiasis mansoni has been paid in earlier studies. Several authors showed *in vitro* that eosinophils, operating via antibody-dependent cytotoxicity, exert damage to schistosomula of S. *mansoni* (16, 17). This effect was attributable, at least in part, to degranulation and release of granule contents, especially to major basic protein (MBP), a typical cationic protein stored pre-synthesized in secretory (specific) granules, onto the surface of the parasite (18). In fact, the toxicity of MBP and Eosinophil Peroxidase/Eosinophil Protein X (EPO/EPX) has given support to the effector function of eosinophils in defense of the host to helminthes, although evidence for such importance have arisen just from *in vitro* data (reviewed in 13). Within *S. mansoni* egg-induced granulomas, there are well-defined clusters of eosinophils and other inflammatory cells embedded in a collagen-rich extracellular matrix around mature parasite eggs (7, 19), but there is a lack of clarity regarding the degranulation patterns of eosinophils and its significance in both humans and mouse models.

Here, we performed a comprehensive *in vivo* study to investigate the secretory processes involved in eosinophil degranulation during the acute phase of schistosomiasis mansoni in mice. By using conventional transmission electron microscopy (TEM), which is the only technique with resolution suitable to clearly identify and distinguish between different modes of cell secretion (20) and immunogold EM for detecting MBP-1 subcellular localization, we identified, for the first time, a major vesicle-mediated secretory process for MBP-1 release underlying the responses of eosinophils to the infection.

MATERIALS AND METHODS

Experimental Infection in Mice

Swiss Webster mice aged 70 days were inoculated or not (12 mice per group) with a single inoculum of cercariae of *S. mansoni* (100 cercariae/mouse), LE strain. Cercariae were harvested from infected *Biomphalaria glabrata* snails, washed, counted, and injected subcutaneously into each mouse by an experienced technician. *S. mansoni* LE strain used in the experiments was originally isolated from a patient in Belo Horizonte, Brazil, and has been maintained in successive passages through *Biomphalaria glabrata* snails and hamsters (*Mesocricetus auratus*) at the Laboratory of Schistosomiasis (Department of Parasitology, UFMG, Brazil). Infected animals and respective uninfected controls from the same age were euthanized at 55 days of infection (acute phase) (5). Infection was confirmed by findings of parasite eggs in the rodent feces at week five of infection (21).

Ethics Statement

This study was carried out in full accordance with all international and Brazilian accepted ethic guidelines and was approved by the Oswaldo Cruz Foundation Ethics Committee on Animal Use [CEUA-*Comissão de Ética no Uso de Animais*,

under the protocol 32/2012). CEUA follows the Brazilian national guidelines recommended by the National Council for Animal Experimentation-CONCEA (*Conselho Nacional de Controle em Experimentação Animal*). Mice experimentally infected and uninfected controls were monitored daily for survival and well-being status (home cage evaluation, body condition, skin lesions, mobility and other general conditions) (22). No animals died prior to the experimental endpoint (55 days of infection).

Collection of Samples

Experimentally infected animals and their respective uninfected controls were anesthetized, euthanized, and blood samples and organ fragments were collected for different studies as below. Animals were euthanized by exsanguination (full bleed) under deep anesthesia by cardiac puncture. The anesthetic protocols included ketamine (100 mg /mL) combined with acepromazine (10 mg /mL) at a ratio of 9:1 (dose of 0.15 mL/100 g body weight) (23). Blood samples were collected by cardiac puncture without anti-coagulant for enzyme determinations or with heparin for cytokine evaluations. Another group of infected and non-infected animals ($n = 4$ for each group) was euthanized in a CO_2 chamber at the same day for peritoneal lavage (PL).

Antibody Reagents

Rat monoclonal anti-mouse IgG2a MBP-1 (clone 14.7.4) (Lee Laboratories; Mayo Clinic, Scottsdale, AZ) whose MBP-1 specificity has been well validated in previous studies (24–26) and irrelevant isotype control monoclonal antibodies IgG (Abcam; Cambridge, MA) were used for the ultrastructural immunodetection studies at concentration of 20 μg/mL. The secondary antibody used for immunoEM was an affinity-purified goat anti-rat Fab fragment conjugated to 1.4-nm gold particles (1:100, Nanogold, Nanoprobes, Stony Brook, NY).

Liver Enzymes Determinations

To evaluate the serum enzymes reflecting liver function, blood samples were centrifuged at 3000 rpm for 10 min and analyzed in a Roche Cobas Mira Plus Chemistry Analyzer (Roche Diagnostics®, IN, USA) as before (27). Assay Kits for measurement of the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Bioclin®, Belo Horizonte, MG, Brazil) were used according to the manufacturer's instructions. A total of 24 samples were evaluated from mice (12 from infected animals and 12 from uninfected of the same age). Results were expressed as units/liter (U/L).

Cytokine Determinations

To investigate the Th2 profile immune response during the acute phase of the disease, plasma from *S. mansoni*-infected and control animals were processed for cytokine (IL-4, IL-10, IL-13) analyzes by ELISA assay (DuoSet® ELISA Development System, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. After blood centrifugation (3000 rpm for 10 min at 4°C), plasma was collected and frozen at −80°C for subsequent processing.

For ELISA assay, the plates were incubated with 50 μL of capture antibody, diluted in PBS (0.15 M NaCl) at RT, overnight. Next day, the plates were washed three times with washing buffer (0.05% Tween® 20 in PBS), blocked with 150 μL of PBS-BSA (bovine serum albumin) 10% for 1 h and washed as before. Samples or cytokine patterns (50 μL) were diluted in PBS-BSA and incubated at 4°C, overnight. Next day, the plates were washed as above and incubated with the detection antibody (50 μL) for 2 h at RT, followed by 50 μL of streptavidin conjugated to peroxidase for 20 min protected from exposure to light. After washing, the reaction was revealed by adding 50 μL of substrate [H_2O_2: tetramethylbenzidine (TMB)] (1:1) for 20–40 min and blocked with 50 μL of sulfuric acid (H_2SO_4 2N). Samples were read in a SPECTRAMAX 190 Microplate Reader (Molecular Devices, San Jose, CA, USA) at 450 nm.

Preparation and Quantification of the Peritoneal Lavage Fluid Cellularity

The numbers and types of cells recruited from the bone marrow to the peritoneal cavity were determined by PL. PL fluid was recovered following injection of 5 mL of 0.02M PBS, pH 7.4, and cells were counted in a Neubauer chamber after dilution (1:40) in Turk solution (2% acetic acid solution). Additionally, cytocentrifuged preparations (100 μL of PL fluid/slide) were obtained in a Cytospin 4 Shandon (Thermo Scientific Corporation, Waltham, MA) at 450 rpm for 5 min at room temperature and stained with a routine hematology stain (Panótico Rápido kit, Laborclin, Pinhais, PR, Brazil). Cells (100/slide) were counted in an Olympus BX-41 microscope (Tokyo, Japan) at a magnification of 1,000x.

Histopathology and Histoquantitative Analyses

Liver samples from uninfected and infected mice (6 animals/group) were removed from the right lobe and divided into approximately 5 mm^3 fragments, which were immediately fixed in 4% paraformaldehyde in buffered phosphate, pH 7.3, 0.1 M overnight at 4°C (28). Next day, the specimens were transferred to a 0.1 M phosphate buffer solution, pH 7.3 and kept in this solution at 4°C for further histological processing. Samples were then dehydrated, embedded in glycolmethacrylate resin (GMA) (Leica Historesin Embedding Kit, Leica Biosystems, Heidelberg, Germany) as before (28) and cut at 3 μm-thick sections using a Leica microtome RM2155. This histological approach combines optimal fixation and processing for visualization and quantification of inflammatory processes. Three sections per animal were obtained at an interval of 300 μm between sections to ensure analysis of different granulomas. Sections were stained with hematoxylin-eosin (Sigma-Aldrich) for qualitative and quantitative evaluation of granulomas and other parameters.

Histological slides from livers were scanned using a *3D Scan Pannoramic Histech* scanner (3D Histech Kft. Budapest, Hungary) connected to a computer (Fujitsu Technology Solutions GmbH, Munich, Germany). This scanner enables a resolution of 0.23 μm per pixel. Tissue section areas were analyzed using *Pannoramic Viewer 1.15.2 SP2 RTM* (3D Histech kft.) and Histoquant (3D Histech kft.) softwares, which provide

a morphometric detailed analysis with precise measurements of different histological parameters, including areas and types of granulomas at high resolution of entire histological slides (19).

The following morphometric parameters were evaluated and quantitated in the liver: (i) Distribution and types of granulomas as previously described (19); (ii) Area taken by granulomas: the total area related to the granulomatous response was measured in three sections per animal. The area occupied by different types of granulomas was also specified. A total of 203 granulomas was recorded. iii) Area taken by non-granulomatous inflammatory infiltrates: the total area occupied by infiltrates was measured in three sections per animal; (iv) Number of parasite eggs in the liver per mm^2 of tissue; (v) number of eosinophils per total area of granuloma.

Additionally, the proportion of eosinophils was estimated in hepatic granuloma types. For this, the number of eosinophils was counted among 400 immune cells in three randomly chosen granulomas per cell section from each animal. Considering that three sections were studied per animal ($n = 6$ animals), a total of 54 granulomas was analyzed for this eosinophil quantification.

Conventional TEM

For conventional TEM, hepatic and intestinal (small and large) fragments were prepared as in previous works (19, 29). Tissue was fixed in a mixture of freshly prepared aldehydes [1% paraformaldehyde and 1.25% glutaraldehyde (EM grade, 50% aqueous, Electron Microscopy Sciences-EMS, Hatfield, PA)] in 0.1 M sodium phosphate buffer, pH 7.4, at room temperature (RT). After 2 h, fragments were sliced into small pieces of 1 mm^3 and fixed in the same fixative overnight at 4°C. Next, the fragments were washed twice in 0.1 M sodium phosphate buffer for 4 h each at 4°C and kept in the same buffer at 4°C for further processing. All fragments were post-fixed in 1% osmium tetroxide in Sym-Collidine buffer, pH 7.4, for 2 h at RT. After washing with sodium maleate buffer, pH 5.2, they were stained en bloc in 2% uranyl acetate (EMS) in 0.05 M sodium maleate buffer, pH 6.0, for 2 h at RT and washed in the same buffer as before prior to dehydration in graded ethanols and acetone, and infiltration and embedding with a propylene oxide-Epon sequence (Eponate 12 Resin; Ted Pella, Redding, CA, USA). After polymerization at 60°C for 16 h, thin sections were cut using a diamond knife on an ultramicrotome (Leica, Bannockburn, IL). Sections were mounted on uncoated 200-mesh copper grids (Ted Pella) before staining with lead citrate and viewed with a transmission electron microscope (CM 10; Philips, or Tecnai G2-20-ThermoFischer Scientific/FEI 2006, Eindhoven, the Netherlands) at 60–80 KV. Electron micrographs from different experiments ($n = 3$) were randomly taken at different magnifications to study the ultrastructural features of secretory granules and other subcellular morphological aspects.

Tissue Preparation for Immunonanogold Electron Microscopy (immunoEM)

For immunoEM, liver fragments were immediately fixed in fresh 4% paraformaldehyde in phosphate-buffered saline (PBS) (0.02 M sodium phosphate buffer, 0.15 M sodium chloride, pH 7.4) (30). Samples were fixed for 4 h at RT, washed in PBS, immersed in 30% sucrose in PBS overnight at 4°C, embedded in OCT compound (Miles, Elkhart, IN), and stored in −180°C liquid nitrogen for subsequent use.

Pre-embedding Immunonanogold EM

As detailed before (30), pre-embedding immunolabeling was carried out before standard EM processing (postfixation, dehydration, infiltration, resin embedding and resin sectioning). All labeling steps were carried out at RT on cryosections as before (30) as follows: a) one wash in 0.02 M PBS, pH 7.4, 5 min; (b) immersion in 50 mM glycine in 0.02 M PBS, pH 7.4, 10 min; (c) incubation in a mixture of PBS and BSA (PBS-BSA buffer; 0.02 M PBS plus 1% BSA) containing 0.1% gelatin (20 min) followed by PBS-BSA plus 10% normal goat serum (NGS) (30 min) – (this step is crucial to block non-specific Ab binding sites); (d) incubation with primary Ab (1 h); (e) blocking with PBS-BSA plus NGS (30 min); (f) incubation with secondary Ab (1 h); (g) washing in PBS-BSA (three times of 5 min each); (h) postfixation in 1% glutaraldehyde (10 min); (i) five washings in distilled water; (j) incubation with HQ silver enhancement solution in a dark room according to the manufacturer's instructions (Nanoprobes) (10 min). This step enables a nucleation of silver ions around gold particles. These ions precipitate as silver metal and the particles grow in size facilitating observation under TEM; (k) three washings in distilled water; (l) immersion in freshly prepared 5% sodium thiosulfate (5 min); (m) postfixation with 1% osmium tetroxide in distilled water (10 min); (n) staining with 2% uranyl acetate in distilled water (5 min); (o) embedding in Eponate (Eponate 12 Resin; Ted Pella); (p) after polymerization at 60°C for 16 h, embedding was performed by inverting eponate-filled plastic capsules over the slide-attached tissue sections; and (q) separation of eponate blocks from glass slides by brief immersion in liquid nitrogen. Thin sections were cut using a diamond knife on an ultramicrotome (Leica). Sections were mounted on uncoated 200-mesh copper grids (Ted Pella) before staining with lead citrate and viewed with a transmission electron microscope (CM 10; Philips) at 60 kV. Two controls were performed: (1) primary Ab was replaced by an irrelevant Ab, and (2) primary Ab was omitted. Electron micrographs were randomly taken at different magnifications to study the entire cell profile and subcellular features.

TEM Quantitative Analysis of Eosinophils

For quantitative studies of secretory granules, electron micrographs randomly taken from eosinophils infiltrated into infected livers were evaluated after conventional preparation for TEM. A total of 148 electron micrographs showing the entire cell profile and nucleus were analyzed to determine the total number of secretory granules per cell section. Moreover, different magnifications from the same cell were taken to identify ultrastructural changes indicative of PMD, classical exocytosis and/or cytolysis. A total of 2,868 secretory granules were counted, and the numbers of intact, emptying and fused granules were established per cell section. Intact granules were observed as membrane-bound organelles full of contents without evident ultrastructural changes. Emptying granules, that is, undergoing losses of their contents, were indicative of the PMD process,

and were identified as enlarged granules with lucent areas in their cores, matrices or both and/or disassembled contents in the absence of granule fusions (20, 31). Granules fused with each other and/or with the plasma membrane were indicative of classical exocytosis (32). The process of cytolysis was recognized by partial or total loss of the plasma membrane integrity and/or extracellular deposition of intact granules (31). For controls, tissue eosinophils randomly distributed in the small and large intestine from uninfected animals were also analyzed by TEM.

Additionally, the total number of specific granules positive for MBP-1 was quantitated in electron micrographs obtained from the liver after ultrastructural immunolabeling for this protein. These analyses were done in clear cross-cell sections (total of 9 cells, $n = 218$ granules) exhibiting the entire eosinophil cell profile, intact plasma membranes and nuclei as previously performed for single-cell analyses at high resolution of immunogold-labeled eosinophils (32). All quantitative studies were done using the *Image J* software (National Institutes of Health, Bethesda, MD).

Finally, the total number of round large, cytoplasmic vesicles was evaluated per cell section. A total of 755 vesicles was enumerated in 19 randomly taken electron micrographs showing the entire cell profile and nucleus from both infected and uninfected tissue eosinophils.

Statistical Analyses

ANOVA followed by Turkey multiple comparisons test, or Kruskal-Wallis test was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California, www.graphpad.com). Significance was $P < 0.05$.

RESULTS

The Liver Pathology Induced by *S. mansoni*

Schistosomiasis mansoni is characterized by a robust egg-induced granulomatous inflammation in the liver [reviewed in (33)]. Granulomas are formed around the eggs that are lodged in the presinusoidal capillary venules of this organ; and hepatomegaly, secondary to granulomatous inflammation, occurs early in the evolution of this disease (33). In the mouse model used in the present work, well-characterized granulomas associated with hepatomegaly were clearly observed (**Supplementary Figure 1**). While the body weights of the infected animals did not show significant difference when compared to the controls (42.7 ± 1.3 g and 40 ± 0.8 g for the infected and control groups respectively, mean ± SEM, $n = 6$ animals/group), the livers were significantly increased (4.3 ± 0.2 g vs. 1.5 ± 0.05 g for infected and control groups respectively, mean ± SEM, $n = 6$ animals/group, $P < 0.0001$).

To study liver histopathology in more detail, we used a histological approach that combines optimal fixation and processing with a plastic resin (glycolmethacrylate) embedding, which provides better tissue resolution than conventional paraffin embedding (28) with optimal visualization of inflammatory processes including granulomas (27). Sections obtained from glycolmethacrylate-embedded liver fragments were then analyzed by whole slide imaging (WSI), which enables scanning and imaging of entire histological slides (**Figure 1A**). The resulting digital images have high resolution and offer access to all areas on the slide, thus allowing reliable assessment of the number, evolutional types, frequency and areas of granulomas (19).

By applying WSI, we found three main types of granulomas: pre-granulomatous exudative (PE); necrotic-exudative (NE) and exudative-productive (EP) (**Figures 1B–D**), as previously demonstrated by our group for this experimental model (19). In the PE stage (**Figure 1B**), inflammatory cells are

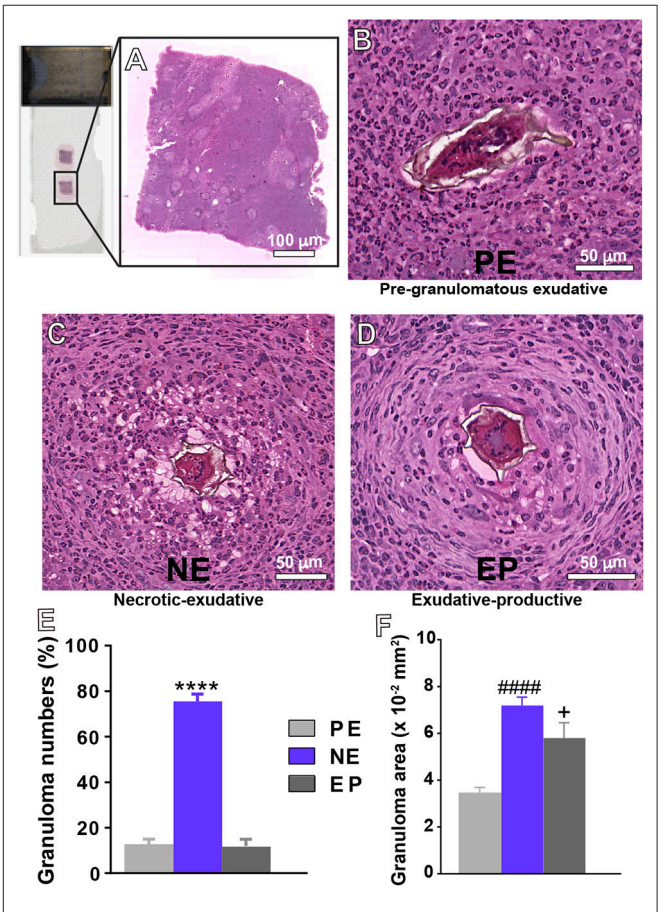

FIGURE 1 | Representative types of granulomas and their frequencies in the livers of mice infected with *S. mansoni*. **(A)** Histological analyses on entire tissue sections identified three types of granulomas: **(B)** Pre-granulomatous exudative (PE), characterized by an infiltrate of inflammatory cells in process of organization around the parasite egg; **(C)** necrotic-exudative (NE), identified by a central halo of necrosis and numerous inflammatory cells distributed irregularly on subsequent layers; and **(D)** exudative-productive (EP), characterized by a rich structure of collagen fibers and inflammatory cells concentrated in the periphery and by a more organized and circumferential aspect. The numbers of granulomas and their areas in the hepatic tissue are shown in **(E,F)**, respectively. Morphometric analyses were performed using Pannoramic Viewer software after whole slide scanning. Data represent mean ± SEM. $^{****}P < 0.0001$ vs. numbers of granulomas PE and EP; $^{\#\#\#\#}P < 0.0001$ area of granuloma PE; $^{+}P = 0.04$ vs. area of granuloma PE. Images are representative of 3 independent experiments.

in process of organization around the egg while the other stages (**Figures 1C,D**) are associated with a more organized circumferential structure in which clusters of inflammatory cells such as eosinophils, lymphocytes and macrophages are intermixed with collagen fibers (34, 35). After recording 203 granulomas, we detected that the NE type, identified by a central halo of necrosis and numerous inflammatory cells distributed irregularly on subsequent layers, was the most frequent type of granuloma (**Figures 1C–E**). Because the sizes of granulomas greatly vary in target tissues, we next measured the tissue area taken by granulomas in the liver. The NE type showed the largest area (**Figure 1F**). The area occupied by the EP type was significantly higher than that occupied by the PE type (**Figure 1F**), although these two types of granulomas did not differ in terms of numbers (**Figure 1E**). Next, the percentage of granuloma area in relation to the entire tissue was obtained. We found that the percentage of hepatic tissue taken by inflammatory granulomatous processes represented 7.7 ± 1.3% (mean ± SEM, $n = 6$ animals) of the liver while the number of parasite eggs in this organ was 2.08 ± 0.5/mm^2 of tissue. Additionally, we measured the area taken by inflammatory infiltrates (non-granulomatous), which represented 35.1 ± 1.1% (mean ± SEM, $n = 6$ animals). In conjunction, the inflammatory response (non-granulomatous and granulomatous) occupied 42.8 ± 2.4% (mean ± SEM) of the hepatic tissue, thus denoting a remarkably compromised liver.

Accordingly, significant increases in the levels of the serum enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) reflecting altered liver function (7, 36, 37) were detected in response to the infection (**Table 1**). We observed approximately 3- and 4-fold elevations, respectively, in response to the acute *S. mansoni* infection (**Table 1**).

Detection of Th2 Cytokines in Response to *S. mansoni* Infection

The continuous antigenic stimulation resulting from the trapped eggs in target organs leads to a pronounced inflammatory response at 6–8 weeks post-infection associated with a dominant CD4+ T cell-dependent immune response [reviewed in (38)]. The interleukins (IL) IL-4, IL-10, and IL-13 are dominant cytokines driving this reaction (38). In fact, by analyzing these cytokines in the plasma, we detected significantly higher levels of them in infected compared to uninfected animals (**Supplementary Figure 2**).

TABLE 1 | Serum transaminases of uninfected and *S. mansoni*-infected animals.

Parameters	Animals				*P*-value**
	Uninfected ($n = 12$)	Fold ↑	Infected ($n = 12$)	Fold ↑	
AST (U/L)	76 ± 12	1.0	322 ± 48	4.24	<0.0001
ALT (U/L)	49 ± 8	1.0	160 ± 24	3.26	<0.0001
De ritis ratio*	1.6 ± 0.1	1.0	2.0 ± 0.1	1.25	<0.0001

*AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase. *De ritis ratio was calculated as [AST/ALT] and indicates the degree of hepatocelular damage. **P-value indicates difference between infected and unifected animals.*

Eosinophils Are Actively Recruited and Represent the Main Inflammatory Cell Population Within *S. mansoni* Infection-Induced Granulomas

During the acute schistosomiasis, increases in the numbers of eosinophils can be detected in the circulation, peritoneal cavity and target tissues (4, 5, 7, 10). Our quantitative analyses of eosinophils in the PL showed that the number of these cells was 15 times higher in the peritoneal fluid collected from infected animals than in controls (4.8 ± 1.1 for infected vs. 0.3 ± 0.02 for control, mean ± SEM, $P = 0.003$, $n = 4$ animals/group) (**Figure 2A**). Eosinophils were also quantitated within NE granulomas, which, as noted here (**Figure 1E**) and before (19), represent the most frequent stage of granuloma found in this model of acute schistosomiasis. A total of 34,432 eosinophils was counted. By applying two morphometric evaluations in whole sections, that is, assessment of the eosinophil numbers per granuloma area and proportion of eosinophils per granuloma, we found that these cells corresponded to 60.1 ± 0.3%

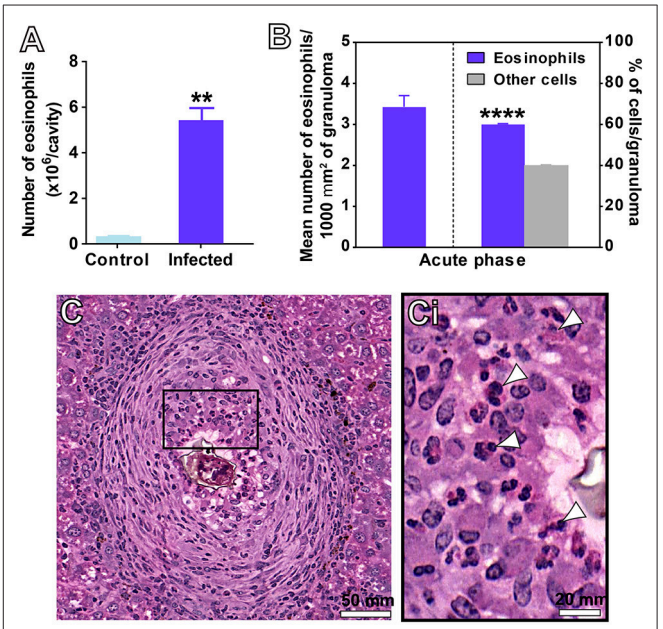

FIGURE 2 | Detection of eosinophils in the peritoneal cavity **(A)** and hepatic granulomas **(B–C)** of mice infected with *S. mansoni*. **(A)** Eosinophil numbers quantitated in the peritoneal lavage. **(B)** Eosinophil numbers quantitated per area of granuloma considering all granulomas, and in the most frequent type of hepatic granuloma (NE type). In **(C)**, a representative NE granuloma. The boxed area in **(C)** is shown in **(Ci)**. Arrowheads indicate examples of eosinophils with characteristic acidophilic cytoplasm. Data represent mean ± SEM. **$P = 0.003$ vs. control uninfected mice; ****$P < 0.0001$ vs. other immune cells within granulomas of infected mice. Morphometric evaluations were done with the use of *Histoquant* software. Cytocentrifuged preparations were stained and analyzed at magnification of 1000x. A total of 34,432 eosinophils were counted in 203 granulomas at a magnification of 20x.

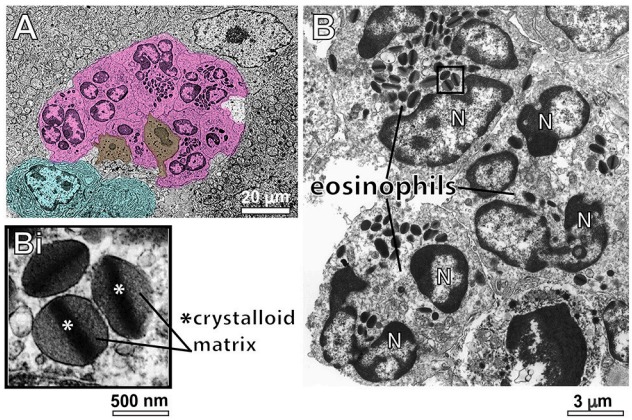

FIGURE 3 | Conventional TEM shows infiltrating eosinophils in the liver of *S. mansoni*-infected mice. **(A)** A representative electron micrograph of the hepatic tissue in low magnification shows a group of eosinophils (colored in pink) in close contact with each other and with neutrophils (brown) and plasma cells (blue). In **(B)**, eosinophils exhibit their typical ultrastructure with a lobulated nucleus (N) and a robust cytoplasmic population of specific granules with a unique morphology-an internal well-defined electron-dense crystalline core and an outer electron-lucent matrix (seen in higher magnification in **Bi**).

(mean ± SEM) of all immune cells within NE granulomas (**Figures 2B,C,Ci**).

Infiltrating Eosinophils Degranulate Through Different Degranulation Patterns

Having confirmed the striking number of eosinophils in hepatic granulomas with WSI, we investigated morphological features of these cells at high resolution. For this, liver fragments were processed for conventional TEM. First, eosinophils with typical ultrastructure, that is, exhibiting a polylobed nucleus and a prominent population of cytoplasmic secretory granules with a electron dense crystalloid core surrounded by an electron lucent matrix were easily identified as isolated cells or forming tight groups of cells (**Figure 3**). Eosinophils were in contact with each other and/or with cells such as neutrophils, plasma cells and lymphocytes (**Figure 3**).

Second, we sought to identify and characterize ultrastructural signs underlying degranulation (as described in material and methods) in infiltrated eosinophils. We found that while 19.1% of eosinophils were intact (**Figure 4A**), most eosinophils (66.4%) exhibited predominantly features of PMD (**Figures 4Ai–iii,B**). Characteristic features of cytolysis (**Figure 4C**) were observed in 13.0% of eosinophils (**Figure 4B**) and just 1.5% (**Figure 4B**) showed classical or compound exocytosis (**Figure 5**).

Third, we wondered if the numbers of intact and emptying secretory granules in infected eosinophils would differ from values shown by resting cells. Because eosinophils are not normally found in the liver, we analyzed the ultrastructure of eosinophils distributed in the intestinal tract of uninfected animals (**Supplementary Figure 3**). A total of 131 cells showing intact plasma membranes, that is, not undergoing cytolysis, was evaluated. Our quantitative analysis revealed a significant increase of emptying, non-fused granules (49.5 ± 1.9/cell section for infected vs. 32.3 ± 6.9/cell section for control, mean ± SEM, $P = 0.003$, $n = 2868$ secretory granules) and a significant reduction of intact granules in response to the acute schistosomiasis ($P = 0.001$) (**Figure 4D**), thus confirming the ability of these cells to secrete mostly through PMD.

PMD Is the Predominant Secretory Process of MBP-1 Release by *S. mansoni*-Activated Eosinophils

MBP-1 is the main cationic protein stored as preformed pool within eosinophil secretory granules and considered a hallmark for these cells. Because MBP-1 has been associated with the immunopathogenesis of various helminthic diseases, including schistosomiasis mansoni [reviewed in (39)], we next investigated the structural mechanism of MBP-1 release by applying pre-embedding immunonanogold EM. This methodology has proved to be very effective in localizing immune mediators in human eosinophils and other cells from the immune system (30, 32, 40).

We observed a clear labeling for MBP-1 in the entire population of inflammatory eosinophils while other infiltrated immune cells were completely negative (**Figure 6**). Infected cells, for which the primary antibody was replaced by an irrelevant antibody or omitted (**Supplementary Figure 4**), were negative. MBP-1 positivity was mostly associated with eosinophil secretory granules (**Figure 7**). By using software for quantitating granules, we detected that the majority of them (84.0 ± 2.5%, mean ± S.E.M, $n = 9$ cells) in each eosinophil section was labeled for MBP-1 and that most of these labeled granules were undergoing PMD (**Figure 7A**), that is, showing disassembled cores, enlargement, matrix coarsening and/or reduced electron-density (**Figures 7B–E**).

Identification of a Vesicular Trafficking of MBP-1 Within *S. mansoni*-Triggered Eosinophils in the Liver

When human eosinophils are activated by inflammatory stimuli, there is a significant increase of cytoplasmic large vesicles termed Eosinophil Sombrero Vesicles (EoSVs), which are involved in the transport of granule-derived products (15, 31, 41). The existence of EoSVs-like vesicles in mouse eosinophils is unknown. By performing conventional TEM in the liver from infected animals, we noticed, for the first time, the presence of numerous round, reasonably large (∼80–150 nm) vesicles in the cytoplasm of inflammatory eosinophils (highlighted in pink in **Figures 4A**, **8B**). By enumerating a total of 755 vesicles in infected and uninfected tissue eosinophils, we found a significant increase of these vesicles in infected compared to uninfected cells (68.1 ± 7.3/cell section for infected vs. 14.2 ± 3.5/cell section for control, mean ± SEM, $P < 0.0001$, $n = 19$ cells) (**Figure 8A**).

Next, we analyzed the population of these vesicles after ultrastructural immunolabeling for MBP-1. Our single-cell analyses at high resolution were revealing in demonstrating MBP-1-positive vesicles distributed in the cytoplasm and attached to or surrounding the surface of emptying granules

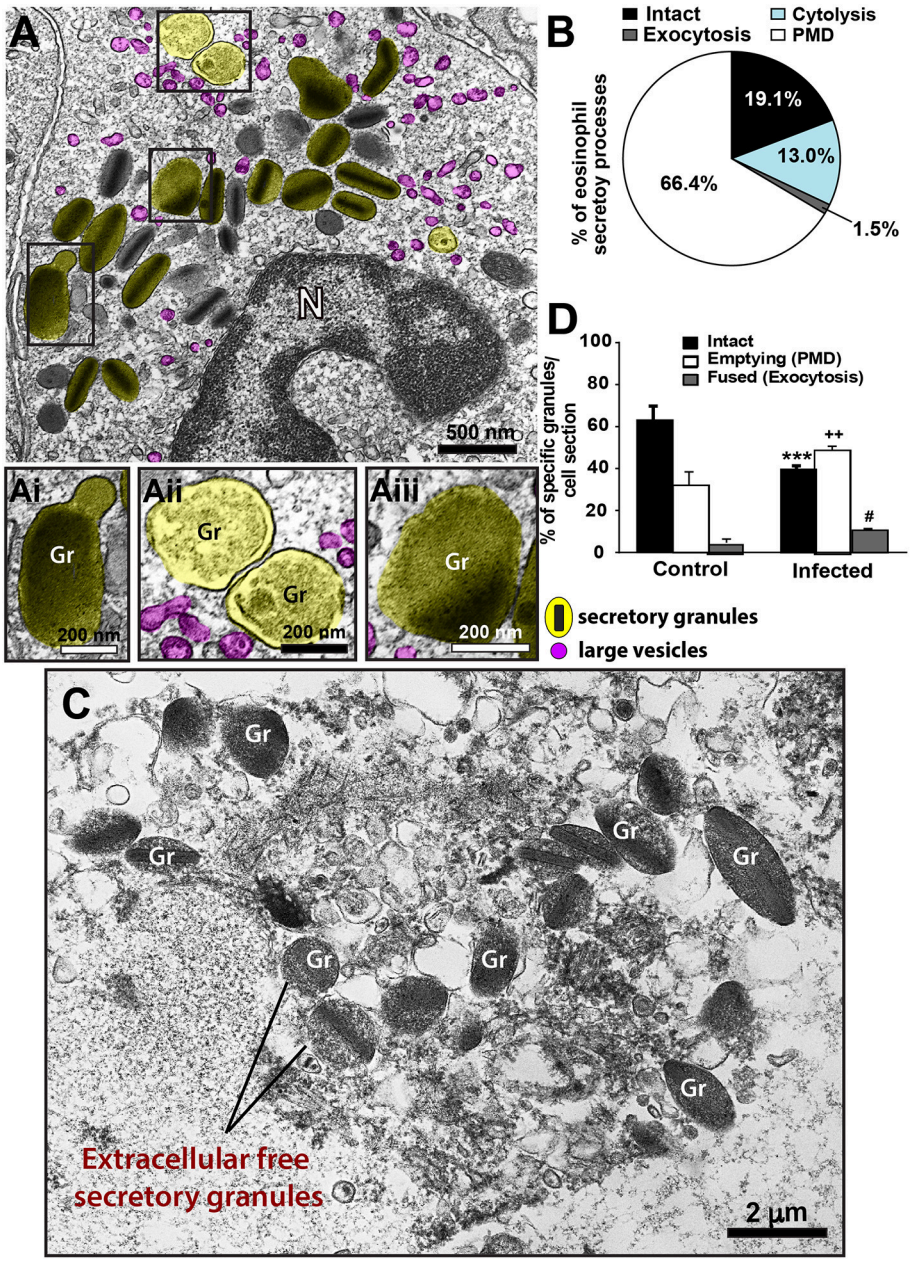

FIGURE 4 | Ultrastructural features of eosinophil degranulation in inflammatory sites of the liver of *S. mansoni*-infected mice. **(A)** A representative eosinophil shows PMD, characterized by the presence of emptying, non-fused secretory granules. The population of eosinophil specific granules is colored in yellow while large vesicles are highlighted in pink. The boxed areas in **(A)** are shown in **(Ai–Aiii)** in higher magnification. **(Ai–Aiii)** Note structural signs of PMD such as granule enlargement and disarrangement of granule cores and matrices. **(B)** Quantification of the secretory patterns shown *in vivo* by hepatic eosinophils in response to the acute infection. In **(C)**, an eosinophil in advanced stage of cytolysis shows extracellular free secretory granules (Gr). **(D)** Most eosinophil secretory granules undergo structural changes indicative of PMD compared to that in uninfected mice. Data represent mean ± SEM. One hundred eight electron micrographs showing the entire cell profile and nucleus were analyzed and 2868 secretory granules were counted. $^{***}P = 0.001$ vs. intact granules, $^{++}P = 0.003$ vs. emptying granules, $^{\#}P = 0.03$ vs. fused granules of uninfected mice. Gr, secretory granule. *N*, nucleus. Fragments of the liver of animals experimentally infected (acute phase) and intestines (for uninfected controls) were prepared for conventional TEM.

(**Figure 8B**). Computational analyses showed that ∼20% of all cytoplasmic vesicles from the same size range were carrying MBP-1 (**Figure 8Bi**). Altogether, our findings consistently demonstrate the occurrence of a secretory process based on vesicular trafficking as a main mechanism for MBP-1 release in response to the acute schistosomiasis in mice. Accordingly, MBP-1 immunolabeling was also detected in the extracellular matrix (**Figure 8B**, arrows). Interestingly, the deposits of MBP-1 were

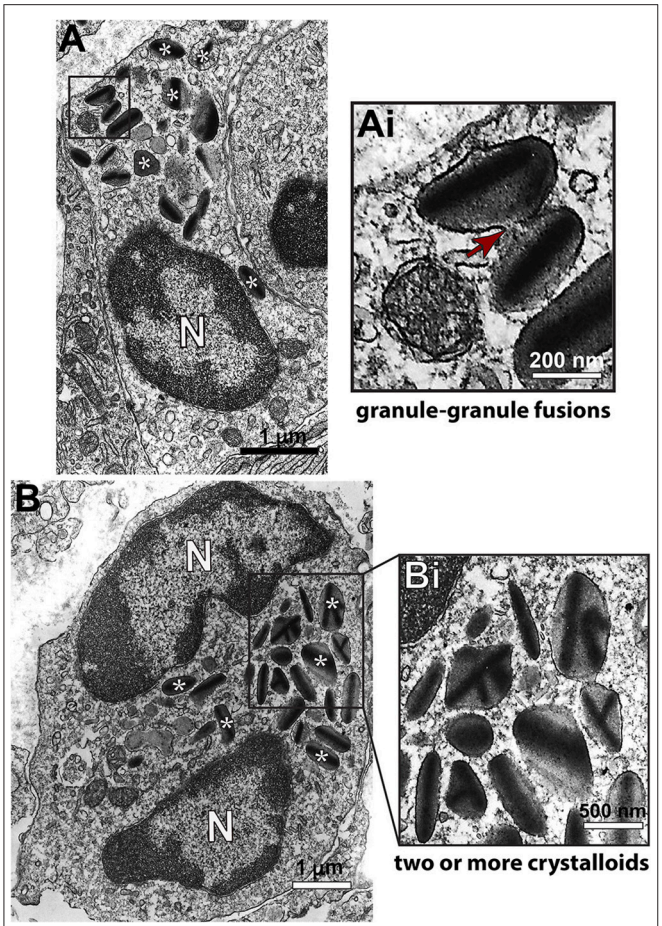

FIGURE 5 | Ultrastructure of eosinophils undergoing compound exocytosis in inflammatory sites of the liver of *S. mansoni*-infected mice. **(A)** A representative electron micrograph shows an eosinophil with granules in process of fusion. In **(B)**, secretory granules show several crystalline cores, a feature also considered as an evidence of fusion. The boxed areas in **(A,B)** are shown in **(Ai,Bi)** in higher magnification. (*) Denotes some of the secretory granules. Arrows indicates a fusion area. *N*, nucleus. Fragments of the liver of animals experimentally infected (acute phase) were prepared for conventional TEM.

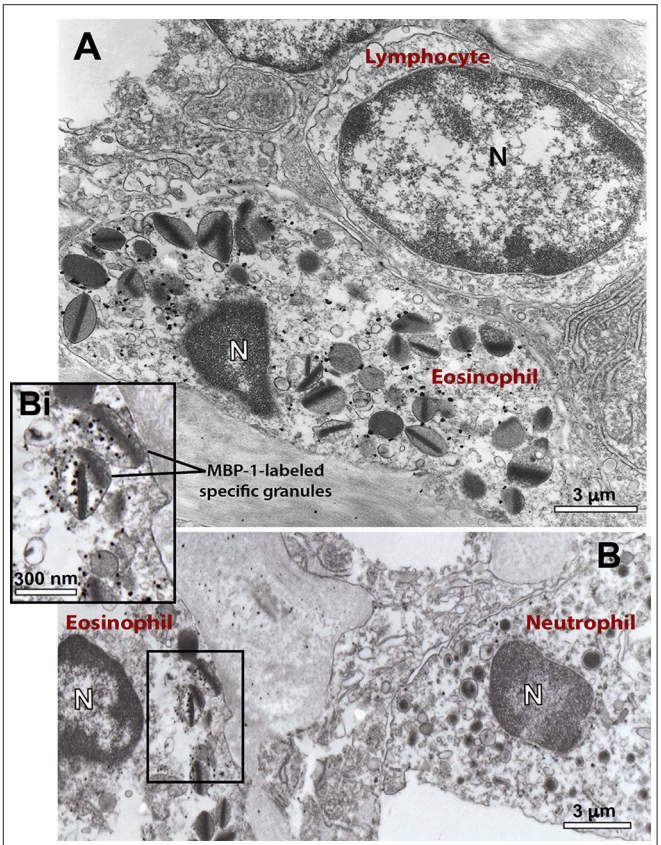

FIGURE 6 | Ultrastructural immunolocalization of MBP-1 in the liver from a *S. mansoni*-infected mouse. **(A,B)** Positivity is seen within representative infiltrating eosinophils while other inflammatory cells such as lymphocyte **(A)** and neutrophil **(B)** are negative. Labeling was associated with secretory (specific) granules, as observed in higher magnification **(Bi).** A close apposition between an eosinophil and a lymphocyte is noted in **(A)**. Liver fragments were prepared for pre-embedding immunonanogold electron microscopy. *N*, nucleus.

not massive, but dispersed in the extracellular matrix (**Figure 8B**, arrows).

DISCUSSION

Expansion and recruitment of eosinophils is a central feature of the host response to the *S. mansoni* infection. How these cells release their products in target organs of this disease is unknown. Our study describes marked eosinophil degranulation *in vivo* in the liver triggered by schistosomiasis mansoni in mice and identifies, for the first time, PMD as the main mode of eosinophil secretion. We also provide direct evidence that MBP-1 is transported in the cytoplasm of infiltrating eosinophils and released through a vesicular trafficking in response to the acute infection.

In this work, we explore the ability of inflamatory eosinophils to degranulate in a murine model of schistosomiasis mansoni. Acute schistosomiasis is characterized by a systemic hypersensitivity reaction against the migrating schistosomula and eggs (42). During the first 3–5 weeks, the host is exposed to migrating immature parasites while at weeks 5–6, the parasite matures and begins to produce eggs, which is associated with a Th2 response (reviewed in 39). All classical parameters confirmatory of the acute infection, such as hepatomegaly, high density of eggs in a target organ; well-characterized granulomatous inflammation around deposited eggs; alteration of liver enzymes, increased levels of Th2 cytokines and extensive eosinophil infiltration were consistently demonstrated in this model. Moreover, our detailed analysis of granuloma formation in infected mice corroborates the prevalence of the NE granuloma, which is greatly enriched in inflammatory cells and it is also the major granuloma type found during the acute schistosomiasis mansoni in humans (42).

Acute schistosomiasis in mice led to substantial degranulation of infiltrating eosinophils in the liver through PMD. PMD is a frequent secretory process of human eosinophils observed *in vivo* in varied human inflammatory and other disorders

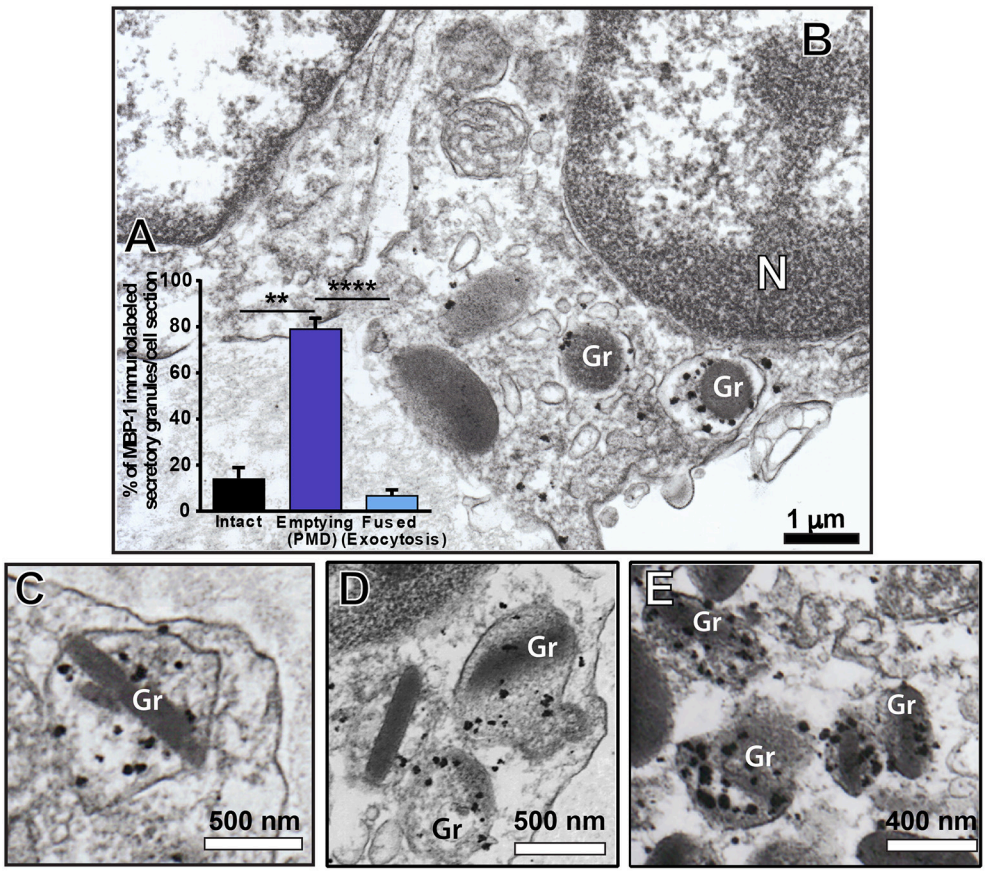

FIGURE 7 | Immunolocalization of MBP-1 on liver-infiltrating eosinophils from *S. mansoni*-infected mice. **(A)** Most MPB-1-positive secretory granules (~80%) showed characteristics of piecemeal degranulation (PMD). **(B–E)** Single-cell analyses at high-resolution reveal robust labeling of MBP-1 within secretory granules (Gr) of activated eosinophils. Note the typical signs of PMD such as enlargement and disarrangement of granule cores and matrices. *N*, nucleus. Data represent mean ± SEM. The numbers of labeled and not labeled granules ($n = 218$ granules) were counted in electron micrographs ($n = 9$). $^{**}P = 0.005$ vs. intact granules; $^{****}P < 0.0001$ vs. fused granules. Liver fragments were prepared for pre-embedding immunonanogold electron microscopy.

such as asthma (43); nasal polyposis (44); allergic rhinitis (44, 45); ulcerative colitis (44); Crohn's disease (44); atopic dermatitis (46); functional dyspepsia (47), gastric carcinoma (48); shigellosis (49) and cholera (50), but this is the first time that it is clearly recognized during the acute infection with *S. mansoni*. In this mode of secretion, eosinophils release granule contents, but retain their granule containers [reviewed in (51)]. PMD is identified mainly by structural disarrangement of the granules cores and matrices within granules delimited by intact membranes, but other subtle signs such as granule matrix coarsening and granule enlargement can additionally indicate PMD occurrence in mouse eosinophils (52) (**Figure 4**). In fact, there have been controversies that mouse eosinophils are not able to "degranulate" in some mouse models of allergic airway inflammation (24, 53, 54). In our work using detailed ultrastructural analyses to examine mouse eosinophils in a "classic" host-response model of murine schistosomiasis, we provide definitive findings for the *in vivo* capacity of mouse eosinophils, like human eosinophils, to undergo PMD.

To compare the extent of the secretory processes shown by infiltrating eosinophils in the liver of infected animals, we used resident intestinal eosinophils as controls since eosinophils are not resident cells of the liver. Hepatic inflammatory eosinophils had significantly higher numbers of secretory granules with PMD features compared to the eosinophil population of the intestinal tract (**Figure 4D**). However, it should be highlighted that this population showed considerable PMD (**Figure 4D**), which can be explained by the fact that intestinal eosinophils are phenotypically distinct from blood eosinophils and exhibit an activated phenotype based on their cytokine expression and degranulation status (55). In fact, eosinophils that reside in the gastrointestinal tract are required for the homeostatic intestinal immune responses, including IgA production through secretion of cytokines (56, 57) and constitutively express antigen presentation markers (58). Thus, intestinal eosinophils are more active and consequently with a higher activity of secretion.

In addition to PMD, our quantitative EM analyses revealed that 13% of liver-infiltrating eosinophils exhibited different degrees of cytolysis (**Figures 4B,C**), which deposits cell-free

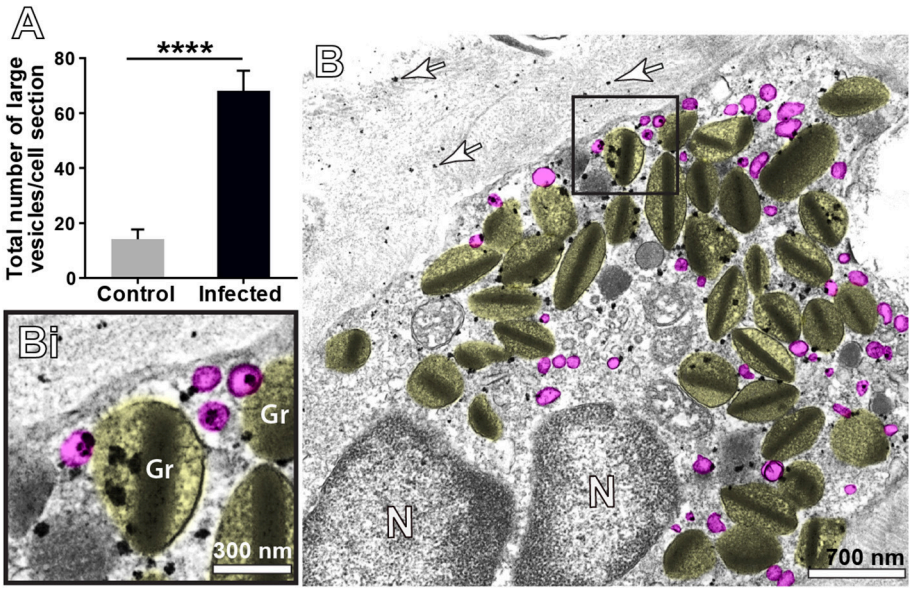

FIGURE 8 | Vesicular trafficking of MBP-1 in the cytoplasm of inflammatory eosinophils in the liver of *S. mansoni*-infected mice. **(A)** The acute infection induces a prominent formation of cytoplasmic, large (80–150 nm) round vesicles (highlighted in pink in **B**. Note in **Bi**) that immunolabeling for MBP-1 is clearly associated with several of these vesicles in addition to secretory granules (Gr, highlighted in yellow). Arrows indicate extracellular deposition of MBP-1. Data represent mean ± SEM. The numbers of vesicles ($n = 755$) were counted in a total of 19 electron micrographs, after conventional processing for TEM. ****$P < 0.0001$ vs. control group. For immunolabeling of MBP-1, liver fragments were prepared for pre-embedding immunonanogold electron microscopy.

secretory granules in the surrounding tissue [reviewed in (31)]. Cytolysis is a physiologically important mode of eosinophil secretion because the specific granules remain active even after cell death (31). Cytolysis is defined ultrastructurally by physical rupture of the cell and is morphologically distinct from both apoptosis and necrosis (46, 59). More recently, another form of cell death–pyroptosis–, which is mediated by caspase-1 (60), was identified in hepatic eosinophils isolated from a mouse model of *S. mansoni* infection (61). Because cell disruption is also a feature of pyroptotic cells (60), we cannot rule out that part of the cytolytic eosinophil population found by our EM analyses may be undergoing pyroptosis.

Formation of large secretory vesicles (EoSVs) that arise from eosinophil specific granules and contain granule proteins and cytokines is another indication of PMD and has been documented in human eosinophils [reviewed in (51, 62)] but heretofore not in mouse eosinophils. Moreover, amplified formation of EoSVs is considered a morphological feature of activated human eosinophils (15, 41). For example, eosinophils stimulated with CCL11/eotaxin-1 or tumor necrosis factor alpha (TNF-α) show increased numbers of cytoplasmic EoSVs (40) as well as do naturally activated eosinophils from patients with hypereosinophilic syndrome when compared to normal donors (63). Our present quantitative EM studies demonstrate, for the first time, that *S. mansoni*-triggered mouse eosinophils have an augmented population of large (80–150 nm) round vesicles, analogously to EoSVs found in human eosinophils (15). As with human EoSVs, these large vesicles from mouse eosinophils are seen distributed in the cytoplasm and clearly associated with secretory granules, but these vesicles do not exhibit the same typical tubular morphology of human EoSVs (**Figures 4A,Aii**, **8B,Bi**).

MBP has been extensively detected extracellularly in inflammatory sites of eosinophil-associated human diseases (64–68), including schistosomiasis mansoni (69). Here, we also observed extracellular localization of MBP in the liver elicited by the acute *S. mansoni* infection in mice (**Figure 8B**). MBP extracellular deposition comes from degranulating eosinophils, which can release their products via, as noted, cytolysis, classical/compound exocytosis and/or PMD. Because PMD was detected in most infiltrated eosinophils (66.4%) and relies on vesicular transport of granule products, we were expecting to find granule-mobilized MBP in association with cytoplasmic vesicles. Indeed, our single-cell analyses using very small gold particles (1.4 nm) for membrane microdomains access enabled labeling of MBP-1 on intracellular vesicles of eosinophils in the livers of *S. mansoni*-infected animals (**Figures 7**, **8**). Therefore, it is clear that part of the cytoplasmic vesicle population is trafficking MBP-1 within inflammatory eosinophils for extracellular release. In fact, deposits of MBP-1 were dispersed in the extracellular matrix (**Figure 8**), which is compatible with gradual vesicle-mediated release of this protein. Vesicular secretion of MBP was also described in human activated eosinophils *in vitro* (63), but this is the first report on this secretory pathway in mice and in association with a parasitic disease *in vivo*.

What is the meaning of PMD and the release of MBP-1 through PMD during acute shistosomiasis? Eosinophils produce an array of granule-stored immune mediators that

are known to be key regulators in diverse physiological and pathological processes (3) and are a source of both pro-inflammatory and immune regulatory cytokines (70). In contrast to classical and compound exocytosis, whereby whole granule contents are extruded *in toto*, PMD enables extracellular delivery of specific mediators in small amounts ("piece-by-piece") through a vesicular trafficking. Gradual release of immune mediators, including MBP-1, by inflammatory eosinophils may be involved with immunoregulatory functions of these cells during schistosomiasis. MBP, in addition to be classically associated with parasite killing, has been implicated with the regulation of cytokine responses during helminth infections (71).

In fact, there is increasing evidence that eosinophils exert a role of immunoregulation in both adaptive and innate immunity including in the context of parasitic diseases [reviewed in (12, 72)]. It is now well documented that eosinophils have key immunoregulatory functions as professional antigen-presenting cells and as modulators of CD4(+) T cell, dendritic cell, B cell, mast cell, neutrophil, and basophil functions(72). Of interest, our comprehensive ultrastructural analyses revealed direct contact of eosinophils undergoing PMD with other immune cells such as neutrophils (**Figure 3A**), plasma cells (**Figure 3A**), and lymphocytes (**Figure 6A**). It is documented that eosinophils are able to modulate the functions of other leukocytes [reviewed in (2)] and our findings showing eosinophil interactions with other immune cells may represent such capacity.

Our results shed light on the ill-understood *in vivo* roles of eosinophils, underlined by their degranulation ability, in target organs of the *S. mansoni* infection. The current view of eosinophils as effector cells, able to kill parasites through massive discharge of granule products, is beginning to change. However, the meaning of eosinophil cell-to-cell interactions and if PMD represent a subtle immunomodulatory contribution of eosinophils in both experimental and human schistosomiais awaits further investigation.

Lastly, it is important to highlight the emerging role of eosinophils in tissue homeostasis and repair (3). Sustained PMD-mediated secretion of infiltrating eosinophils during acute schistosomiasis might additionally be associated with the competence of these cells to promote a tissue repair response. Particularly in the liver, the functions of eosinophils and type-2 cytokine signaling were studied in the context of experimental tissue regeneration (73). It was demonstrated that type 2 immune responses related to eosinophils and IL-4/IL-13, via IL-4Rα in hepatocytes, stimulated liver regeneration after experimental injury (73). Thus, it is clear that eosinophils have unanticipated functions *in vivo* and their roles during eosinophilic diseases such as schistosomiasis have been more difficult to establish.

In summary, our findings identify PMD as the main secretory process of inflammatory eosinophils in the liver of *S. mansoni*-infected mice, with detection of granule-derived vesicular transport of MBP-1 in response to the infection. Vesicle-mediated release of MBP-1 and other immune mediators, such as cytokines stored within eosinophil granules may be associated with an immunoregulatory function of eosinophils, but the definitive roles of these cells in the parasitic immune response remains to be recognized. Our present work also expands our understanding of the ultrastructural aspects of mouse eosinophils, their ability to degranulate and the basic mechanisms that underlie the functioning of these cells in this experimental model.

AUTHOR CONTRIBUTIONS

RM provided the study supervision and prepared the manuscript. FD, KA, KM, and TS performed experiments. FD prepared the figures. GSCR, FD, VC, and GOLR performed cytokine analyses. RM, PW, HC-G, and FR provided critical editing of the manuscript. All authors contributed in part to writing and editing the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.03019/full#supplementary-material

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Leptin Elicits LTC_4 Synthesis by Eosinophils Mediated by Sequential Two-Step Autocrine Activation of CCR3 and PGD_2 Receptors

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Leptin is a cytokine, produced mainly by mature adipocytes, that regulates the central nervous system, mainly to suppress appetite and stimulate energy expenditure. Leptin also regulates the immune response by controlling activation of immunomodulatory cells, including eosinophils. While emerging as immune regulatory cells with roles in adipose tissue homeostasis, eosinophils have a well-established ability to synthesize pro-inflammatory molecules such as lipid mediators, a key event in several inflammatory pathologies. Here, we investigated the impact and mechanisms involved in leptin-driven activation of eicosanoid-synthesizing machinery within eosinophils. Direct *in vitro* activation of human or mouse eosinophils with leptin elicited synthesis of lipoxygenase as well as cyclooxygenase products. Displaying selectivity, leptin triggered synthesis of LTC_4 and PGD_2, but not PGE_2, in parallel to dose-dependent induction of lipid body/lipid droplets biogenesis. While dependent on PI3K activation, leptin-driven eosinophil activation was also sensitive to pertussis toxin, indicating the involvement of G-protein coupled receptors on leptin effects. Leptin-induced lipid body-driven LTC_4 synthesis appeared to be mediated through autocrine activation of G-coupled CCR3 receptors by eosinophil-derived CCL5, inasmuch as leptin was able to trigger rapid CCL5 secretion, and neutralizing anti-RANTES or anti-CCR3 antibodies blocked lipid body assembly and LTC_4 synthesis induced by leptin. Remarkably, autocrine activation of PGD_2 G-coupled receptors DP1 and DP2 also contributes to leptin-elicited lipid body-driven LTC_4 synthesis by eosinophils in a PGD_2-dependent fashion. Blockade of leptin-induced PGD_2 autocrine/paracrine activity by a specific synthesis inhibitor or DP1 and DP2 receptor antagonists, inhibited both lipid body biogenesis and LTC_4 synthesis induced by leptin stimulation within eosinophils. In addition, CCL5-driven CCR3 activation appears to precede PGD_2

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receptor activation within eosinophils, since neutralizing anti-CCL5 or anti-CCR3 antibodies inhibited leptin-induced PGD_2 secretion, while it failed to alter PGD_2-induced LTC_4 synthesis. Altogether, sequential activation of CCR3 and then PGD_2 receptors by autocrine ligands in response to leptin stimulation of eosinophils culminates with eosinophil activation, characterized here by assembly of lipidic cytoplasmic platforms synthesis and secretion of the pleiotropic lipid mediators, PGD_2, and LTC_4.

Keywords: leptin, eosinophil, prostaglandin D_2, leukotriene C_4, CCR3, CCL5, lipid body, lipid droplet

INTRODUCTION

Classically, eosinophils are perceived as innate leukocytes with important roles in allergic conditions and parasitic infection. More recently, the understanding of eosinophil biological significance has evolved from disease-driven inflammatory effector cells to immunomodulatory housekeepers (1–3). Eosinophils are now recognized to be resident cells in different tissues where they play homeostatic roles, including uterine priming for pregnancy (4) or supporting mammary gland development (5, 6). In healthy white adipose tissue, sentinel eosinophils preserve adipose homeostatic baseline and metabolic regulation, and mitigate obesity. Specifically, adipose tissue-associated eosinophils mediate M2 polarization of adipose macrophages in a paracrine fashion by releasing cytokines, such as IL-4 (7). M2 macrophage-enriched adipose tissue is a tolerogenic environment, which favors browning and limits adipose expansion (7–9). In eosinophil-deficient mice, loss of adipose tissue eosinophil population allows phenotypic switch of macrophages from M2 to M1, setting up an inflammatory environment that culminates with weight gain and systemic insulin resistance (7). Reduction of eosinophil numbers in obese adipose tissue reinforces the notion that eosinophil population is actively regulated across different physiological states of the adipose tissue (10, 11).

Amongst physiological mediators promoting eosinophil localization within adipose tissue, locally IL-5 released by innate lymphoid type 2 cells (ILC2s) (12), as well as extracellular matrix molecules (10), emerged as chief regulators of adipose tissue eosinophilia development and survival. Besides interacting with adipose resident immune cells, like ILC2 and M2 macrophages, eosinophils also adjoin lean adipocytes within healthy adipose tissue. Therefore, eosinophil/adipocyte cross-talk may also take place to maintain homeostasis and regulation of adipocyte lipid handling and storage. In agreement, adipose eosinophils were identified as cellular sources of cathecholamines, which activate adipocyte-expressed β3 adrenoceptors triggering release of adiponectin, a key regulator of adipose vascular functionality (13). However, even though it is becoming clear that eosinophil activation represents a lead-off event of steady state adipose tissue maintenance, very little is known about the local molecular signals that control cellular activity of eosinophils within lean adipose tissue.

Adipokines are constitutively secreted by adipocytes, with both hormonal and *in situ* functions. They may significantly modulate adipose eosinophil roles since eosinophils express specific adipokine receptors, like adiponectin AdipoRs (14) and leptin ObRs receptors (15). Like other leukocytes, eosinophils express the active isoform of leptin receptors ObRb (15–17), which typically signals via PI3K-activated pathways (18–20). Acting in a variety of tissues, adipocyte-derived leptin has pleiotropic effects, notably the regulation of lipid metabolism. In eosinophils, ObRb activation by leptin is known to increase cell survival, chemokinesis and secretion of pro-inflammatory cytokines (15–17). Of note, eosinophils have diverse immune functional capabilities, not restricted to cytokine secretion. Eosinophils are particularly capable of producing bioactive lipids from arachidonic acid metabolism within their cytoplasmic lipid bodies, including prostaglandin $(PG)E_2$ and PGD_2 and leukotriene $(LT)C_4$ (21, 22). Acting on specific receptors with widespread tissue expression (including adipose tissue; (23), these lipid mediators can mediate functions, from homeostatic to pro-inflammatory, as diverse as eosinophils themselves. Pertinent here, leptin prompts 5-lipoxygenase-mediated synthesis of LTB_4 within newly formed cytoplasmic lipid bodies in macrophages (24). Studies of eosinophil activation by adipocyte-derived factors, like leptin, are germane for full characterization of the potential mechanisms involved in eosinophil-driven contribution to adipose tissue homeostasis. Here, we investigated leptin's ability to elicit arachidonic acid metabolism within eosinophils, evaluating the cellular signaling involved. Specifically, by studying the mechanisms of leptin-induced LTC_4 synthesis in both human and mouse eosinophils, we uncovered a leptin-triggered complex signaling pathway, which comprises two consecutive and rapid autocrine loops within eosinophils, including up-stream CCL5 release/CCR3 activation followed by PGD_2 release/DP receptor activation.

MATERIALS AND METHODS

Isolation of Human Blood Eosinophils

Peripheral blood was obtained with informed consent from normal donors. Briefly, after dextran sedimentation and Ficoll gradient steps, eosinophils were isolated from contaminating neutrophils by negative immunomagnetic selection using the

Abbreviations: 5-LO, 5-lipoxygenase; COX, cyclooxygenase; EIA, enzyme immuno assay; LTC4, leukotriene C4; PGD2, prostaglandin D2; RANTES, Regulated on Activation Normal T Cell Expressed and Secreted.

EasySep™ system (StemCell Technologies Inc.) (cell purity ∼99%; cell viability ∼95%). The protocol was approved by ethical review boards of both the Federal University of Rio de Janeiro and the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil).

In vitro Eosinophil Differentiation From Mouse Bone Marrow Cells

BALB/c mice from both sexes were used. Animals were obtained from the Oswaldo Cruz Foundation breeding unit (Rio de Janeiro, Brazil). The protocols were approved by both Federal University of Rio de Janeiro Animal Use and Oswaldo Cruz Foundation Animal Welfare Committees. Eosinophils were differentiated *in vitro* from mouse bone marrow cells as previously described (25). Briefly, bone marrow cells were collected from femurs and tibiae of wild-type BALB/c mice with RPMI 1640 containing 20% FBS. Cells were cultured at 10^6 cells/mL in RPMI 1640 containing 20% FBS (VitroCell), 100 U/mL penicillin, 10 μg/ml streptomycin, 2 mM glutamine and 1 mM sodium pyruvate (Sigma), 100 ng/mL stem cell factor (SCF; PeproTech) and 100 ng/mL FLT3 ligand (PeproTech) from days 0 to 4. On day 4, SCF and FLT3-L were replaced with IL-5 (10 ng/mL; Peprotech). On day 14, eosinophils were enumerated (purity ≥ 90%).

In vitro Eosinophil Stimulation and Treatments

Purified human eosinophils or mouse eosinophils at 2×10^6 cells/mL or 3×10^6 cells/mL in Ca^{2+}/Mg^{2+} HBSS ($HBSS^{+/+}$; pH 7.4) were pre-treated with the PI3K inhibitors wortmannin (1 μM; Biomol) and LY294002 (10 μM; Cayman Chemicals), PKC inhibitor calphostin C (1 μM; Biomol), pertussis toxin (PTX; 100 ng/mL), neutralizing monoclonal antibodies anti-CCL5 (10 μg/mL) and anti-CCR3 (10 μg/mL) (both from R&D), the PAF receptor antagonist BN52021 (10 μM), PGD_2 receptor antagonists BWA868c (200 nM; DP1 receptor) and Cay10471 (200 nM; DP2 receptor, or PGD_2 synthesis inhibitors HQL-79 (10 μM; H-PGDS) and AT-56 (10 μM; L-PGDS) (all from Cayman Chemicals) at 37°C for 30 min before stimulation with human recombinant (hr) or mouse recombinant (mr) leptin (0.5, 5, or 50 nM, as indicated; Peprotech) for 15 or 60 min in a water bath (37°C). Alternatively, eosinophils were also stimulated with PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine; 1 μM; Cayman Chemicals), hr CCL5 (also known as RANTES—100 ng/mL; R&D) or PGD_2 (25 nM; Cayman Chemicals). Each experimental condition was repeated at least three times with eosinophils purified from different donors or differentiated *in vitro* from different mouse bone marrows.

EicosaCell for Intracellular Immunodetection of LTC_4

EicosaCell methodology (26, 27) was used to immunolocalize intracellular LTC_4 at its synthesis sites. *In vitro*-stimulated human eosinophils were mixed with an equal volume of water-soluble 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC; 0.5% in HBSS for 10 min) (Sigma), used to cross-link eicosanoid carboxyl groups to amines in adjacent proteins. After, eosinophils were cytospun onto glass slides and after block step, they were incubated with rabbit anti-LTC_4 Abs (Cayman Chemicals) overnight and secondary DyLight488 green fluorochrome anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for 1 h. As specificity controls for the immunolocalization of LTC_4, rabbit IgG (Sigma) was routinely included as a nonimmune control for the primary anti-LTC_4 and eosinophils were pre-treated with PI3K inhibitor LY294002 (10 μM), for 30 min prior leptin stimulation. Mounting medium containing DAPI was applied to each slide before coverslip attachment to allow visualization of blue-stained eosinophil nuclei. Images were obtained using an Olympus BX51 fluorescence microscope at 100x magnification and photographs were taken with the Olympus 72 digital camera (Olympus Optical Co., Tokyo, Japan) in conjunction with CellF Imaging Software for Life Science Microscopy (Olympus Life Science Europa GMBH, Hamburg, Germany).

Lipid Body Staining and Enumeration

For lipid body enumeration within eosinophil cytoplasm, cytospun cells were fixed in 3.7% formaldehyde (diluted in $HBSS^{-/-}$), rinsed in 0.1 M cacodylate buffer (pH 7.4), stained with 1.5% OsO_4 for 30 min, rinsed in distilled H_2O, immersed in 1.0% thiocarbohydrazide for 5 min, rinsed in 0.1 M cacodylate buffer, restained with 1.5%OsO_4 for 3 min, rinsed in distilled water, and mounted. Lipid bodies were enumerated by light (osmium staining) microscopy. Fifty consecutively scanned eosinophils were evaluated in a blinded fashion by more than one individual, and the results were expressed as the number of lipid bodies *per* eosinophil. Alternatively, cells were stained with Oil Red O (lipid fluorescent stain) for better visualization of the cytoplasmic distribution of eosinophil lipid bodies. Briefly, cytospun cells were fixed in 3.7% formaldehyde (diluted in $HBSS^{-/-}$), rinsed with 60% isopropanol, stained with 0.3% Oil Red O and rinsed with 60% isopropanol. Finally, cytospun cells were washed with water and mounted with DAPI for visualization of blue-stained eosinophil nuclei.

Eicosanoids and CCL5 Quantification

PGE_2, PGD_2, or LTC_4 found in eosinophil supernatants were measured by commercial EIA kits, according to the manufacturer's instructions (Cayman), while mouse and human CCL5 were measured by commercial ELISA kits, according to the manufacturer's instructions (Petrotech). Of note, we found about 150 ng of preformed stores of CCL5 within non-stimulated *in vitro* differentiated mouse eosinophils analyzed in whole cells lysates of 3×10^6 pelleted mouse eosinophils by ELISA.

Statistical Analysis

Results are expressed as the mean ± SEM and were analyzed statistically by means of ANOVA, followed by Student-Newman-Keuls test, with the level of significance set at $p < 0.05$.

RESULTS

Leptin Activates LTC_4-Synthesizing Machinery Within Eosinophils *in vitro*

Human eosinophils upon stimulation preferentially synthesize LTC_4 as an arachidonic acid metabolite by activating the 5-LO pathway, although it is well established that proper intracellular signaling within eosinophils can also couple to COX-driven prostanoid synthesis (22). Here, we initially examined whether leptin can directly activate LTC_4 synthesis within human as well as mouse eosinophils *in vitro*, also evaluating its potential effect on prostanoid synthesis. As shown in **Figure 1**, while leptin (50 nM) failed to trigger PGE_2 synthesis within both human and mouse eosinophils (**Figures 1A,D**), the adipokine induced rapid (within 1 h) PGD_2 (**Figures 1B,E**) and LTC_4 synthesis (**Figures 1C,F**) by both eosinophil species studied, indicating that leptin-elicited intracellular signaling in eosinophils activates both leukotriene- and prostanoid-synthesizing pathways. However, under leptin-stimulation eosinophil COX preferentially couples to H-PGDS rather than E series-synthesizing isomerases. Of note, eosinophils are capable of PGE_2 synthesis upon proper stimulation, such as with PAF–an eosinophil stimulus that triggers a different profile of eicosanoid synthesis by eosinophils with production of PGE_2 (**Figure 1A**) and LTC_4 (**Figure 1C**), but not PGD_2 (**Figure 1B**) (28, 29). On the other hand, **Figure 1** also shows that CCL5 stimulation display a panel of synthesized eicosanoids (including secretion of PGD_2 and LTC_4, but not PGE_2) similar to that triggered by leptin in human or mouse eosinophils. Altogether, the data illustrate the complexity and stimulus-dependent specificity of receptor-initiated arachidonic acid metabolic pathways within eosinophils, while also demonstrating that human and mouse eosinophils, at least to what concerns the ability to synthesize eicosanoids, present the same general patterns of response.

In parallel with increased PGD_2 and LTC_4 synthesis within 1 h of stimulation, leptin (50 nM) was able to directly increase the number of cytoplasmic lipid bodies within human eosinophils (**Figure 2A**). Morphology and distribution analysis of newly assembled lipid bodies within leptin-stimulated eosinophils visualized in either osmium (not shown) or Oil Red O stained cells (**Figure 2B**) revealed discrete punctate organelles with cytoplasmic localizations both adjacent to and far from nuclei. Such leptin-induced rapid lipid body biogenesis was dose-dependent and displayed levels similar to those induced by well-established eosinophil-relevant stimuli, such as CCL5 and PAF within human eosinophils (**Figure 2A**). **Figure 2C** shows that, similar to human eosinophils, mouse eosinophils also respond directly to leptin stimulation with increased assembly of these organelles—cytoplasmic platforms responsible for compartmentalization of bioactive enzymatic machinery of eicosanoid synthesis under inflammatory stimulations and markers of leukocyte activation (22). Of note, we have previously shown that *in vitro* differentiated mouse eosinophils also respond to PAF or CCR3 activation with rapid (1 h) assembly of lipid bodies, which function as intracellular compartments of eicosanoid synthesis (29).

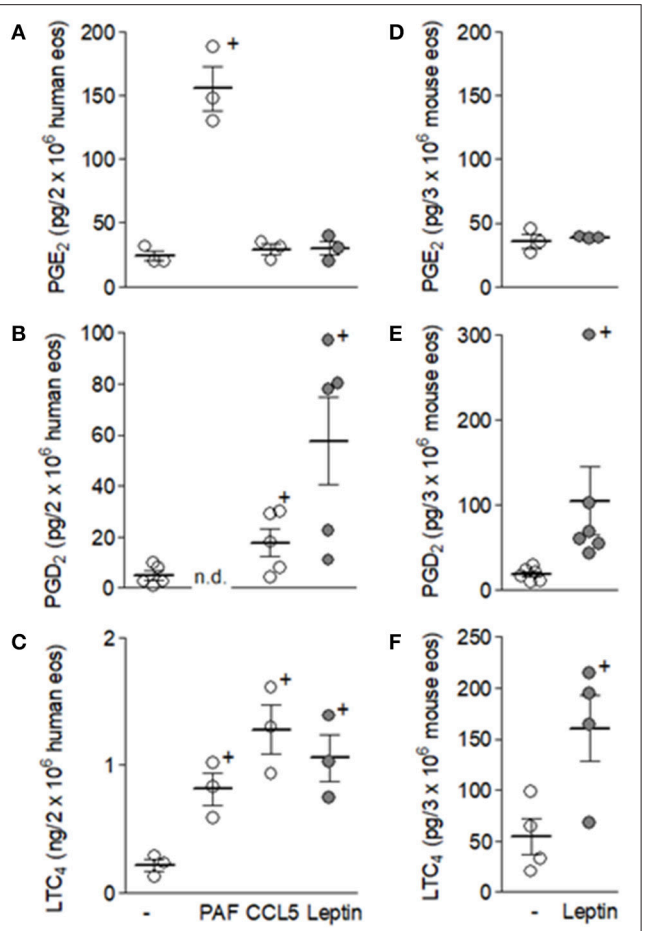

FIGURE 1 | Leptin triggers PGD_2 and LTC_4 synthesis, but not PGE_2, within eosinophils. Human eosinophils **(A–C)** were stimulated with PAF (1 μM), hrCCL5 (100 ng/mL), or hrleptin (50 nM) for 1 h. Mouse eosinophils **(B–F)** were stimulated with mrleptin (50 nM) for 1 h. Eicosanoid PGE_2, PGD_2, or LTC_4 production by eosinophils were quantified in cell supernatants by specific EIA kits. Values are expressed as the mean ± SEM of at least three distinct donors or three different mouse bone marrow cultures. + $p < 0.05$ compared with non-stimulated eosinophils.

Leptin-Induced Activation of Lipid Body-Driven LTC_4-Synthesizing Machinery Within Eosinophils Is a PI3K-Dependent Phenomenon

Inasmuch as activation of PI3K represents an ubiquitous event of ObR-elicited intracellular signaling pathways mediating activation of different leukocyte functions (20), we evaluated PI3K involvement in leptin-induced lipid body-driven LTC4 synthesis by eosinophils. As shown in **Figure 3A**, leptin-stimulated human eosinophils pre-treated with two structurally non-related PI3K inhibitors (30), wortmannin or LY294002, exhibited decreased lipid body biogenesis and LTC4 production. Of note, inhibition of PKC activation by calphostin C showed no effect on leptin-induced lipid body-driven LTC4 synthesis within eosinophils (**Figure 3A**), even though leptin effects on macrophages depend on PKC (31). To further study

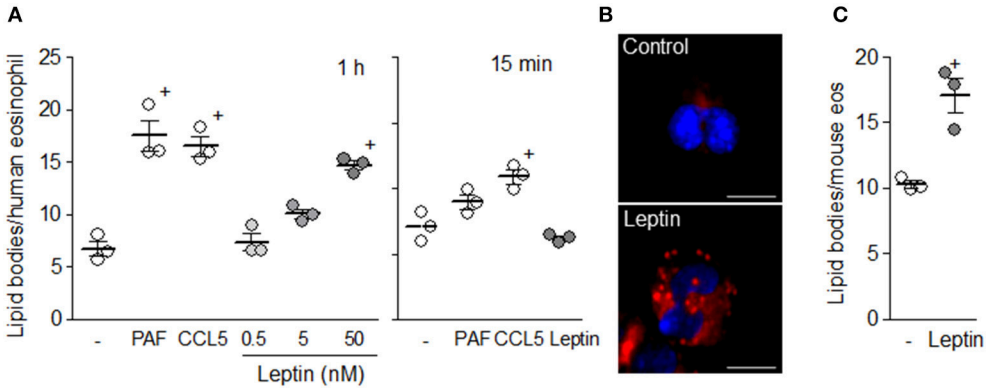

FIGURE 2 | Leptin triggers lipid body biogenesis within eosinophils. In **(A)** human eosinophils were stimulated with PAF (1 μM), hr CCL5 (100 ng/mL), or hr leptin (0.5–50 nM) for 1 h or 15 min, as indicated. **(B)** shows lipid bodies stained by Oil Red O within eosinophils stimulated with hr leptin (50 nM) for 1 h. In **(C)** mouse eosinophils were stimulated with mr leptin (50 nM) for 1 h. In **(A)** and **(C)** lipid bodies were enumerated in 50 consecutive osmium-stained cells. Values are expressed as the mean ± SEM of at least three distinct donors or three different mouse bone marrow cultures. + $p < 0.05$ compared with non-stimulated eosinophils.

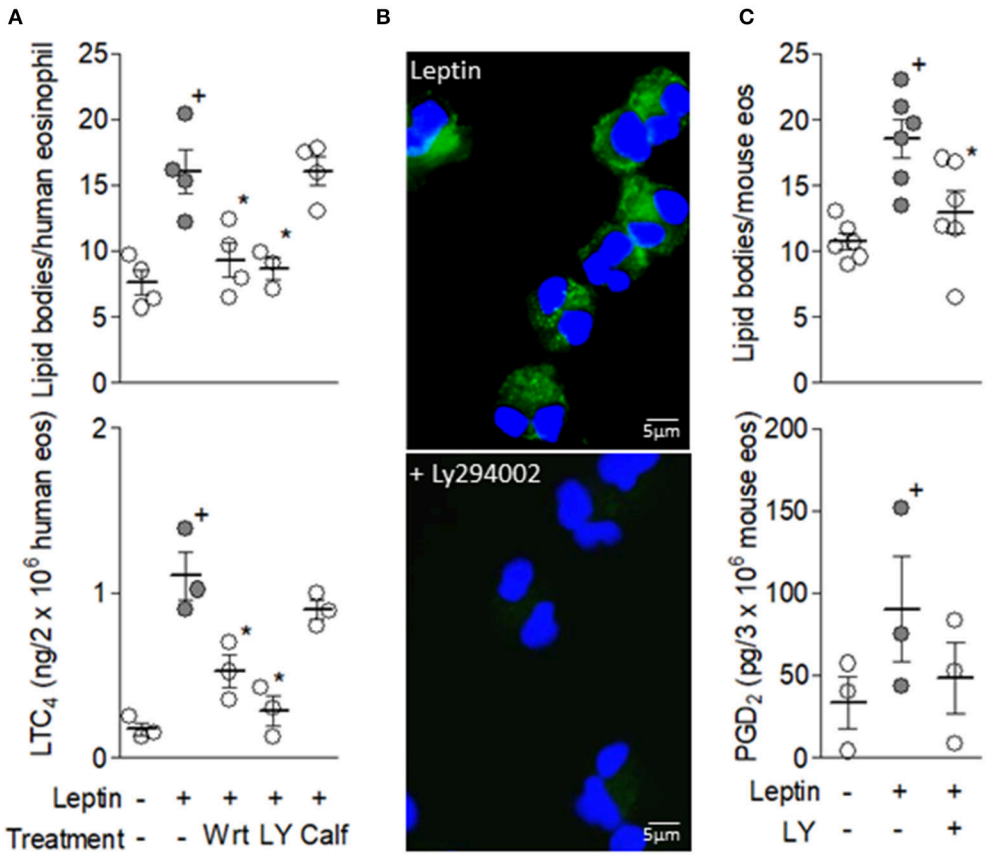

FIGURE 3 | Leptin-induced lipid body-driven LTC4 synthesis depends on PI3K activation. In **(A)** human eosinophils were pretreated with PI3K inhibitors (1 μM wortmannin or 10 μM LY294002) or a PKC inhibitor (calphostin C; 1 mM) 30 min before stimulation with hr leptin (50 nM) for 1 h. **(B)** shows confocal images overlays of intracellular EicosaCell immuno-detection of newly formed LTC_4 (green) and DAPI stained nuclei (blue) within hr leptin-stimulated (upper image) or LY294002-treated hr leptin-stimulated (bottom image) human eosinophils. In **(C)** mouse eosinophils were pretreated with PI3K inhibitor LY294002 (10 μM) for 30 min before stimulation with mr leptin (50 nM) for 1 h. Lipid body count was evaluated in osmium-stained cells and LTC_4 production in cell-free supernatants by EIA kits. Values are expressed as the mean ± SEM of at least three distinct donors or three different mouse bone marrow cultures. + $p < 0.05$ compared with control. *$p < 0.05$ compared with leptin-stimulated eosinophils.

the role of PI3K in LTC4 production triggered by leptin within human eosinophils, we employed the EicosaCell methodology—an imaging—and immunofluorescent-based assay that identifies spatiotemporal intracellular synthesis of lipid mediators (27). Inhibition of PI3K by LY294002 blocked immunolocalization of leptin-induced newly synthesized LTC4 (detected by a green fluorochrome labeled anti-LTC4 specific antibody) within punctate cytoplasmic compartments, which are compatible with eosinophil lipid bodies in size, form and intracellular distribution (**Figure 3B**), therefore establishing that lipid body-compartmentalized LTC4 synthesis is the target of PI3K inhibition. Moreover, LY294002 was also able to inhibit leptin-induced cytoplasmic lipid body biogenesis and PGD2 synthesis (**Figure 3C**) within mouse eosinophils, indicating that leptin-elicited lipid body-driven eicosanoid synthesis is an ObR-driven PI3K-dependent phenomenon that is conserved in both human and mouse eosinophils. Comparison between eosinophils from both species is relevant since at times, mouse and human eosinophils demonstrate differing functional responses to the same stimulatory condition (32).

Activation of G Protein-Coupled CCR3 Receptor by Eosinophil-Derived RANTES Mediates Leptin-Induced Lipid Body-Driven LTC_4 Synthesis

Pretreatment with PTX, a G protein inhibitor, was able to block both lipid body biogenesis and LTC_4 synthesis induced by leptin within human eosinophils (**Figure 4A**). Whereas PI3K inhibitors effect on an ObR-mediated phenomenon was anticipated, inhibition of leptin-induced eosinophil activation by the protein pertussis (PTX) would be an unexpected outcome, since ObR is not a G protein-coupled receptor (33). In an attempt to explain PTX effect, we investigated the potential role of autocrine loops mediated by G protein-coupled receptors in leptin-induced eosinophil activation. Indeed, induction of eosinophil functions, including lipid body-driven LTC_4 synthesis, has been shown to depend on cross-talk between eosinophil-derived mediators in an autocrine fashion (34, 35). Initially, we studied the involvement of CCL5 and PAF–two agonists of distinct G protein-coupled receptors expressed on eosinophils that are known to induce both lipid body biogenesis and LTC_4 synthesis (22, 28) and participate in autocrine phenomena (35, 36). As shown in **Figure 4B**, while pretreatment with the PAF receptor antagonist, BN52021, did not interfere with lipid body biogenesis or LTC_4 synthesis, pretreatment with anti-CCR3 or anti-CCL5 neutralizing antibodies decreased these parameters of eosinophil activation (**Figure 3B**). These results not only explain PTX effect on an ObR-mediated activity, but also demonstrate the involvement of CCR3/CCL5-mediated autocrine activity in leptin-induced lipid body-driven LTC_4 synthesis within eosinophils. Moreover, this data also demonstrated that leptin triggers release of CCL5 from human eosinophils, since only an extracellular neutralization of released CCL5 by the antibody pretreatment would inhibit leptin effects. Of note, rapid CCL5 release was firstly shown within interferon-gamma-stimulated human eosinophils and further confirmed under CD4 activation by IL-16 stimulation. These studies demonstrated that CCL5 secretion was due rapid mobilization from intracellular pre-formed stores of CCL5, which is packaged within cytoplasmic granules and vesicles within eosinophils, ready for prompt secretion (35, 37).

Co-operative Signaling Through DP1 and DP2 PGD_2 Receptors Is Also Required to Lipid Body-Driven LTC_4 Synthesis Induced by Leptin in Eosinophils

Autocrine PGD_2 participation on leptin-induced lipid-body driven LTC_4 synthesis was also investigated, considering: (i) the rapid PGD_2 synthesis and secretion triggered by leptin stimulation of human or mouse eosinophils (**Figure 1**); (ii) that PGD_2 is a potent inducer of both lipid body biogenesis and LTC_4 synthesis within human or mouse eosinophils (38, 39); (iii) PGD_2-induced effects comprise PTX-sensitive activation of G protein-coupled receptor on eosinophils (39); and (iv) eosinophil-derived PGD_2 have previously shown autocrine

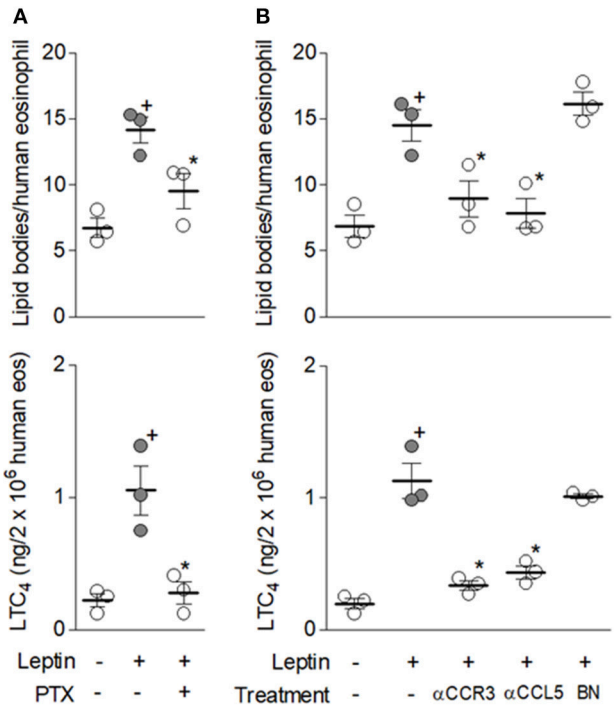

FIGURE 4 | G protein-coupled CCR3 receptor activation by endogenous CCL5 mediates leptin-induced lipid body-driven LTC_4 synthesis. In **(A)** human eosinophils were pretreated with pertussis toxin (PTX; 100 ng/mL). In **(B)** human eosinophils were pretreated with neutralizing anti-CCR3 or anti-CCL5 antibodies (both at 10 μg/mL) or with the PAF receptor antagonist BN52021 (10 μM). All pretreatments were added 30 min before stimulation with hr leptin (50 nM) for 1 h. Lipid body biogenesis was evaluated in osmium-stained cells and LTC_4 production in cell-free supernatants by EIA kits. Values are expressed as the mean ± SEM of at least three distinct donors. + $p < 0.05$ compared with non-stimulated cells. * $p < 0.05$ compared with leptin-stimulated eosinophils.

activity on CCR3-activated eosinophils (29). Identical to the inhibitory pattern observed on eosinophils stimulated by PGD_2 itself (39), lipid body biogenesis induced by leptin was inhibited only by DP1 receptor antagonist BWA868c, while LTC_4 synthesis was blocked by both BWA868c and DP2 receptor antagonist Cay10741 within leptin-stimulated human (**Figure 5A**) or mouse eosinophils (**Figure 5B**), indicating the same cooperation between DP1 and DP2 receptors responsible for eosinophil activation triggered by exogenous PGD_2 appears to take place under leptin stimulation. To ascertain that an endogenously-produced PGD_2 was mediating leptin-induced lipid body-driven LTC_4 synthesis, eosinophils were pretreated with the specific inhibitor of hematopoietic prostaglandin D (PGD) synthase, HQL-79, which was able to inhibit both lipid body biogenesis and LTC_4 production induced by leptin stimulation of human (**Figure 6A**) or mouse eosinophils (**Figure 6B**). Of note, an inhibitor of lipocalin-type prostaglandin D synthase (L-PGDS), AT-56, had no impact on both lipid bodies biogenesis and LTC_4 production (**Figure 6B**). Altogether the data prove that simultaneous activation of PGD_2 G-coupled receptors DP1 and DP2 by an eosinophil-derived PGD_2 corresponds to a second autocrine loop mediating leptin-elicited lipid body-driven LTC_4 synthesis by eosinophils.

Activation of CCR3 by Endogenous CCL5 Precedes and Triggers Autocrine Activity of PGD_2 That Culminates in Leptin-Elicited LTC_4 Synthesis

Inasmuch as we have established that leptin stimulation triggers signaling pathways comprising at least two extracellular stimulatory events of G protein coupled-receptors by molecules secreted by the eosinophils themselves, specifically CCL5- and PGD_2-activating CCR3 and DP1/DP2 receptors, the sequence of these events was then studied. The data shown in **Figures 7**, **8** collectively identify CCL5/CCR3 step as an initial cellular event that determines subsequent PGD_2/DP receptors-mediated step of the leptin-induced lipid body-driven LTC_4 synthesis. First, we found that leptin, but not PGD_2, appears to be able to induce PI3K activation-dependent secretion of CCL5 to the extracellular space of mouse eosinophils (**Figure 7A**). **Figure 7B** shows similar phenomenon for leptin-stimulated human eosinophils. Even though leptin-induced CCL5 release by human eosinophils was not found statistically significant, a clear

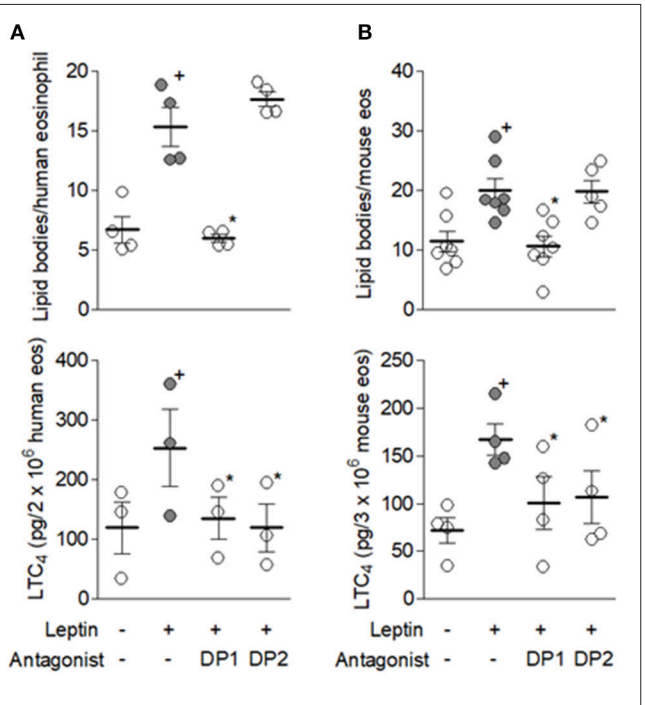

FIGURE 5 | Cooperative DP1/DP2 receptor activation mediates leptin-induced lipid body-driven LTC_4 synthesis. Human **(A)** and mouse **(B)** eosinophils were pretreated with DP1 (BWA868c; 200 nM) or DP2 (CAY10471; 200 nM) receptor antagonists for 30 min before stimulation with respectively hr leptin or mr leptin (50 nM) for 1 h. Lipid body count was evaluated in osmium-stained cells and LTC_4 production in cell-free supernatants by EIA kits. Values are expressed as the mean ± SEM of at least three distinct donors or three different mouse bone marrow cultures. + $p < 0.05$ compared with control. *$p < 0.05$ compared with leptin-stimulated eosinophils.

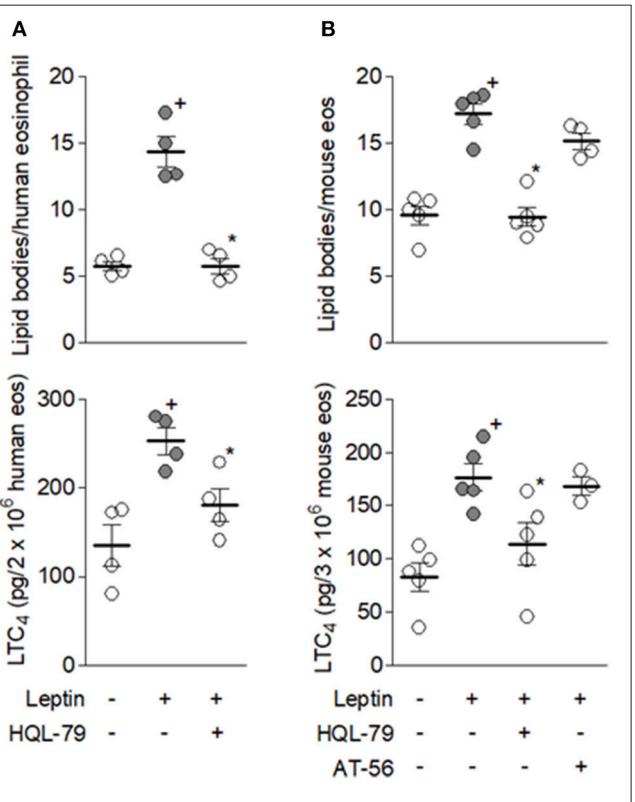

FIGURE 6 | Endogenous eosinophil-derived PGD_2 mediates leptin-induced lipid body-driven LTC_4 synthesis. In **(A)** human eosinophils were pretreated with PGD_2 synthesis inhibitor HQL-79 (10 μM) for 30 min before stimulation with hr leptin (50 nM) for 1 h. In **(B)** mouse eosinophils were pretreated with PGD_2 synthesis inhibitors HQL-79 (10 μM) or AT-56 (10 μM) for 30 min before stimulation with mr leptin (50 nM) for 1 h. Lipid body count was evaluated in osmium-stained cells and LTC_4 production in cell-free supernatants by EIA kits. Values are expressed as the mean ± SEM of at least three distinct donors or three different mouse bone marrow cultures. + $p < 0.05$ compared with control. * $p < 0.05$ compared with leptin-stimulated eosinophils.

tendency of CCL5 secretion triggered by leptin can be observed (**Figure 7B**). Indeed, CCL5 secretion by leptin-stimulated human eosinophils had been shown here in an indirect manner by the effectiveness of anti-CCL5 antibody treatment in inhibiting leptin-induced human eosinophil activation (**Figure 4B**); addition of neutralizing antibody molecules to viable and bioactive cells, such as leptin-stimulated eosinophils, can only target secreted/extracellular CCL5, inasmuch as intact cells are impermeable to antibody molecules. Second, pretreatment with HQL-79 failed to inhibit leptin ability to induce CCL5 release from human eosinophils (**Figure 7B**), while pretreatment with anti-CCR3 antibody inhibits leptin ability to induce PGD_2 production (**Figure 7C**). Finally, the treatment with anti-CCR3 antibody failed to inhibit PGD_2-induced lipid body formation and LTC_4 production (**Figure 8**), indicating that the PGD_2-induced eosinophil activation, a phenomenon known to depend on cooperation between both DP1 and DP2 expressed on eosinophils (39), is not mediated by CCR3-driven autocrine loop.

DISCUSSION

Eosinophils have emerged recently as key housekeeping cells of adipose tissue physiology (2, 11). The mechanisms of homeostatic eosinophils on healthy adipose tissue depend on eosinophils' abilities to secrete mediators that control resident adipose tissue cells, including IL-4/macrophages (7–9) and cathecolamines/adipocytes (13) paracrine circuits. However, the physiologically relevant adipose tissue-derived stimuli for proper eosinophil activation remain elusive. Leptin emerges as preferential candidate in view of the understanding that (i) eosinophils are resident cells within adipose tissue (7), (ii) leptin is continuously produced by adipocytes, and (iii) eosinophils express biologically active leptin receptors (15). Thus, studies characterizing the role of leptin in the activation and effector function of eosinophils may unveil new pathways and molecules involved in the eosinophil-driven immune-regulation of adipose tissue.

Here we demonstrated that leptin has distinctive regulatory roles in activating arachidonic acid metabolism within eosinophils. Leptin activates eosinophils through a multi-step pathway that sequentially involves secretion and autocrine signaling of RANTES and PGD_2, through CCR3 and DP1/DP2 activation respectively, for LTC_4 production (**Figure 9**). It is noteworthy that the whole picture of eosinophil secretory capability upon stimulation with leptin or other adipose tissue-derived molecules is far from fully characterized. Therefore, the primary relevance of our study is the identification of eicosanoids as leptin-induced eosinophil-derived mediators.

Our study is the first to unveil the ability of leptin to induce synthesis of a prostanoid. PGD_2 secreted by eosinophils upon leptin stimulation proved to be a bioactive molecule displaying autocrine activity able to trigger rapid LTC_4 synthesis. The sensitivity to HQL-79 (but not to ATL-56) treatment showed the involvement of H-PGDS, rather than L-PGDS, in leptin-induced synthesis of PGD_2 by eosinophils. Of note, expression of H-PGDS by adipose macrophages was shown to positively correlate with healthy adipose tissue features (40), while PGD_2 synthesis dependent on adipocyte-expressed L-PGDS are related to inflammatory pro-adipogenic functions (41–44). Similar to leptin, CCR3 direct activation by exogenous chemokines, including eotaxin or RANTES, also induces HQL-79-sensitive H-PGDS-driven PGD_2-synthesizing activity, a rapid (within few minutes) phenomenon which is followed by PGD_2-driven autocrine induction of LTC_4 synthesis by eosinophils (29). This piece of information delivers the cellular event lacking to complete the sequence of leptin-elicited signaling steps of LTC_4 synthesis within eosinophils: (i) leptin receptor

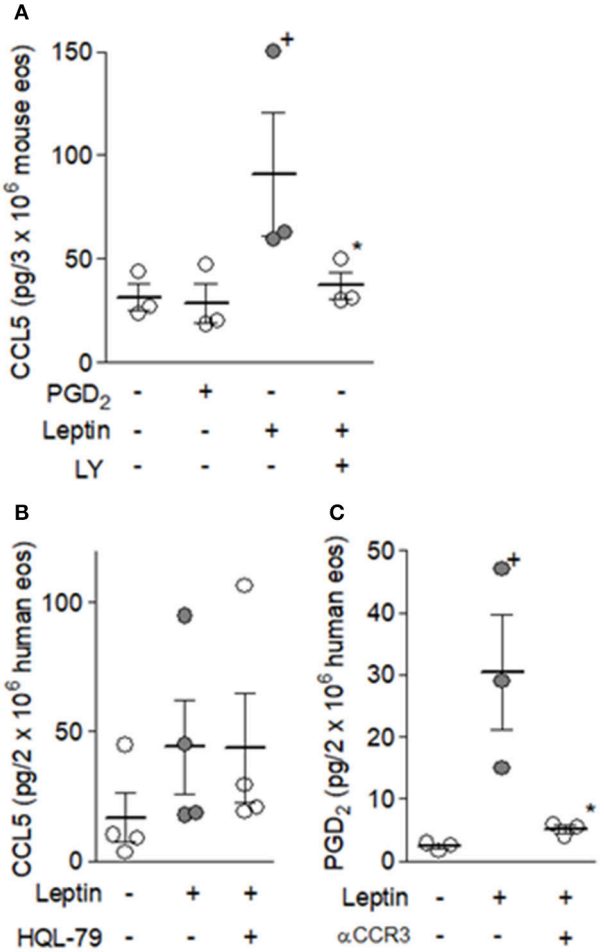

FIGURE 7 | Autocrine CCR3 activation by eosinophil-derived CCL5 elicits PGD_2 synthesis in response to leptin stimulation of eosinophils**.** In **(A)** mouse eosinophils were stimulated for 1 h with PGD_2 (25 nM) or mr leptin (50 nM); mr leptin-stimulated cells were pretreated LY294002 (10 μM) for 30 min. In **(B)** and **(C)** human eosinophils were pretreated with HQL-79 (10 μM) or neutralizing anti-CCR3 antibody (10 μg/mL) for 30 min before stimulation with respectively hr leptin (50 m nM) for 1 h. CCL5 secretion was evaluated in cell free supernatants by ELISAand PGD_2 production in cell-free supernatants by EIA kits. Values are expressed as the mean ± SEM of at least three distinct donors or three different mouse bone marrow cultures. + $p < 0.05$ compared with control. *$p < 0.05$ compared with leptin-stimulated eosinophils.

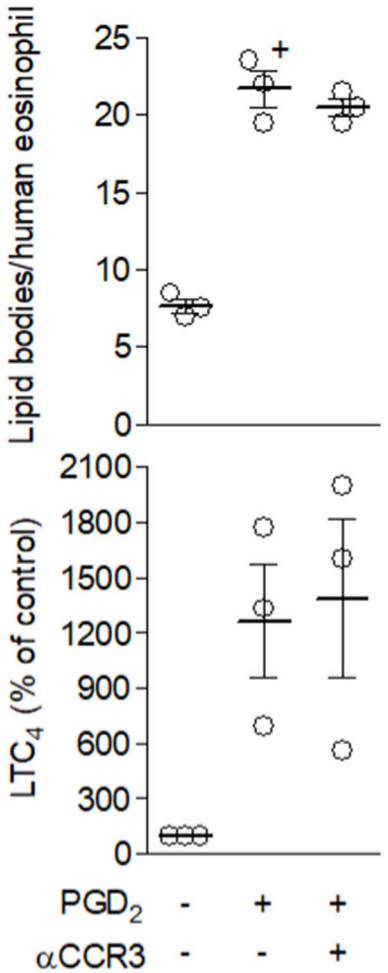

FIGURE 8 | Autocrine CCR3 activation does not play a role in eosinophil activation by PGD2. Human eosinophils were pretreated with neutralizing anti-CCR3 antibody (10 μg/mL) for 30 min before stimulation with PGD_2 (25 nM) for 1 h. Lipid body count was analyzed in osmium-stained cells and LTC_4 production in cell-free supernatants by EIA kits. Values of top panel are expressed as the mean ± SEM of at least three distinct donors. + $p < 0.05$ compared with control. In bottom panel, normalized values show mean ± SEM and individual % percentage of LTC_4 control levels, which were for each of the three donors analyzed 10, 75, and 656 pg/2 × 10^6 human eosinophils.

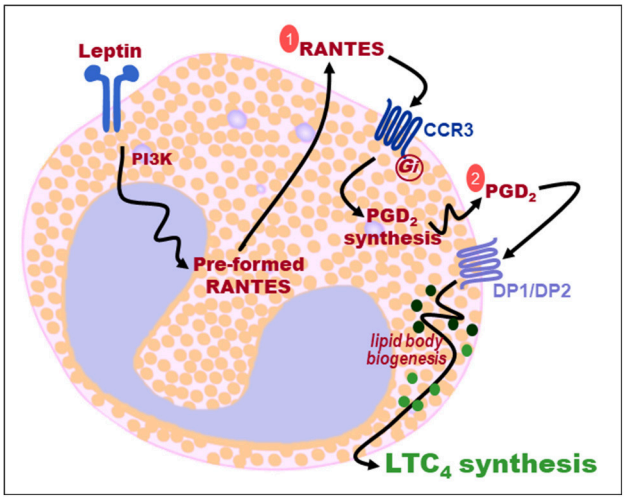

FIGURE 9 | Sequential two-step autocrine mechanism of leptin-induced lipid body-driven LTC_4 synthesis within eosinophils. Leptin stimulation of eosinophils activates PI3K signaling, rapid RANTES secretion, autocrine activation of CCR3 receptors by RANTES (step 1), PGD_2 synthesis, autocrine activation by PGD_2 of both DP1 and DP2 receptors (step 2), that cooperate to trigger lipid body biogenesis and lipid body-compartmentalized LTC_4 synthesis.

stimulation, (ii) PI3K activation, (iii) rapid secretion of pre-formed RANTES, (iv) autocrine CCR3 activation by extracellular RANTES, (v) rapid H-PGDS-driven synthesis of PGD_2, (vi) autocrine activation of PGD_2 receptors DP1/DP2, and finally (vii) lipid body-compartmentalized LTC_4 synthesis (**Figure 8**). Therefore, while studying LTC_4 synthesis, we found out that leptin stimulation triggers secretion of at least two more active molecules, RANTES and PGD_2, which may have additional functions besides triggering LTC_4 synthesis described here.

Within adipose tissue, leptin produced by mature adipocytes continuously may induce synthesis of bioactive PGD_2 by eosinophils. Secreted PGD_2 may act to activate eosinophils to release LTC_4 in autocrine fashion, and may also stimulate nearby adipose tissue cells in a paracrine fashion. Acting on DP receptors expressed by resident cells in adipose tissue, leptin-induced PGD_2 can down-regulate production of leptin (45), trigger secretion of Th2 cytokines IL-5 as well as IL-4 by ILC2s (46, 47), or polarize macrophages toward a M2 anti-inflammatory state in an autocrine fashion (40)—all adipose housekeeping mechanisms of metabolic syndrome evasion.

In peritoneal macrophages, stimulation of leptin receptors and subsequent PI3K activation regulates arachidonic acid metabolism by 5-LO to synthesize LTB_4–a pro-inflammatory mediator known for its potent neutrophilotactic activity (48). Distinctly, 5-LO couples with LTC_4 synthase to generate LTC_4 within properly stimulated eosinophils, a highly regulated intracellular event that is known to be compartmentalized within eosinophil cytoplasmic lipid bodies. Concurring, enhanced LTC_4 synthesis by circulating granulocytes (including eosinophils) positively correlates with increased leptin levels (49). Here, we showed that leptin stimulation of eosinophils is capable of rapid assembly of the enzymatic LTC_4-synthesizing machinery within newly formed lipid bodies that culminates with detection of extracellular LTC_4 within 1 h of stimulation. PI3K activation by leptin, follows through two sequential steps of autocrine activity, involving CCR3 and then DP1/DP2 activation, already described as capable individually to trigger lipid body-driven LTC_4 synthesis within eosinophils (26, 29, 39). It is noteworthy that PI3K activation also mediates CCR3-driven LTC_4 synthesis (26), reinforcing the role of this pathway to leptin-induced lipid body-driven arachidonic acid metabolism.

LTC_4 and its extracellular metabolites LTD_4 and LTE_4 are classically recognized by their major pro-inflammatory effector roles in allergic conditions and asthma (50). There are very few reports addressing LTC_4 roles on adipose tissue

regulation, but they indicate potential homeostatic functions. It has been shown that LTC_4 is able to: (i) potentiate ILC2 activation with increased release of IL-5 (51), which can control homeostatic eosinophilia of adipose tissue (12); and (ii) induce IL-4 secretion from eosinophils (52, 53), therefore with indirect stimulatory impact in M2 macrophage phenotype.

Our findings point to the need for detailed studies considering the adipose tissue environment as a source of molecules that trigger fine-tuned eosinophil activation, that instead of inducing release of eosinophil potentially pro-inflammatory, or even toxic, granule contents (2, 54), do elicit secretion of eosinophil-derived immunomodulatory molecules with homeostatic impact on adipose tissue, like IL-4, cathecolamines and possibly eicosanoids, H-PGDS-driven PGD_2 and LTC_4. Leptin appears to be one of these very special eosinophil activators that may also include PGD_2 and LTC_4 themselves. Indeed, even intracellular LTC_4 can function as an intracrine signal that regulates IL-4 secretion from eosinophils (53, 55), therefore placing the leptin/eosinophil/PGD_2/LTC_4 axis as a potential determining factor in immune-mediated homeostasis of adipose tissue.

AUTHOR CONTRIBUTIONS

All authors had critically revised and approved the final version of the manuscript. NA, TL-G, CM-M, and CB-M performed the conception, designed and performed the experiments, analyzed and interpreted data, and wrote the manuscript draft. MG-A and GS-A participated in the data acquisition, analysis, and interpretation of the data. CC, BD, PW and PB participated in the conception, design, analysis, and interpretation of the work.

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Metabolic Profiling of Human Eosinophils

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Immune cells face constant changes in their microenvironment, which requires rapid metabolic adaptation. In contrast to neutrophils, which are known to rely near exclusively on glycolysis, the metabolic profile of human eosinophils has not been characterized. Here, we assess the key metabolic parameters of peripheral blood-derived human eosinophils using real-time extracellular flux analysis to measure extracellular acidification rate and oxygen consumption rate, and compare these parameters to human neutrophils. Using this methodology, we demonstrate that eosinophils and neutrophils have a similar glycolytic capacity, albeit with a minimal glycolytic reserve. However, compared to neutrophils, eosinophils exhibit significantly greater basal mitochondrial respiration, ATP-linked respiration, maximum respiratory capacity, and spare respiratory capacity. Of note, the glucose oxidation pathway is also utilized by eosinophils, something not evident in neutrophils. Furthermore, using a colorimetric enzymatic assay, we show that eosinophils have much reduced glycogen stores compared to neutrophils. We also show that physiologically relevant levels of hypoxia (PO_2 3 kPa), by suppressing oxygen consumption rates, have a profound effect on basal and phorbol–myristate–acetate-stimulated eosinophil and neutrophil metabolism. Finally, we compared the metabolic profile of eosinophils purified from atopic and non-atopic subjects and show that, despite a difference in the activation status of eosinophils derived from atopic subjects, these cells exhibit comparable oxygen consumption rates upon priming with IL-5 and stimulation with fMLP. In summary, our findings show that eosinophils display far greater metabolic flexibility compared to neutrophils, with the potential to use glycolysis, glucose oxidation, and oxidative phosphorylation. This flexibility may allow eosinophils to adapt better to diverse roles in host defense, homeostasis, and immunomodulation.

Keywords: eosinophil, neutrophil, oxidative phosphorylation, metabolism, glycolysis, hypoxia, atopy, real-time deformability cytometry

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INTRODUCTION

Eosinophils are innate immune cells that represent approximately 5% of the circulating leukocyte population (1). Traditionally, eosinophils are thought to play a role in host defense against parasites but also play a major role in promoting allergic inflammation (2, 3). Eosinophils fulfill this pro-inflammatory role *via* release of their granule proteins, leukotrienes, chemokines, and

cytokines (4). However, newly emerging functions of eosinophils include a broader participation in homeostasis and immunomodulatory roles in the liver, adipose tissue, and the small intestine (5–7). The recent identification of a resident lung eosinophil population (rEos) and a separate allergen induced inflammatory eosinophil population (iEos) indicates that phenotypically distinct eosinophils exist, and that these subsets likely perform differing roles (8).

Crucial to our understanding of eosinophil biology is an insight into their energy and metabolic pathways. The metabolic requirements of immune cells are wide-ranging as these cells need to be able to migrate and adapt their metabolic machinery to mount the required immunological response. It is known that naive T cells can switch their metabolism when exposed to foreign antigens (9), macrophages likewise exhibit notable metabolic plasticity depending on their M1/M2 status (10), and neutrophils are reliant on glycolysis to perform phagocytosis and neutrophil extracellular trap formation (11, 12). While there is extensive literature on the bioenergetic profile of these immune cells, data on eosinophil metabolism remains extremely limited, and there are no investigations on eosinophils in disease (13).

The aim of our work was to assess key metabolic parameters (glycolysis, glucose oxidation, and mitochondrial oxidative phosphorylation) in purified human blood-derived eosinophils and compare their metabolic profile to neutrophils. To assess these parameters, we measured eosinophil and neutrophil extracellular acidification rates (ECAR) and oxygen consumption rates (OCR) in an extracellular flux analyser. This technology allows the real-time assessment of live cell metabolism and has previously been used to assess neutrophil, monocyte, lymphocyte, and platelet bioenergetics (14). Our data suggest that while eosinophils and neutrophils both use glycolysis, eosinophils also access additional pathways such as glucose oxidation and mitochondrial oxidative phosphorylation. This greater metabolic flexibility may allow eosinophils to adapt better to their different tissue locations and perform both pro-inflammatory and homeostatic roles.

MATERIALS AND METHODS

Study Subjects

Human eosinophils and neutrophils were isolated using blood from atopic and non-atopic volunteers. Atopy was defined as volunteers with appropriate history and a positive skin prick test to one or more aeroallergens (the majority of the volunteers tested positive for timothy-grass pollen). The subjects all gave written informed consent and the study was approved by the Cambridgeshire 2 Research Ethics Committee (06/Q0108/281).

Neutrophil and Eosinophil Isolation

Neutrophils were isolated from human peripheral blood using discontinuous plasma-Percoll gradients as previously described (15). Eosinophils were obtained from human peripheral blood using the RoboSep system (StemCell Technologies, Vancouver, BC, Canada) as described (16). We routinely purify neutrophils to ≥95% purity and eosinophils to ≥98% purity, with viability of both cell types ≥99% (as assessed by trypan blue exclusion).

Measurement of ECAR and Oxygen Consumption Rate (OCR)

Sensor cartridges (96-well) were hydrated for a minimum period of 12 h with XF Calibrant solution according to manufacturer's instructions (Seahorse Biosciences, North Billerica, MA, USA). XF Assay Base Media (Seahorse Biosciences) was supplemented with L-glutamine (2 mM) for ECAR measurement or supplemented with L-glutamine (2 mM), sodium pyruvate (2 mM), and glucose (10 mM) for OCR measurement. All media was pH 7.35 ± 0.05 and 0.2-μm sterile filtered prior to use.

Cell culture plates (96-well) (Seahorse Biosciences) were coated with 30 μl of Cell-Tak (Corning) (22.4 μg/ml) and left for a minimum period of 12 h at 4°C. Cell-Tak was removed and wells washed with the appropriate XF Base Media before addition of cells. Granulocytes (3×10^6/ml in XF Assay Base Media) were spun (acceleration 2 and brake 0, 58 *g* for 30 s) onto 96-well plates before incubation in a non-CO_2 incubator at 37°C for 1 h. Measurement of ECAR and OCR was performed in a 96-well XF Extracellular Flux Analyser (Seahorse Biosciences).

Sensor cartridge injection compounds or XF Base Media controls were injected in a total volume of 25 μl. Injection compound concentrations were as follows: glucose (10 mM), oligomycin (OLIGO) (mitochondrial ATP synthase inhibitor) (2.5 μM), 2-deoxy-D-glucose (2-DG) (100 mM), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (1.5 μM), the mitochondrial complex I inhibitor rotenone (1 μM), glutamine (2 mM), phorbol 12-myristate 13-acetate (PMA) (200 nM), and the mitochondrial complex III inhibitor antimycin A (1 μM) unless otherwise stated in the figure legend.

Glycolysis parameters [glycolysis, glycolytic capacity (GC), glycolytic reserve (GR), and non-glycolytic acidification (NGA)] and oxidative phosphorylation parameters [basal respiration (BR), proton leak, ATP-linked production, maximum respiration capacity, spare respiratory capacity, and non-mitochondrial-derived OCR] were calculated as previously described (17). A summary of these calculations is provided in **Table 1** [adapted from literature provided by Agilent Technologies (18)].

ECAR and OCR Measurements Under Hypoxia

The Extracellular Flux Analyser was placed in an *Invivo* 400 hypoxic chamber (Baker Ruskinn, UK) to provide ECAR and OCR measurements under hypoxia (oxygen concentration of 0.8% and 0.1% CO_2). The Extracellular Flux Analyser, sensor cartridges, XF Base Media, and Cell-Tak coated plates were allowed to equilibrate in the *Invivo* 400 chamber for at least 12 h prior to start of experiment. Granulocytes were harvested as described above, plated onto Cell-Tak coated plates, and incubated in the *Invivo* 400 hypoxic chamber for 1 h prior to OCR and ECAR measurements. A hypoxic environment was confirmed using an ABL5 blood gas analyser (Radiometer, Denmark). Additional sodium sulfite (Sigma-Aldrich, UK) injections (100 mM) were added to provide a 0 oxygen reference and the data analyzed using XF Hypoxia Rate Calculator software (Seahorse Biosciences) according to manufacturer's instructions.

TABLE 1 | Calculation of glycolytic and oxidative parameters.

Glycolytic parameter	Calculation
Glycolysis (G)	(Maximal rate measurement after glucose injection through measurement prior to oligomycin (OLIGO) injection) minus (measurement prior to glucose injection)
Glycolytic capacity (GC)	(Maximal rate measurement after OLIGO injection through measurement prior to 2-DG injection) minus (measurement prior to glucose injection)
Glycolytic reserve	(GC) minus (glycolysis)
Non-glycolytic acidification (NGA)	Measurement prior to glucose injection
Oxidative parameter	**Calculation**
Basal respiration (BR)	(Last measurement prior to OLIGO injection) minus (non-mitochondrial-derived OCR value)
Proton leak (PL)	(Minimum rate measurement after OLIGO injection) minus (non-mitochondrial-derived OCR value)
ATP-linked production (ATP LINK)	(Basal rate) minus (PL)
Maximum respiration capacity (MAX CAP)	(Maximal rate measurement after FCCP injection) minus (non-mitochondrial-derived OCR value)
Non-mitochondrial-derived OCR (NON-MITO)	Minimum rate measurement after addition of rotenone/antimycin A through to the end of the assay
Spare respiratory capacity (SPA CAP)	(Maximum respiration capacity) minus (BR)

This table is adapted from data provided by Agilent Technologies (18).

Measurement of Reactive Oxygen Species

Granulocytes (3×10^6/ml) were primed with tumor necrosis factor-alpha (TNF-α), 20 ng/ml; GM-CSF 10 ng/ml (R&D Systems, Abingdon, UK), or phosphate-buffered saline (PBS) vehicle control for 30 min and reactive oxygen species generation subsequently measured as peak *N*-formyl-methionyl–leucyl–phenylalanine (100 nM) (fMLP; Sigma-Aldrich, UK)-stimulated, horseradish peroxidase (HRP; Sigma-Aldrich, UK)-catalyzed luminol (Sigma-Aldrich, UK) chemiluminescence as described previously (19).

Real-Time Deformability Cytometry (RT-DC)

RT-DC measurements were performed to determine the morpho-rheological properties of cells as described previously (20). Eosinophils were resuspended at 2.5×10^7 cells/ml in 1× PBS containing 0.5% methylcellulose to adjust the viscosity to 15 mPas (CellCarrier, Zellmechanik Dresden, Germany). Cell suspensions were drawn into 1-ml syringes connected to polymer tubing, which was attached to a microfluidic chip composed of cured polydimethylsiloxane bonded to a cover glass (Flic20, Zellmechanik Dresden, Germany). At a sample flow rate of 0.03 µl/s and a sheath flow of 0.09 µl/s, cells were acquired within the channel area of the chip at a square measurement channel cross section of 20 µm × 20 µm. Cells were detected in a region of interest of 250 × 80 pixels at a frame rate of 2,000 frames per second using an image analysis algorithm that detects the contour of each cell, and its convex hull, in real time (19). A minimum of 5,000 events were collected and analysis of the convex hull contour of the cells performed using the open source software ShapeOut available on GitHub (https://github.com/ZELLMECHANIK-DRESDEN/ShapeOut). The ratio of convex hull area to cell contour area was defined as the area ratio, a novel and very sensitive surrogate quantifying shape changes as defined by Toepfner and colleagues (21).

Glycogen Assay

The glycogen content of neutrophils, eosinophils, and HepG2 cells was determined using a glycogen assay kit (Abcam, UK) and normalized to protein content (BCA, Bio-Rad, UK).

Statistical Analyses

All data represent the mean (±SEM) of (*n*) separate experiments unless otherwise stated. Statistical analyses were performed using GraphPad Prism (6.0d, San Diego, CA, USA) as detailed in the figure legends. Data were tested for normality using the Shapiro–Wilk test. For non-parametric data a Mann–Whitney *U* test was applied and for parametric data a one-way ANOVA (with Tukey *post hoc* test) or Student's *t*-test was used. $P < 0.05$ was considered significant.

RESULTS

Optimization of Extracellular Flux Measurement in Eosinophils

To determine the optimal conditions required to study purified blood eosinophils in an extracellular flux assay, we first determined the optimal cell concentration required for flux measurements (**Figure 1A**). As shown in **Figure 1B**, the glycolytic response of eosinophils was cell concentration-dependent with a linear relationship between cell concentration and basal or post-glucose ECAR (**Figure 1C**). After visual inspection of the adherent cells, a cell concentration of 3×10^6/ml (or 150×10^3 per well) was chosen as this resulted in an evenly distributed monolayer of eosinophils with no observable shape change (**Figure 1A**). Cell concentrations above 3×10^6/ml were found to result in eosinophil clumping. A concentration of 3×10^6/ml was also found to be optimal for neutrophil ECAR responses (data not shown) and this concentration was used for both cell types throughout the study. Given the well-described susceptibility of neutrophils to activation and that granulocytes are adherent to Cell-Tak throughout the flux assay, we assessed the contribution of Cell-Tak adherence to neutrophil function. We found that contact with Cell-Tak had surprisingly little impact on luminol-dependent chemiluminescence even in GM-CSF or TNF-α primed neutrophils (Figure S1 in Supplementary Material).

Eosinophils and Neutrophils Display Similar Glycolytic Responses but Differing Oxygen Consumption Rates

Having established the optimal flux assay conditions, we then sought to compare the metabolic profile of eosinophils and neutrophils in terms of glycolysis and oxygen consumption. Using

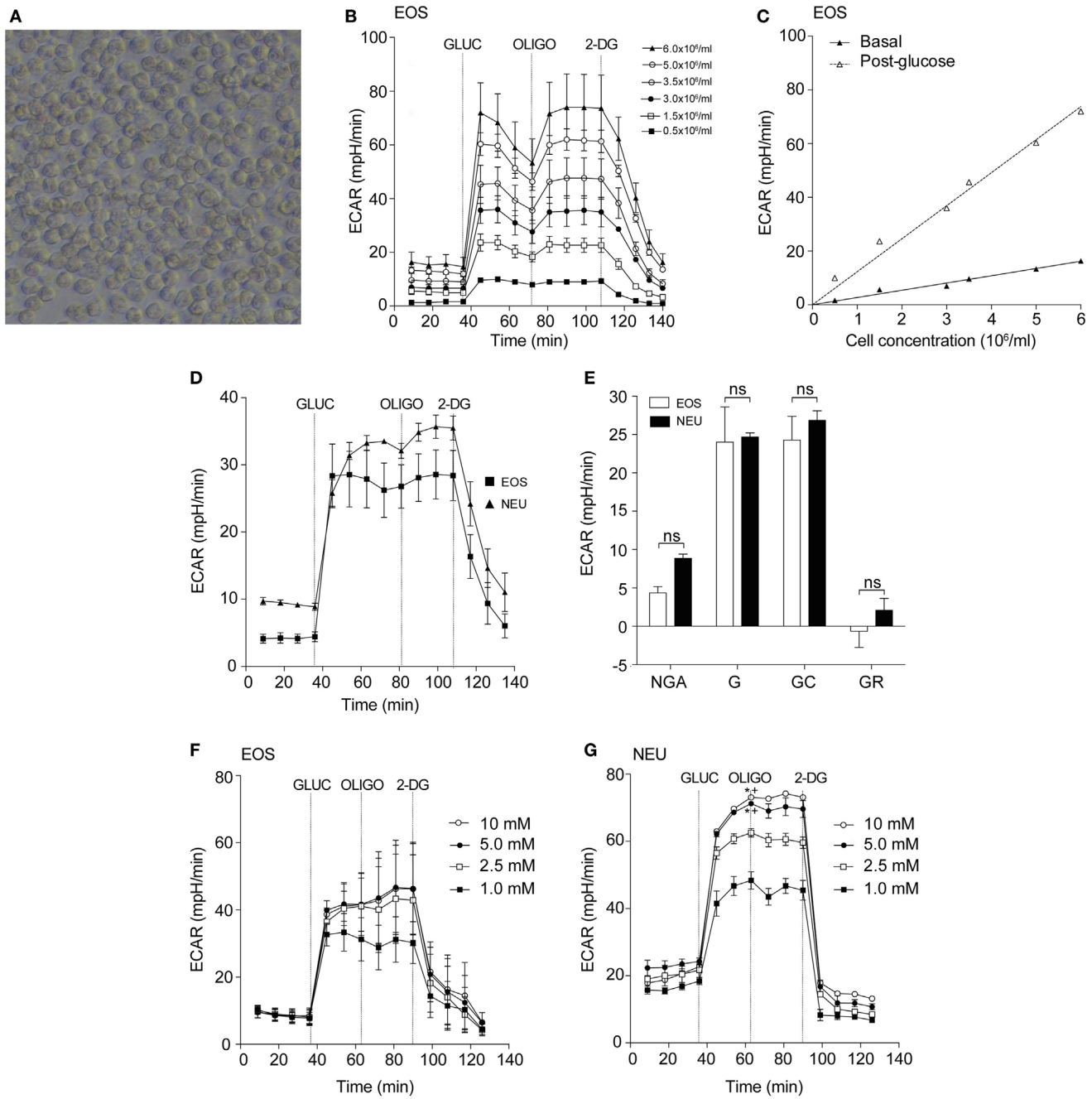

FIGURE 1 | Optimization of eosinophil seeding density and measurement of glycolysis in eosinophils and neutrophils. **(A)** Representative light microscopy of eosinophils (3×10^6/ml) adhered to a Cell-Tak-coated 96-well plate for 1 h. Magnification 40×. **(B)** Kinetic extracellular acidification rate (ECAR) response of eosinophils to glucose injection at 36 min (GLUC, 10 mM), the mitochondrial ATP synthase inhibitor oligomycin (OLIGO) injection at 72 min (OLIGO, 2.5 μM), and the inhibitor of glucose 2-DG (100 mM) injection at 108 min, using 0.5–6 × 10^6 eosinophils/ml. Data represent the mean of a single experiment with ± SD of triplicate wells. **(C)** Eosinophil seeding density titration using basal and post-glucose ECAR levels. Data represent the mean ± SD of a single experiment. **(D)** Kinetic ECAR response of eosinophils (black squares) and neutrophils (black triangles) to stimulation with glucose (GLUC) at 36 min, OLIGO at 81 min, and 2-DG at 108 min. Data represent the mean ± SEM of three independent experiments. Eosinophil and neutrophil ECAR responses were measured simultaneously on the same assay plate. **(E)** Measurement of glycolytic rates in eosinophils (white bars) and neutrophils (black bars). Non-glycolytic acidification (NGA), glycolysis (G), glycolytic capacity (GC), and glycolytic reserve (GR). Data represent the mean ± SEM of three independent experiments, analyzed using one-way ANOVA test with Tukey *post hoc* test. Ns indicates a non-significant difference. **(F)** Eosinophil ECAR following stimulation with glucose (GLUC) (1–10 mM) at 36 min, OLIGO (2.5 μM) at 72 min, and 2-DG (100 mM) at 90 min. Data represent the mean ± SEM of three independent experiments, analyzed using one-way ANOVA with Tukey *post hoc* test. **(G)** Neutrophil ECAR following stimulation with glucose (GLUC) (1–10 mM) at 36 min, OLIGO (2.5 μM) at 72 min, and 2-DG (100 mM) at 90 min. Data represent the mean ± SEM of three independent experiments, analyzed using one-way ANOVA test with Tukey *post hoc* test. $^*p < 0.05$ compared with 1 mM glucose and $^+p < 0.05$ compared with 2.5 mM glucose. Eosinophil and neutrophil ECAR responses were measured simultaneously on the same assay plate **(F,G)**.

the flux analyser we demonstrate that eosinophils and neutrophils have similar ECAR responses in terms of glycolysis rates, GR, GC, and NGA (**Figures 1D,E**). To determine the influence of glucose concentration on ECAR responses, we tested different concentrations of glucose (1–10 mM) in the assay medium. The eosinophil ECAR response was not greatly influenced by external glucose levels as illustrated in **Figure 1F**; eosinophil post-glucose ECAR peak at 1 mM glucose is 31.2 ± 6.4 mpH/min at 1 mM glucose compared to 41.6 ± 7.9 mpH/min at 10 mM glucose. In contrast, neutrophils exhibited a concentration-dependent ECAR response to glucose (**Figure 1G**). The neutrophil post-glucose ECAR peak was 48.4 ± 2.6 mpH/min at 1 mM glucose compared to 73.1 ± 0.4 mpH/min at 10 mM glucose ($p < 0.05$). Given the distinctive reliance of neutrophils on glycolysis we also compared the abundance of glycogen stores between neutrophils and eosinophils. Figures S2A,B in Supplementary Material illustrates that freshly isolated neutrophils have ~3-fold higher glycogen stores compared to eosinophils, a value significantly higher than HepG2 cells.

Figure 2A shows the real-time OCR responses of eosinophils and neutrophils using reagents that disrupt the mitochondrial respiratory chain such as the uncoupling agent FCCP, the mitochondrial ATP synthase inhibitor OLIGO and Rot/AA (mitochondrial complex I and III inhibitors, respectively). When these responses were compared between the cell types, we observed that basal respiration, ATP-linked respiration, maximum respiratory capacity, and spare respiratory capacity were all significantly increased in eosinophils compared to neutrophils (**Figure 2B**). Of note, eosinophil OCR increased significantly from 22.9 ± 1.9 pmol/min at baseline to 62.1 ± 3.2 pmol/min ($p < 0.0001$, **Figure 2C**) following the addition of glucose, whereas neutrophil OCR remained unchanged. This response is attributable to eosinophil glucose oxidation as the levels of OCR decreased substantially following the early (18 min post-glucose) injection of OLIGO (**Figure 2D**). Given that our assay media contained glutamine, we also examined whether eosinophils could undergo glutamine oxidation (17). However, as shown in Figure S3A in Supplementary Material, addition of 2 mM glutamine to eosinophils in glucose-free and glutamine-free media had no impact on OCR or ECAR, suggesting this is not a major energy pathway. Similarly, neutrophil OCR and ECAR remained unchanged upon injection of glutamine (Figure S3B in Supplementary Material).

It is well known that hypoxia can influence metabolism in a wide variety of cell types (22, 23). We, therefore, examined eosinophil and neutrophil OCR responses under hypoxia (3 kPa; 0.8% O_2, and 0.1% CO_2). Granulocytes were exposed to hypoxia for 2 h prior to assessment of mitochondrial respiration. Addition of FCCP increased eosinophil OCR under normoxia (63.8 ± 0.7 pmol/min) but this response was reduced to 9.4 ± 1.6 pmol/min under hypoxia (**Figure 2E**). Although less pronounced, neutrophil OCR was also reduced as shown in **Figure 2F**. The post-FCCP neutrophil OCR decreased from 43.8 ± 8.5 pmol/min during normoxia to 17.1 ± 2.4 pmol/min ($p < 0.001$) under hypoxia.

Priming Agents Increase Eosinophil Oxygen Consumption Rates

Granulocyte priming by agents such as GM-CSF or IL-5 are known to markedly enhance agonist-stimulated respiratory burst (24, 25). We sought to investigate the effect of these priming agents on eosinophil and neutrophil oxygen consumption rates. Eosinophils incubated inside the flux analyser were stimulated with IL-5 for 15 min before addition of PAF or fMLP and OCR measurements acquired (**Figures 3A,B**). Eosinophils exhibited enhanced OCR from 38.5 ± 4.8 pmol/min to 150.9 ± 65.1 pmol/min when primed with IL-5 and stimulated with PAF ($p < 0.05$). The PKC activator PMA also increased OCR by eosinophils from 24.5 ± 5.4 to 572.8 ± 15.8 pmol/min ($p < 0.0001$) as measured by the peak height OCR. Similarly, eosinophil OCR increased from 38.6 ± 6.8 to 112.1 ± 22.3 pmol/min when primed with IL-5 and stimulated with fMLP ($p < 0.05$). Injection of IL-5 alone had no effect on baseline OCR or ECAR (Figure S4 in Supplementary Material). As shown in **Figure 3C** GM-CSF primed neutrophils stimulated with fMLP also show an increase in OCR from 12.5 ± 1.9 to 84.3 ± 25.5 pmol/min in GM-CSF-primed neutrophils ($p < 0.05$). Neutrophils exhibited an uplift in OCR when stimulated with PMA (from 13.9 ± 2.2 to 513 ± 28.8 pmol/min with PMA ($p < 0.0001$)). Notably however, the kinetics of the eosinophil and neutrophil OCR response to PMA differed markedly; neutrophil OCR returned close to baseline levels after 135 min, whereas eosinophils displayed a biphasic and more sustained OCR over the equivalent time period. **Figure 3D** summarizes the peak height oxygen consumption rates in eosinophils and neutrophils in response to PAF, fMLP, and PMA.

We next evaluated the influence of hypoxia on eosinophil and neutrophil OCR in response to PMA treatment (**Figures 3E,F**). As anticipated, basal and PMA-induced OCR in eosinophils was profoundly attenuated under hypoxic conditions (27.0 ± 3.0 pmol/min under hypoxia compared to 574.4 ± 14.2 pmol/min under normoxia). In a similar manner neutrophil OCR responses to PMA were also reduced by approximately 95% under hypoxia (389.7 ± 32.3 vs 17.8 ± 0.8 pmol/min) (**Figure 3F**).

Eosinophil Morpho-Rheological Profile and Oxygen Consumption Rates in Atopic and Non-Atopic Subjects

To determine whether the morpho-rheological and metabolic properties of eosinophils are altered in atopic conditions, eosinophils were isolated from atopic subjects and compared to eosinophils from non-atopic subjects. We first compared the morpho-rheological properties of atopic and non-atopic eosinophils using RT-DC (detailed in Figures S5A–C in Supplementary Material). The measurement of total area ratio by RT-DC acts as a sensitive, label-free, marker of granulocyte shape change (21). **Figure 4A** shows that eosinophils from atopic subjects display a significant increase in the total area ratio compared to eosinophils from non-atopic subjects ($p = 0.008$), comparable with basal priming. Additional morpho-rheological parameters were also assessed, including eosinophil deformability and area, but these parameters were unchanged between the atopic and non-atopic eosinophils (Figures S5D,E in Supplementary Material). When the peak height OCR was measured (**Figure 4B**), we found that there was no difference between IL-5-primed and fMLP-stimulated eosinophils from atopic subjects compared to non-atopic eosinophils ($p = 0.66$).

DISCUSSION

Human neutrophils are known to be primarily glycolytic cells, harnessing aerobic glycolysis to perform their critical functions (11, 12). While a lot is known about the dependence of neutrophils on glycolysis, the metabolic characteristics of eosinophils have been less well defined. Recent technological advances have enabled the real-time assessment of cellular bioenergetics in intact primary cells (14, 26). These improvements avoid many of the artifacts previously associated with mitochondrial isolation or cell permeabilization and thus have far greater physiological relevance. In this study, we have used a real-time extracellular flux analyser to characterize for the first time the metabolic profile of human blood-derived eosinophils and compared directly their profile to neutrophils.

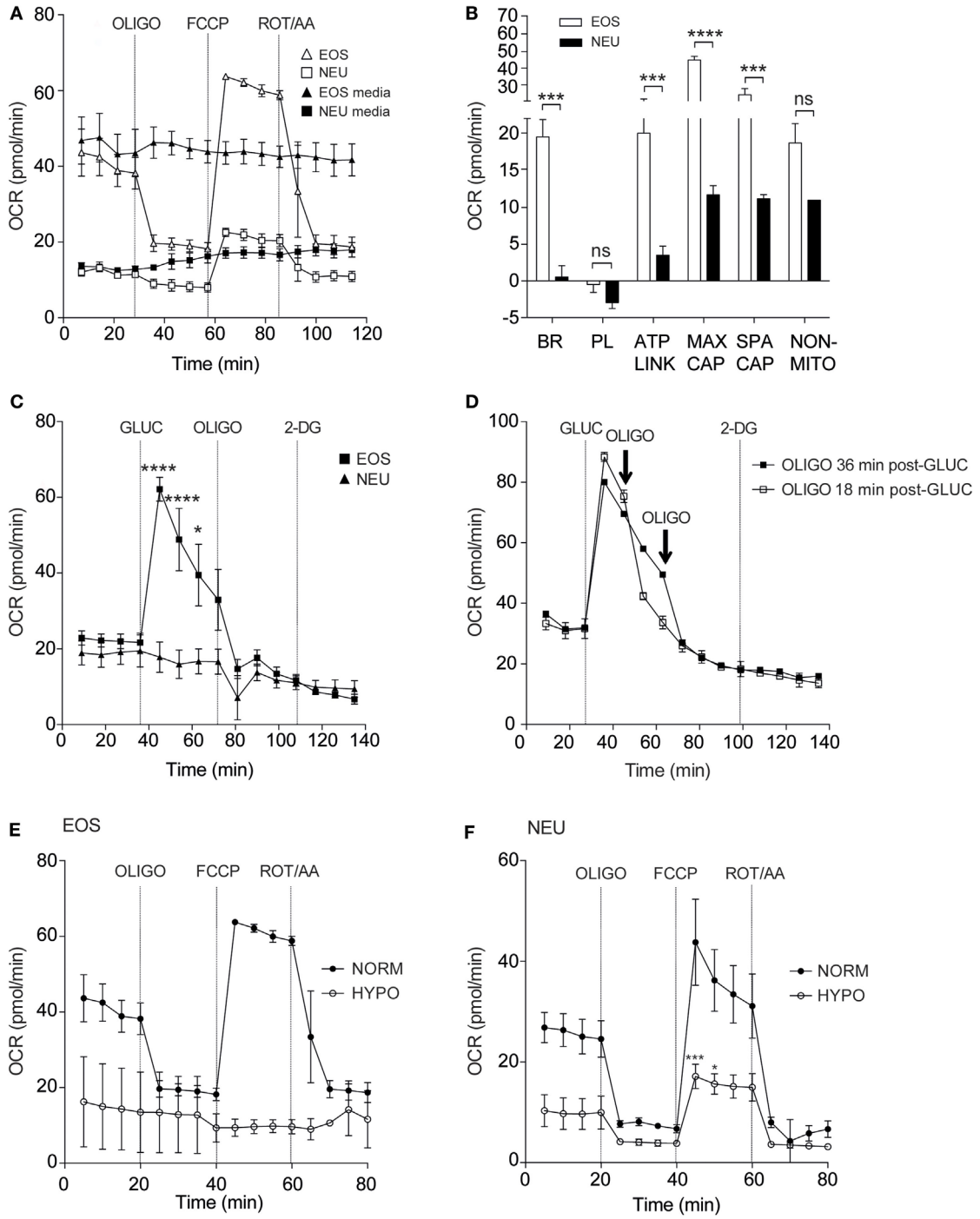

FIGURE 2 | Continued

FIGURE 2 | Eosinophil and neutrophil oxygen consumption rates under normoxia and hypoxia. **(A)** Kinetic oxygen consumption rates (OCR) of eosinophils (open triangles) and neutrophils (open squares) following injection of oligomycin (OLIGO) at 28 min, FCCP at 57 min and rotenone with antimycin A (mitochondrial complex I and III inhibitors, respectively) (ROT/AA) at 86 min. Closed triangles and closed squares represent the media only injections for eosinophils and neutrophils, respectively. Data represent the mean ± SEM of three independent experiments. **(B)** Comparison of oxidative parameters in eosinophils (white bars) and neutrophils (black bars). Basal respiration (BR), proton leak (PL), ATP-linked production (ATP LINK), maximum respiration capacity (MAX CAP), spare respiratory capacity (SPA CAP), and non-mitochondrial-derived OCR (NON-MITO). Eosinophil and neutrophil OCR responses were measured simultaneously on the same assay plate. Data represent the mean ± SEM of three independent experiments, $^{***}p < 0.001$ and $^{****}p < 0.0001$ compared with equivalent neutrophil values (one-way ANOVA test with Tukey *post hoc* test). Ns indicates a non-significant difference. **(C)** Measurement of OCR in eosinophils (closed squares) and neutrophils (closed triangles) in response to glucose (GLUC) injection at 36 min, OLIGO injection at 73 min and 2-DG injection at 108 min. Eosinophil and neutrophil OCR responses were measured simultaneously on the same assay plate. Data represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$ and $^{****}p < 0.0001$ compared with equivalent neutrophil values (one-way ANOVA test with Tukey *post hoc* test). **(D)** Eosinophil OCR in response to the addition of OLIGO 18 (open squares) or 36 min (closed squares) after GLUC injection. Data represent the mean ± SD of a single representative experiment (representative of 3). **(E)** Eosinophil OCR under normoxia (NORM) or hypoxia (HYPO) in response to stimulation by OLIGO at 20 min, FCCP at 40 min, and rotenone with antimycin A (ROT/AA) injection at 60 min. Data represent the mean ± SEM of three independent experiments under normoxia and a single independent experiment under hypoxia (mean ± SD). **(F)** Neutrophil OCR under normoxia (NORM) or hypoxia (HYPO) in response to stimulation with OLIGO at 20 min, FCCP injection at 40 min, and rotenone with antimycin A (ROT/AA) injection at 60 min. Data represent the mean ± SEM of ≥3 independent experiments. $^{***}p < 0.001$ and $^{*}p < 0.05$ compared with normoxia 45 and 50 min values (one-way ANOVA test with Tukey *post hoc* test). Eosinophil and neutrophil responses were measured simultaneously on the same assay plate **(E,F)**.

Eosinophils demonstrated a number of bioenergetic differences compared to neutrophils. First, they consume more oxygen than neutrophils as demonstrated by their enhanced basal rate of OCR, ATP-linked OCR, maximal capacity, and spare capacity. These data are consistent with previous studies, which show that neutrophils are primarily glycolytic, and have fewer mitochondria than other circulating leukocytes (27). In fact, Peachman and colleagues have calculated that there are only 5–6 mitochondria per neutrophil, while a similar quantification in eosinophils has identified 24–36 mitochondria per eosinophil (28). Second, our studies reveal that eosinophils can utilize glucose oxidation as an alternative energy pathway. By undergoing glucose oxidation, glucose-derived pyruvate can enter the mitochondria and thus participate in the tricarboxylic acid cycle. Previous work has shown that eosinophils display enhanced glucose oxidation compared to neutrophils as measured by oxidation of ^{14}C-glucose (29). However, these results were somewhat provisional as they relied on eosinophils derived from a single patient with eosinophilia. Glutamine oxidation has been observed in rat neutrophils (30); however, in our experiments, human eosinophil and neutrophil OCR and ECAR were unchanged in response to injection of glutamine.

Our study demonstrates that eosinophils, like neutrophils, also utilize glycolysis as an energy pathway. Although not directly indicative of active glycolysis, Venge and colleagues have shown uptake of 18-FDG by human eosinophils and describe the involvement of the glucose transporter molecules (GLUTs) 1, 3, and 4 in this process (31). While neutrophils and eosinophils both undergo glycolysis, we noted that eosinophils were less responsive to increasing concentrations of glucose compared to neutrophils, perhaps due to the reliance of neutrophils on glycolysis. Future metabolomics analysis and/or glucose uptake assays will help further determine the glucose dependency of eosinophils compared to neutrophils. Of interest, neutrophils display a threefold higher glycogen content compared to eosinophils, and this finding may reflect the additional ability of neutrophils to utilize glycogen stores for energy production (glycogenolysis). Moreover, Robinson and colleagues have shown that guinea-pig neutrophils can increase their glycogen content as they leave the circulation and enter sites of inflammation (32).

Environmental signals, such as cytokines, growth factors, and oxygen availability, can all contribute to metabolic regulation. In the case of eosinophils the cytokine IL-5 enhances eosinophil activation and differentiation, and acts as an anti-apoptotic (pro-survival) signal (33). Moreover, eosinophils obtained from the bronchial lavage of atopic asthmatics show increased expression of IL-5 mRNA following allergen challenge (34) and there is a correlation between the IL-5 concentration in the serum and severity of asthma (35). Our data show that IL-5 primed and fMLP-stimulated eosinophils undergo a profound increase in their OCR. This response highlights how the inflammatory milieu can alter the metabolic profile of eosinophils, and conversely, how these cells might contribute to local tissue hypoxia.

Previous studies have demonstrated that, like neutrophils, stimulated eosinophils can undergo a respiratory burst (36, 37). The highly reactive oxygen metabolites thus generated perform a protective function in both granulocytes; principally bactericidal in neutrophils and anti-parasitic in eosinophils. Petreccia and colleagues (36) showed that relative to neutrophils, eosinophils displayed higher levels of superoxide anion production both at rest and following PMA stimulation. Consistent with these findings, not only did eosinophils have a higher basal OCR but also PMA stimulation promoted a far more sustained increase in oxygen consumption in eosinophils relative to neutrophils, although the maximal OCRs achieved was not too dissimilar.

Inflamed lesions are often severely hypoxic due to increased cellular oxygen demand and reduced oxygen availability (38–40). Hypoxia is a potent pro-inflammatory stimulus for both neutrophils and eosinophils, contributing to an anti-apoptotic and a hyper-secretory profile in both cell types (notably enhanced release of neutrophil elastase and myeloperoxidase from neutrophils and Charcot–Leyden crystal/Galectin-10 production by eosinophils) (22, 40). In our study, hypoxia profoundly suppressed mitochondrial oxidative phosphorylation in eosinophils, indicative of a metabolic pathway shift from oxidative phosphorylation to glycolysis. This finding is supported by our previously published data, showing upregulation of GLUT1 in eosinophils under hypoxic conditions (40). Such metabolic changes are likely attributable to the activation of hypoxia-inducible factor-1-alpha (HIF-1α) signaling, which is known to increase the levels of glycolytic enzymes and glucose transporters through HIF-1α responsive elements, while downregulating mitochondrial metabolism (39). This shift to glycolysis may allow eosinophils to operate in an inflammatory environment where the oxygen availability is restricted.

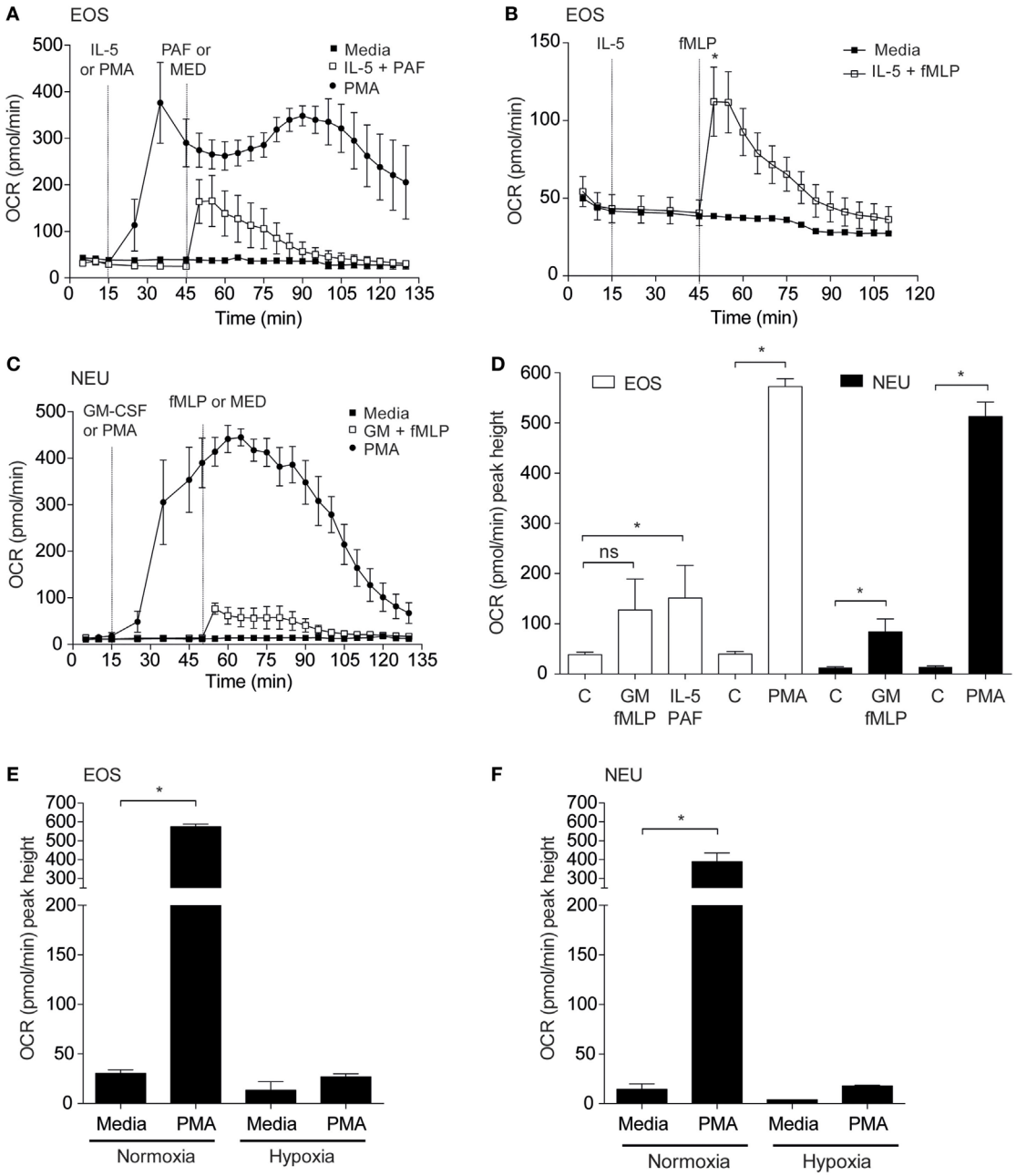

FIGURE 3 | Effect of priming agents on eosinophil and neutrophil oxygen consumption rates. **(A)** Kinetic oxygen consumption rates (OCR) of eosinophils following injection of PMA (closed circles, 200 nM) at 15 min or PAF (open squares, 5 μM) at 45 min. Eosinophils stimulated with PAF were exposed to IL-5 (10 ng/ml) for 30 min prior to injection of PAF. Closed squares represent the media only injections. Data represent the mean ± SEM of three independent experiments. **(B)** Kinetic oxygen consumption rates (OCR) of eosinophils following injection of IL-5 alone at 15 min followed by fMLP injection (open squares, 100 nM) at 45 min. Closed squares represent the media only injections. Data represent the mean ± SEM of seven independent experiments (*$p < 0.05$ compared with media alone using a Student's *t*-test). **(C)** Kinetic OCR responses of neutrophils following injection of PMA (closed circles, 200 nM) at 15 min or fMLP (open squares, 100 nM) at 50 min. Neutrophils stimulated with fMLP were injected with GM-CSF (10 ng/ml) for 35 min prior to fMLP treatment. Black squares represent the media only injections. Data represent the mean ± SEM of three independent experiments. **(D)** Comparison of the peak height OCR by eosinophils (white bars) and peak height OCR by neutrophils (black bars). Treatments were performed as described in **(A,C)**. Data represent the mean ± SEM of four independent experiments for GM-CSF/fMLP stimulation, five independent experiments for PAF/fMLP, and ≥4 independent experiments for PMA (*$p < 0.05$ compared with media alone using a Student's *t*-test). Ns indicates a non-significant difference. **(E)** Comparison of eosinophil peak height OCR following PMA (200 nM) injection under normoxia or hypoxia. Data represent the mean ± SEM of ≥4 independent experiments under normoxia (*$p < 0.05$ compared with media alone using a Student's *t*-test) and mean ± SD of two independent experiments under hypoxia. **(F)** Comparison of neutrophil peak height OCR following PMA (200 nM) injection under normoxia or hypoxia. Data represent the mean ± SEM of ≥4 independent experiments under normoxia (*$p < 0.05$ compared with media alone using a Student's *t*-test) and mean ± SD of two independent experiments under hypoxia. Eosinophil and neutrophil OCR responses were measured simultaneously on the same assay plate **(D,E)**.

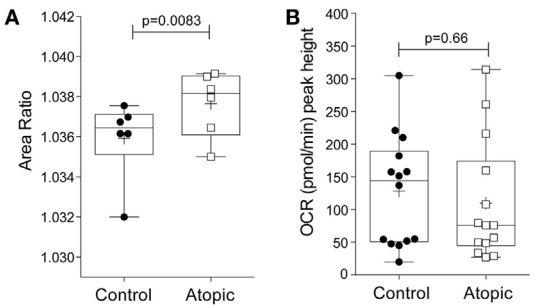

FIGURE 4 | Morpho-rheological properties and oxygen consumption rates in eosinophils from atopic and non-atopic subjects. **(A)** RT-DC was performed on freshly isolated eosinophils from non-atopic volunteers (controls) and atopic volunteers to calculate the area ratio, as described in Section "Materials and Methods." Box-and-whiskers plot shows median ± range of six independent experiments with mean shown as +, $^*p < 0.05$ compared with non-atopic controls (Student's *t* test). **(B)** Comparison of the peak height OCR by eosinophils from non-atopic and atopic subjects primed with IL-5 (10 ng/ml) for 15 min and then stimulated with fMLP (100 nM). Box-and-whiskers plot shows median ± range of 14 independent experiments with mean shown as +, $^*p < 0.05$ compared with non-atopic controls (Mann–Whitney test).

Our study had certain limitations. First, we focused on glucose and glutamine as the primary substrates and did not explore extensively alternative energy sources such as fatty acids and alanine. Few studies have investigated the role of these alternative substrates in granulocyte biology but a role for fatty acid metabolism in neutrophil differentiation has been recently identified (41). Second, we only considered blood-derived eosinophils and the metabolic profile of tissue-resident eosinophils may differ. Mesnil and colleagues have identified lung resident eosinophils (rEos) that are distinguished from "influxing" inflammatory eosinophils (iEos) by their higher CD62L expression, low CD101 expression, and notable expression of immunoregulatory genes (8).

Eosinophils obtained from atopic donors are known to be functionally distinct from cells in non-atopic donors, demonstrating enhanced respiratory burst and a greater proportion of hypodense granules (25, 42). In agreement with these data, we find alterations in the shape or external contour of atopic eosinophils compared to non-atopic eosinophils. Changes in eosinophil shape may occur because eosinophils from atopic volunteers are thought to be primed or partially primed *in vivo* (43). Eosinophils undergo shape change *in vitro* in response to agonists, such as IL-5, eotaxin, and platelet-activating factor (44, 45), but differences in baseline shape change have not been previously demonstrated between atopic and non-atopic eosinophils. Given these attributes, we predicted that eosinophils from atopic donors would be more metabolically active. However, we found no significant difference in the oxygen consumption between atopic and non-atopic eosinophils following treatment with IL-5 and fMLP. Whether there are differences in the basal glycolytic rate or oxidative phosphorylation levels between atopic and non-atopic eosinophils remains to be investigated. Intriguingly, a study comparing the transcriptional profile of circulating eosinophils in asthmatics and healthy subjects found that acyl-CoA thioesterase 4 was upregulated in asthmatics (46). It is, therefore, tempting to speculate that alterations in fatty acid metabolism may contribute to the phenotypic changes exhibited by eosinophils from asthmatics (47).

Overall, our data indicate that eosinophils are more metabolically "flexible" than neutrophils, and that eosinophils can adapt more readily under conditions of altered energy demands. This flexibility may allow the eosinophil to operate not only as a cytotoxic effector cell but also as a participant in immune homeostasis.

ETHICS STATEMENT

This study was approved by the Cambridgeshire 2 Research Ethics Committee (06/Q0108/281). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

EC, NF, and MA conceived and designed the experiments. LP, NF, NT, and KB performed the experiments and analyzed the data. NF wrote the manuscript with input from LP, NT, KB, JG, MA, and EC. All authors contributed to the drafting and revising of the manuscript and have approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01404/full#supplementary-material.

FIGURE S1 | Effect of Cell-Tak-coated wells on reactive oxygen species generation by neutrophils. Freshly isolated neutrophils were left to adhere onto Cell-Tak coated wells for 5 min after priming in suspension (control) or primed while adhered to Cell-Tak coated wells (pre-prime + Cell Tak). Data represent the mean ± SD of two independent experiments.

FIGURE S2 | Glycogen content of freshly isolated eosinophils, neutrophils, and HepG2 cells. **(A)** Glycogen content (pg/cell) was measured by enzymatic assay as described in the Section "Materials and Methods." Data represent the mean ± SEM of ≥3 independent experiments. $^*p < 0.05$ compared with HepG2 samples (one-way ANOVA with Tukey *post hoc* test). **(B)** Glycogen content expressed per milligram protein concentration. Data represent the

mean ± SD of ≥2 independent experiments. Ns indicates a non-significant difference.

FIGURE S3 | Kinetic extracellular acidification rate (ECAR) and OCR response of eosinophils and neutrophils to glutamine stimulation. **(A)** Eosinophil ECAR and OCR response to glutamine injection (GLUT, 2 mM) at 36 min, oligomycin (OLIGO, 2.5 μM) at 72 min and 2-DG (100 mM) at 108 min. **(B)** Neutrophil ECAR and OCR response to glutamine injection (GLUT, 2 mM) at 36 min, OLIGO (2.5 μM) at 72 min, and 2-DG (100 mM) at 108 min. Data represent the mean ± SD of a single experiment, representative of two. Eosinophil and neutrophil ECAR and OCR responses were measured simultaneously on the same assay plate.

FIGURE S4 | Kinetic extracellular acidification rate (ECAR) and OCR response of eosinophils to IL-5 stimulation. Eosinophil OCR and ECAR response to IL-5 injection (10 ng/ml) at 20 min, glucose injection (GLUC, 10 mM) at 35 min, oligomycin (OLIGO, 2.5 μM) at 60 min, and 2-DG (100 mM) at 75 min. Data represent the mean ± SD of two independent experiments.

FIGURE S5 | Analysis of eosinophil cross-sectional area, deformation, and area ratio using RT-DC. Analysis was performed on freshly isolated eosinophils as described in Section "Materials and Methods." **(A)** Within a constriction channel, shear stress and pressure act to deform the eosinophil. **(B)** An image analysis algorithm determines the contour of individual eosinophils (left) and its convex hull (right), and quantifies deformation and area ratio in real-time. The cross-sectional area is defined *via* cell contour detection as shown and convex cell contour and convex hull area are calculated. **(C)** Analysis reveals cell deformation and area ratio. Eosinophils with area ratios between 1 and 1.05 are used for calculation of cellular deformation. **(D)** Comparison of eosinophil cross-sectional area in eosinophils from atopic and non-atopic subjects. Box-and-whiskers plot shows median ± range of six independent experiments with mean shown as +, *p* values calculated using a Student's *t*-test. **(E)** Comparison of eosinophil deformation in eosinophils from atopic and non-atopic subjects. Box-and-whiskers plot shows median ± range of six independent experiments with mean shown as +, *p* values calculated using a Student's *t*-test.

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Non-redundant Functions of IL-6 Produced by Macrophages and Dendritic Cells in Allergic Airway Inflammation

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Asthma is a common inflammatory disease of the airway caused by a combination of genetic and environmental factors and characterized by airflow obstruction, wheezing, eosinophilia, and neutrophilia of lungs and sputum. Similar to other proinflammatory cytokines, IL-6 is elevated in asthma and plays an active role in this disease. However, the exact molecular mechanism of IL-6 involvement in the pathogenesis of asthma remains largely unknown and the major cellular source of pathogenic IL-6 has not been defined. In the present study, we used conditional gene targeting to demonstrate that macrophages and dendritic cells are the critical sources of pathogenic IL-6 in acute HDM-induced asthma in mice. Complete genetic inactivation of IL-6 ameliorated the disease with significant decrease in eosinophilia in the lungs. Specific ablation of IL-6 in macrophages reduced key indicators of type 2 allergic inflammation, including eosinophil and Th2 cell accumulation in the lungs, production of IgE and expression of asthma-associated inflammatory mediators. In contrast, mice with deficiency of IL-6 in dendritic cells demonstrated attenuated neutrophilic, but regular eosinophilic response in HDM-induced asthma. Taken together, our results indicate that IL-6 plays a pathogenic role in the HDM-induced asthma model and that lung macrophages and dendritic cells are the predominant sources of pathogenic IL-6 but contribute differently to the disease.

Keywords: HDM-induced asthma, eosinophils, neutrophils, mouse models, house dust mite (HDM)

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INTRODUCTION

Allergic asthma is a chronic inflammatory disease of the airways that occurs in response to inhaled allergens, such as pollen, house dust mites, and fungi. The incidence and the severity of chronic lung diseases, such as allergic asthma, are growing and affect between 200 and 300 million people worldwide. It is also associated with a significant mortality rate (1). Asthma is characterized by swelling and narrowing of the airways, infiltration of eosinophils to the lungs and activation of Th2 cells (2). Unfortunately, the initial cause that triggers most chronic and acute lung diseases remains unknown, and currently available therapies only ameliorate the symptoms, but do not cure the disease. Thus, there is a pressing need to identify new targets and develop novel therapies, especially, for those most severely affected.

IL-6 is an inflammatory cytokine with pleiotropic functions, ranging from hematopoietic regulation and tissue regeneration to the induction of chronic inflammation (3, 4), sustaining autoimmunity (5) and tumorigenesis (6). IL-6, like other inflammatory cytokines, is elevated in acute and chronic asthma, perhaps as a byproduct of the ongoing inflammation. However, recent studies (7–13) provide evidence that IL-6, rather than being critically involved in lung inflammation, is actually playing a key role in the pathogenesis of asthma. Therefore, IL-6 should be considered as a potential target for the treatment of this disease.

In recent years significant efforts were made to develop mouse models for allergic airway disease, since this would allow for the role of selected genes and gene products in asthma pathophysiology to be established (14). Although mouse models do not replicate human asthma exactly, the uncovered pathogenic mechanisms of allergic airway inflammation may be generally applicable to humans (15). The combination of various molecularly defined allergens found within the house dust mite (HDM) *Dermatophagoides pteronyssinus* is the most common trigger of allergic asthma worldwide (16). For example, HDM extract contains proteases, which cause local damage to the epithelium. Therefore, it directly activates the epithelium, and the resulting Th2 inflammatory cascade, characterized by the infiltration of Th2 lymphocytes, eosinophils, and mast cells, closely reflects the sequence of events observed in humans. Thus, HDM-induced asthma presents the most clinically relevant mouse model to date.

Despite the fact that a number of mouse and human studies implicated IL-6 in the pathogenesis of allergic asthma, the exact molecular mechanism allowing IL-6 to interfere with the lung functions, as well as, the major cellular sources of pathogenic IL-6 (17) remain largely unknown. In the present study, using clinically relevant low-dose (10 μg) acute HDM asthma mouse model (18, 19), we applied reverse genetics to document the active role of IL-6 in the pathogenesis of acute asthma and uncover non-redundant contributions from two important cellular sources of IL-6: macrophages and dendritic cells.

MATERIALS AND METHODS

Mice

IL-6 KO mice were generated by crossing IL-6 floxed mice (IL-6fl/fl) (20) with CMV-Cre mice (21). Mice with ablation of IL-6 in myeloid cells (Mlys-IL-6 KO) were generated by crossing IL-6fl/fl mice with Mlys-Cre knock-in mice (22). Generation of mice with IL-6 deficiency in $CD11c^+$ dendritic cells (CD11c-IL-6 KO) has previously been described (23). Mice were genotyped by genomic PCR of tail DNA: primers for Mlys-Cre transgene Mlys1 5′-CTTGGGCTGCCAGAATTTCTC-3′, Cre8 5′-CCCAGAAATGCCAGATTACG-3′; primers for CD11c-Cre transgene CD11c-Cre F 5′-ACTTGGCAGCTGTCTCCAAG-3′, CD11c-Cre R 5′-GCGAACATCTTCAGGTTCTG-3′. Animals with age of 8–12 weeks were used for experiments. All manipulations with animals were carried out in accordance with recommendations in the Guide for the Care and use of Laboratory Animals (NRC 2011), the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes, Council of Europe (ETS 123), and "The Guidelines for Manipulations with Experimental Animals" (the decree of the Presidium of the Russian Academy of Sciences of April 02, 1980, no. 12000-496). All animal procedures were approved by the Scientific Council of the Engelhardt Institute of Molecular Biology, Russian Academy of Sciences.

Induction of Asthma Using HDM

Purified House dust mite (HDM) (*Dermatophagoides pteronyssinus*) extract (Greer Labs, US) was used in the experiments. For HDM-induced allergic inflammation, mice were anesthetized using mild anesthesia and sensitized intranasally (i.n.) using 1 μg HDM or with saline serving as a control. After 1 week mice were challenged daily for 7 consecutive days with 10 μg HDM or saline (i.n.). Forty-eight hours after the last HDM challenge mice were sacrificed for analysis.

Isolation of Tissue Cells

The trachea was cannulated and bronchoalveolar lavage fluid (BALF) was obtained twice via lavage with 0.8 ml sterile PBS each time. Blood was drawn to screen for serum antibodies and cytokines.

Whole lungs were cleared of blood via ventricular perfusion of the heart with 0.9% NaCl. The lungs were excised and minced with scissors. Reproducible lung tissue dissociation in 5 ml/column of HEPES digestion cocktail (Collagenase D-100 mg/ml, DNAse I-20,000 U/ml, HEPES buffer-10 mM HEPES in PBS) was obtained by using the gentleMACS Octo Dissociator (Miltenyi Biotec, Germany) program "lung_01" (36 s, 165 rpr), and then incubated for 25 min at 37°C. After that samples were further dissociated with gentleMACS Octo Dissociator program "lung_02" (37 s, 2,079 rpr) and centrifuged at 300 g for 5 min at 4°C. The lung suspension and any remaining lung tissue chunks were pushed through a 70 μm filter and centrifuged at 300 g for 7 min at 4°C. Lungs were layered over Percoll (GE Healthcare, Sweden) (80/40%) gradient and centrifuged at 330 g for 25 min at 4°C without braking, and the cells contained in the interphase were recovered.

Lung (tracheobronchial) lymph nodes (lung LN), peripheral lymph nodes (per. LN) and spleen were dissected by passing cell suspension through a 100 μm filter (for lung LN) and a 70 μm filter (for per. LN and spleen). Samples were centrifuged at 300 g for 7 min at 4°C. Lymph node cells were resuspended in 0.25 ml 2% PBS/FBS. Splenocytes were resuspended in 1 ml of ACK lysis buffer, washed twice and resuspended in 1 ml PBS/FBS.

RNA Isolation and cDNA Preparation

For RNA extraction lung samples were homogenized in TRK Lysis buffer [20 μL 2-mercaptoethanol per 1 mL GTC Lysis Buffer (OMEGA biotek, US)] using IKA T10 basic Ultra Turrax Homogenizer (Germany), then centrifuged at maximum speed for 5 min. RNA was extracted using the RNA isolation kit (E.Z.N.A.® Total RNA Kit I, USA). The concentration and

purity of RNA was defined by absorbance measurements at 260 and 280 nm with a NanoDrop spectrophotometer (Thermo). RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase (200 U/μl).

RT-PCR

cDNA was then used for real-time quantitative PCR using 7500 Real Time PCR System amplificator (Apllied Biosystems). A SYBR Green Master PCR mix was employed to amplify *Actb, Il17a, Gob5, Il4, Il5, Il10, Il10, Il33, Il13, Tgfb1, Tslp, Il6, Ifng, Muc5ac, Muc5b, Il1b* using gene-specific primers (Eurogene, primer sequences are shown in **Table 1**).

Comparative CT method ($2^{-\Delta\Delta Ct}$) was used. mRNA levels for the genes of interest relative to the expression of *actin beta* as housekeeping gene were obtained (ΔCt). ΔΔCt values were then obtained by subtracting the ΔCt value from a given reference sample as a calibrator to the rest of the samples. The mean of the ΔCT value within each group was used as a calibrator. The final relative expression data were obtained as $2^{-\Delta\Delta CT}$, defined as RQ value (relative quantitation).

TABLE 1 | Primers for qPCR analysis.

Actb	F 5′-TAAAACGCAGCTCAGTAACAGTCC-3′
	R 5′-CTCCTGAGCGCAAGTACTCTGTG-3′
Il17a	F 5′-GGACTCTCCACCGCAATGA-3′
	R 5′-GGACTCTCCACCGCAATGA-3′
Gob5	F 5′-ACTAAGGTGGCCTACCTCCAA-3′
	R 5′-GGAGGTGACAGTCAAGGTGAG-3′
Il4	F 5′-GGTCTCAACCCCCAGCTAGT-3′
	R 5′-GCCGATGATCTCTCTCAAGTGAT-3′
Il5	F 5′-AGCACAGTGGTGAAAGAGACCTT-3′
	R 5′-TCCAATGCATAGCTGGTGATTT-3′
Il10	F 5′-ATTTGAATTCCCTGGGTGAGAAG-3′
	R 5′-CACAGGGGAGAAATCGATGACA-3′
Il33	F 5′-TGCTCAATGTGTCAACAGACG-3′,
	R 5′-TCCTTGCTTGGCAGTATCCA-3′
Il13	F 5′-CCTGGCTCTTGCTTGCCTT-3′,
	R 5′-GGTCTTGTGTGATGTTGCTCA-3′
Tgfb1	F 5′-ACCATGCCAACTTCTGTCTG-3′,
	R 5′-CGGGTTGTGTTGGTTGTAGA-3′
Tslp	F 5′-TCGAGCAAATCGAGGACTGTG-3′
	R 5′-CAAATGTTTTGTCGGGGAGTGA-3′
Il6	F 5′-CTGATGCTGGTGACAACCAC-3′
	R 5′-GCCACTCCTTCTGTGACTCC-3′
Ifng	F 5′-TCAAGTGGCATAGATGTGGAAGAA-3′,
	R 5′-TGGCTCTGCAGGATTTTCATG-3′
Muc5ac	F 5′-AGAATATCTTTCAGGACCCCTGCT-3′,
	R 5′-ACACCAGTGCTGAGCATACTTT-3′
Muc5b	F 5′-TCCTGCTCTGGAATATCCAAG-3′
	R 5′-GCCTCGGGGAGCTTGCCTGCC-3′
Il1b	F 5′-CAACCAACAAGTGATATTCTCCATG-3′
	R 5′-GATCCACACTCTCCAGCTGCA-3′

ELISA

The supernatants from BAL fluid and serum were collected for the measurement of cytokines and IgE using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen, Austria). IgE, IFNγ, IL-6, IL-13, and IL-1β levels in serially diluted serum and BALF samples were analyzed using HRP-conjugated antibodies specific for these cytokines. IgE levels in serially diluted serum and BALF samples (1:250 for serum and 1:20 for BALF) were determined with anti-mouse IgE as capture antibody (Invitrogen, Austria) and horseradish peroxidase (HRP)-conjugated anti-mouse IgE as detection antibody (Invitrogen, Austria), with mouse IgE as a standard (Invitrogen, Austria). Procedures were performed according to the manufacturer's instructions. Multiscan Go spectrophotometer (Thermo Scientific) was used to measure optical density at 450 nm, calculations of protein levels in serum and BALF were performed using SkanIt Software 4.0 (Thermo Scientific).

Flow Cytometry Analysis

For flow cytometry, FcRs were blocked with Ab 2.4G2 (10 μg/ml), followed by staining with Abs against various surface markers. Myeloid cells were stained with Fixable Viability Dye-eFluor 780 (eBioscience), MHCII-PE (NIMR-4, eBioscience), CD11c-APC or CD11c-AmCyan (N418, BioLegend), CD11b-AmCyan or CD11b-PerCP-Cy5.5 (M1/70, eBioscience), Ly6G-FITC or Ly6G-Pacific Blue (RB6-8C5, eBioscience), Ly6C-PE-Cy7(HK1.4, eBioscience), CD45-PerCP-Cy5.5 or CD45-FITC (both 30-F11, eBioscience), F4/80-Pacific Blue or F4/80-PE-Cy7 (both BM8, eBioscience), SiglecF-PE (1RNM44N, eBioscience), CD103-APC (2E7, eBioscience). Lymphocytes were stained with Fixable Viability Dye-eFluor 780 (eBioscience), TCRβ-PE (H57-597, eBioscience), CD8-APC (53-6.7, BioLegend), CD4-PerCP-Cy5.5 (GK1.5, eBioscience), CD25-Pacific Blue (PC61.5, eBioscience), NK1.1-AmCyan (PK136, eBioscience), ST2-FITC (RMST2-2, eBioscience).

For intracellular staining of IL-6, cells were stimulated with 50 ng/mL phorbol myristate acetate (PMA) and 500 ng/mL ionomycin in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 at 37°C for 4 h. Brefeldin A was used to block protein transport to Golgi apparatus and accumulate proteins in the endoplasmic reticulum. Immediately after activation, cells were washed and stained for surface markers. Distinct populations of lymphocytes were distinguished with the following Ab panel: Fixable Viability Dye-eFluor 780, anti-CD45 (30-F11), anti-Ly6G (RB6-8C5), anti-CD103 (2E7), anti-CD11b (M1/70), anti-CD11c (N418), anti-SiglecF (1RNM44N), anti-CD4(GK1.5, eBioscience), anti-CD8(53-6.7, BioLegend), anti-ST2(RMST2-2, eBioscience), anti-CD19 (6D5, eBioscience) conjugated with FITC, PE, APC, PE-Cy7, PerCP-Cy5.5 and AmCyan. Cells were fixed in Permeabilization buffer and incubated at 4°C for 1 h. Myeloid cells and lymphocytes were washed, centrifuged at 400 g for 5 min and stained with anti-IL-6 PB-conjugated antibody (MP5-20F3, eBioscience) in 1X Permeabilization buffer at 4°C for 1 h. Mouse IgG1 Ab-PB (MOPC-21, eBioscience) was used as isotype control. Data were acquired using flow cytometer

FACSCanto II (BD Biosciences). Data analysis was performed using FlowJo software (Tree Star).

Histology

Lung tissue samples from each experimental group were fixed with 10% neutral buffered formalin solution and embedded in paraffin. After deparaffinization, sections of 4 μm-thickness were stained with periodic acid-Schiff (PAS) to identify degree of expression of mucosal glycoproteins. Slides were examined by light microscopy to evaluate the degree of airway inflammation.

Statistical Analysis

All experiments described were performed 2–4 times. Statistical analyses were performed using Prism 7 (GraphPad Software). Datasets were first tested for Gaussian distribution with the D'Agostino & Pearson omnibus normality test. Statistical significance was determined using Two-tailed, Mann-Whitney test or Two-way ANOVA, followed by Bonferroni post-test analysis for multiple comparisons. Results are expressed as mean ± SEM or ± Mean. $P < 0.05$ was considered statistically significant.

RESULTS

IL-6 Deficiency Attenuates Eosinophilic Inflammatory Response to *Dermatophagoides Pteronyssinus* Extract

Although IL-6 was implicated in the pathogenesis of allergic asthma both in human patients and in several mouse models of asthma (11, 24, 25), the contribution of this cytokine in the most clinically relevant mouse model—administration of HDM at low doses—has not been addressed.

To investigate the role of IL-6 in allergic airway inflammation, acute asthma was induced in WT and IL-6 deficient mice by intranasally administering HDM extract−10 μg of protein for 7 days following sensitization with 1 μg of protein 1 week prior to the main course as shown on **Figure 1A**. Serum was collected 24 h after the last challenge, and 48 h after the last HDM administration mice were euthanized and BAL fluid, lungs, spleens, and draining lymph nodes were harvested for gene expression, cytokine production and FACS analysis (**Figure 1** and **Supplementary Figure 1**).

Mice with genetic IL-6 deficiency mounted a significantly impaired airway inflammatory response as compared to WT mice. Flow cytometry of BAL cells revealed global decrease in inflammation, i.e., reduction in all cell types, in both the airway and lung tissue of IL-6 KO mice. Genetic ablation of IL-6 signaling was associated with abrogated infiltration of eosinophils and Th2 cells to the airways and the lung tissue (**Figure 1B**). This impaired inflammatory response was characterized by decreased numbers of lymphocytes in BAL fluid in IL-6 KO mice (**Figure 1B**). Furthermore, IL-6-deficient mice showed a marked decrease in IgE levels, a signature asthma antibody, in both BALF and serum as compared with their WT counterparts (**Figure 1C**). Interestingly, IFNγ level in serum was decreased in IL-6 KO mice as compared to WT mice (**Figure 1C**). Expression levels of TSLP, the master-regulator of airway remodeling during asthma, and of TGFβ1, which is involved in airway inflammation and hyper-responsiveness, were also decreased (**Figure 1D**) in IL-6 KO mice as compared to WT mice. Notably, inhibition of eosinophilic inflammation was associated with considerably lower expression levels of *Il4* (**Figure 1E**). Moreover, mucus production was reduced in mice with IL-6 deficiency, since the expression level of *Muc5ac*, the major airway mucin, and *Gob5*, a goblet cell marker, were significantly lower as compared to WT mice (**Figure 1E**). Finally, the expression level of *Il17a* was reduced in IL-6 KO mice compared to WT controls (**Figure 1E**). The inflammatory cells infiltrate around the bronchioles and vessels, as well as, the mucus layer (**Supplementary Figure 3**), were also reduced in IL-6 KO mice. These results are in accordance with earlier findings that IL-6 may enhance airway hyper responsiveness, allergic inflammation and development of airway remodeling in the high dose HDM-induced asthma model (24).

Asthma is associated with a broad spectrum of clinical manifestations, ranging from mild to a severe disease onset, as well as, intractable disease. In general, pathogenesis of asthma is based on several interrelated processes and molecular cascades (**Figure 2**). Transcriptomic profiles of bronchoscopic samples led to identification of molecular phenotypes consistent with high type 2 immunity (26) and low type 2 immunity asthma (27). Eosinophilic (Th2-high) airway inflammation (**Figure 2,** central panel) is present in more than 50% of adults with asthma and arises after sensitization to allergen (28). Eosinophilic asthma is characterized by Th2 cell activation, IL-4, IL-5, and IL-13 production, high IgE levels and strong eosinophilia (29). Low-eosinophilic, neutrophil-predominant asthma (30) (**Figure 2,** right panel) is less common, but often presents a more severe disease that does not respond to corticosteroid therapy (31–34). Airway hyper-responsiveness and remodeling are the features present in all asthma subtypes (34).

IL-6 is known for its capability to promote differentiation of Th2 cells (35), to inhibit Th1 and Treg differentiation and expansion in response to allergen, to activate immunoglobulin class-switching in plasma cells and to enhance the differentiation of Th17 cells (36), which are engaged in amplification of severe asthma, and to control airway remodeling (37). Our observations in mice with systemic inactivation of IL-6 (**Figure 1**) provided a rational basis to address the impact of IL-6 from distinct cellular sources in clinically relevant low dose HDM-induced airway inflammation.

Leukocytes Are the Major Cellular Source of IL-6 in the Lungs at Steady State and After HDM Administration

At steady state, IL-6 is produced by macrophages, dendritic cells, neutrophils, B cells and by some $CD4^+$ T cells. In addition, IL-6 can also be secreted by endothelial cells, fibroblasts and epithelial cells (**Figure 2**). To determine the cell type that makes the most significant contribution to the production of IL-6 during asthma, we first examined whether the lymphoid or non-lymphoid cells produce higher amounts of IL-6 in steady state and in response to HDM. Allergic asthma was induced in wild-type mice in accordance with the protocol described above (**Figure 1A**) and

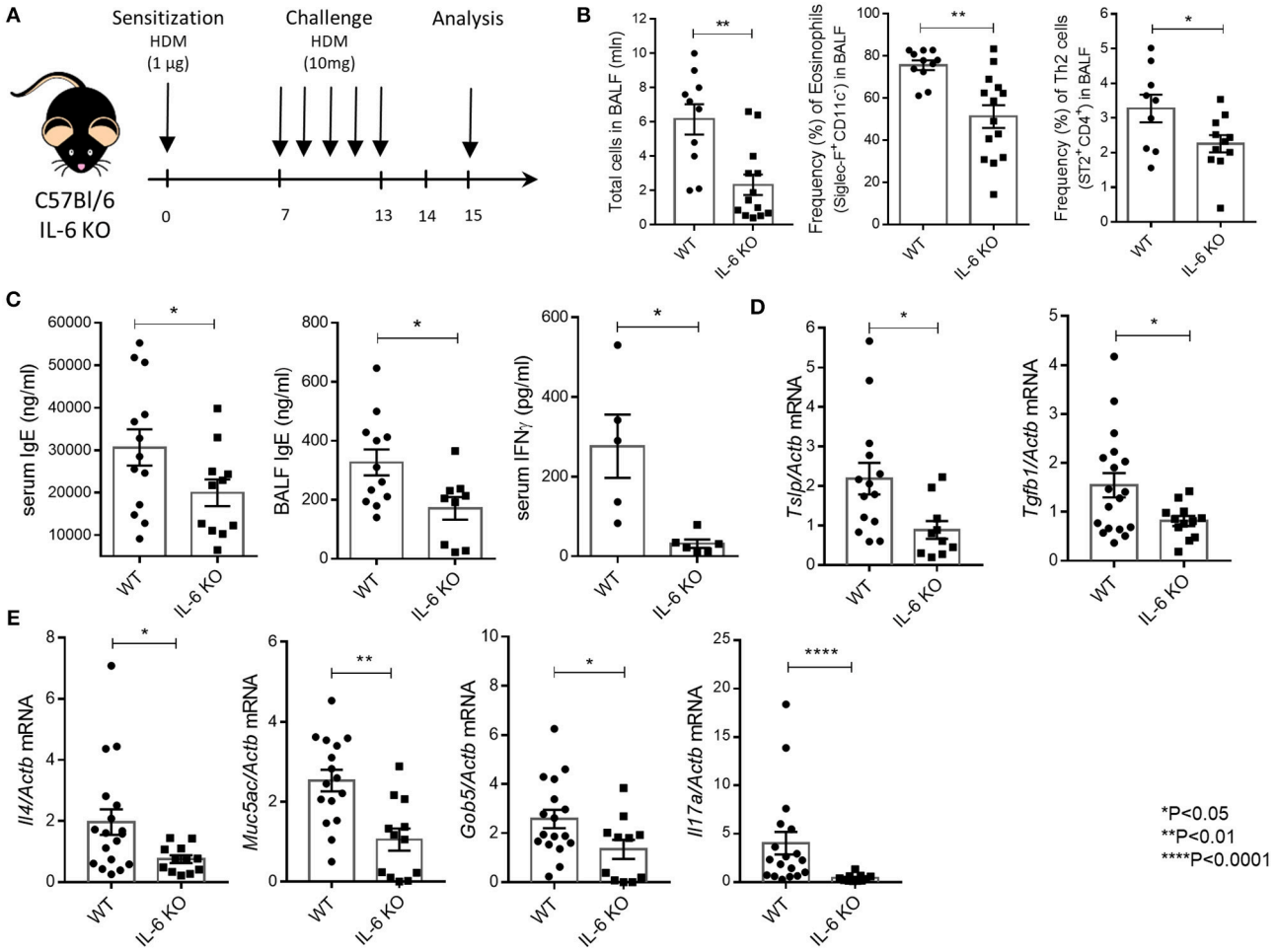

FIGURE 1 | IL-6 deficient mice are resistant to HDM-induced asthma. **(A)** Scheme of the experiment. 8–12-weeks-old mice were sensitized intranasally with HDM extract (Greer Labs) on day 0 (1 μg of total protein in 10 μl). On days 7–13 mice were intranasally challenged with HDM extract (10 μg in 10 μl) and analyses were performed 48 h after the last challenge. All mice were euthanized by intraperitoneal injection of a lethal dose of the Zoletil/Rometar cocktail for anesthesia, and BAL fluid was collected. Lungs were then perfused by cardiac puncture using 10–15 ml DPBS, and lungs, spleens, and draining lymph nodes were harvested for RNA isolation and FACS analysis. **(B)** Total number of cells in BAL fluid and the frequency (%) of eosinophils ($CD45^+$ $SiglecF^+$ $CD11c^-$) and Th2 cells ($CD45^+CD3^+CD4^+CD8^-ST2^+$) in the BAL of WT and IL-6 KO mice assessed by flow cytometry. **(C)** Serum was collected 24 h after the last HDM challenge. The IgE levels in serum and BAL fluid and IFNγ levels in serum were determined by ELISA. **(D)** Quantitative RT-PCR analysis of *Tslp* and *Tgfb1* mRNAs in lungs 48 h after immunization. Expression of each gene was normalized to *Actβ*. **(E)** Quantitative RT-PCR analysis of *Muc5ac, Gob5, Il4*, and *Il17a* mRNAs in lungs 48 h after last HDM challenge. Expression of each gene was normalized to *Actb*. Datasets were first tested for Gaussian distribution with the D'Agostino & Pearson omnibus normality test. Parametric or non-parametric comparison tests was applied where appropriated. Data represent means ± SEM, 5–17 mice in each group.

mice receiving saline instead of HDM were used as a control group. Following 48 h after the last HDM injection, mice were euthanized, BAL and lung cells were isolated and analyzed for IL-6 by flow cytometry. Flow cytometry of BAL and isolated lung cells revealed that in steady state, as well as, in response to HDM, leukocytes ($CD45^+$ cells) were the main source of IL-6 both in the BAL fluid and in the lungs (**Figure 3A**).

It should be noted that HDM administration resulted in the increase of a fraction of non-lymphoid IL-6-producing cells (**Figure 3A**). However, the percentage and fluorescence intensity of the IL-6-expressing lymphoid cell population remained significantly higher following asthma induction (**Figure 3B**). Immune cell counts as assessed by FACS analysis of IL-6 positive cell populations indicated that the absolute numbers of IL-6 positive cells sharply increased both in the airway and in the lung tissue of mice in response to HDM (**Figure 3C**), consistent with the notion that IL-6 is actively involved in the pathogenesis of allergic asthma.

To assess which leukocyte population is involved in IL-6 production in the low dose HDM asthma model, we induced asthma in wild-type mice, as shown on **Figure 1A**. Following 48 h after the last HDM injection, infiltrating lung lymphocytes were obtained and subjected to FACS analysis. The main sources of IL-6 in the lungs appeared to be monocytes, dendritic cells, macrophages, B cells, T cells and $CD8^+$ T cells (**Figure 3D**; **Supplementary Figure 2**). Interestingly, at

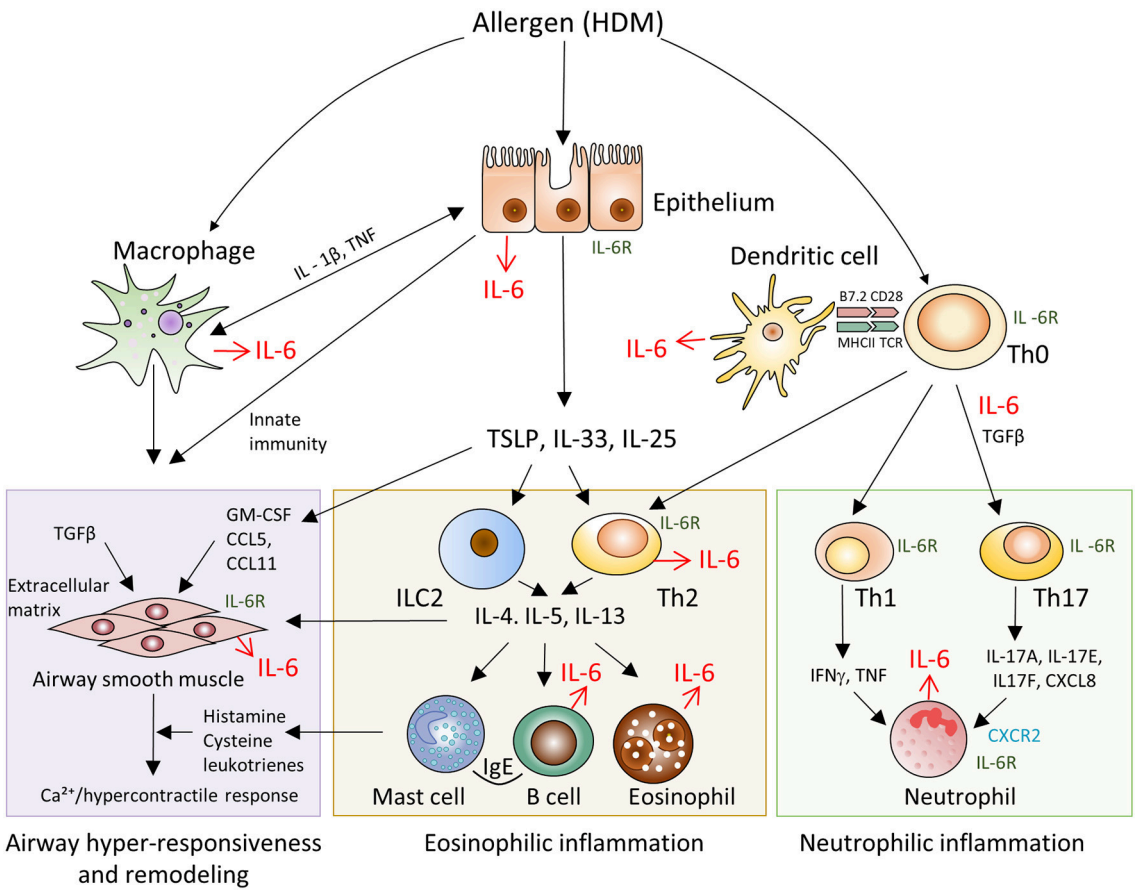

FIGURE 2 | Schematic representation of IL-6 driven pathways and cell types involved in the pathogenesis of allergen-induced asthma. Pathogenesis of asthma is based on several interrelated processes and molecular cascades. Eosinophilic (Th2-high) airway inflammation (central panel) is present in more than 50% of adults with asthma and arises after sensitization to allergen. Eosinophilic asthma is characterized by Th2 cell activation, IL-4, IL-5, IL-13 production, high IgE levels and strong eosinophilia. Low-eosinophilic, neutrophil-predominant asthma (right panel) is less common, but often presents a more severe disease onset that does not respond to corticosteroid therapy. Airway hyper-responsiveness and remodeling (left panel) are the features present in all asthma subtypes.

steady state conditions, most of the IL-6 producing cells were represented by macrophages and monocytes (i.e., myeloid cells), whereas after the induction of asthma, the contribution of the T cell fraction increased (**Figure 3D**). In summary, these results suggested that IL-6 from subtypes of myeloid cells, such as dendritic cells and macrophages, may play a pathogenic role in the development of allergic asthma.

Mice With IL-6 Deficiency in Macrophages Demonstrate Attenuated Th2 Response, Eosinophilic Inflammation and IgE Production in HDM-Induced Asthma

To specifically address the role of IL-6 produced by macrophages in allergic airway inflammation, we generated mice with tissue-restricted inactivation of IL-6 in myeloid (Mlys-IL-6 KO) cells. It should be noted that, despite the fact that Mlys-Cre mediated deletion removes IL-6 not only in macrophages, but also in other myeloid cells (38), in our model neutrophils do not have any impact on IL-6 production either in steady state or after HDM is administered to wild-type mice, as shown in **Figure 3D**. Therefore, results obtained with Mlys-IL-6 KO mice, defined the contribution of IL-6 produced by macrophages in pathogenesis of acute HDM-induced asthma.

Mlys-IL-6 KO mice, as well as, IL-6 KO mice and littermate WT control mice, were subjected to intranasal administration of HDM extract for 7 days with additional sensitization treatment 1 week prior to the main course (**Figure 1A**). Serum was collected 24 h after the last HDM challenge, and the IgE in serum and in BAL fluid were determined by ELISA. Following 48 h after the last injection, mice were sacrificed and BAL fluid, lung infiltrating lymphocytes and splenocytes were collected for qPCR and FACS analysis.

Unexpectedly, we found that genetic ablation of macrophage-derived IL-6 ameliorated the disease with significant decrease in the number of total BAL fluid lymphocytes and in the eosinophilia of the respiratory tract (**Figure 4A**). To establish whether the downshift in the local lung inflammation affected the systemic accumulation of Th2 cells, we determined the frequency of Th2 cells in the spleens of mice from different

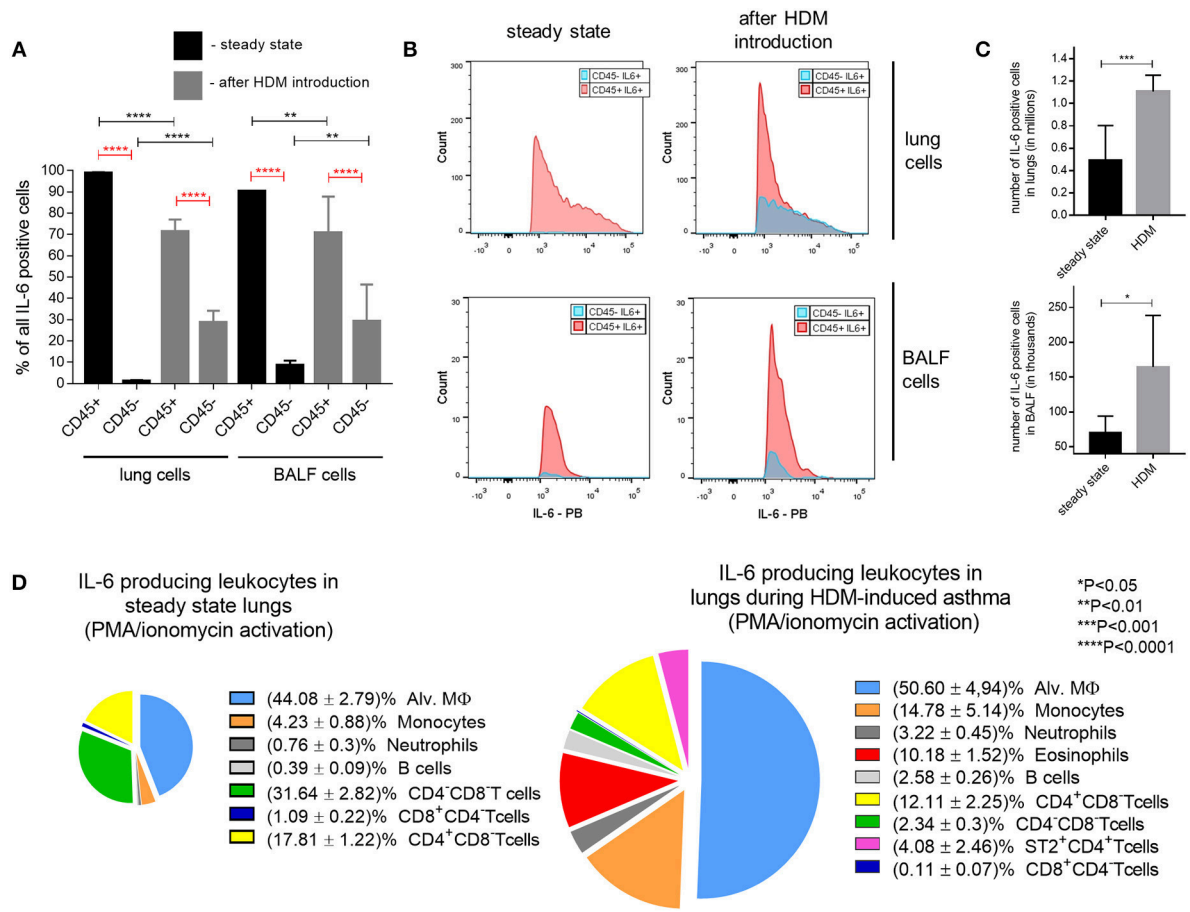

FIGURE 3 | Cellular source of IL-6 in HDM-induced asthma. **(A)** Frequency (%) of lung and BAL fluid CD45$^+$ (leukocytes) and CD45$^-$ (non-leukocytes) IL-6 producing cells in steady state (black bars) and 48 h after last HDM introduction (gray bars) in WT mice assessed by flow cytometry. **(B)** Mean fluorescence intensity (MFI) of non-hematopoietic (CD45$^-$, blue histograms) and hematopoietic (CD45$^+$, red histograms) IL-6 expressing cells in lungs and BAL fluid in steady state and after HDM introduction. **(C)** Number of IL-6 producing cells in BALF and lungs in steady state (black bar) and 48 h after last HDM introduction (gray bar) in WT mice. **(D)** Representative percentage ratio of IL-6 producing cells of different lung leukocyte populations in steady state (left) and 48 h after last HDM introduction (right) in WT mice assessed by flow cytometry. Datasets were first tested for Gaussian distribution with the D'Agostino & Pearson omnibus normality test. Parametric or non-parametric One-way ANOVA with multiple comparisons tests was applied where appropriated. Data represent means ± SEM, 5–11mice in each group.

experimental groups. We found that the removal of IL-6 from macrophages not only reduced local inflammation in the airways, but also diminished the systemic type 2 response, previously characterized by the expansion of Th2 cells (**Figure 4B**). Moreover, Mlys-IL-6 KO mice displayed a significant reduction in IgE, a key indicator of atopy and of allergic inflammatory processes in the respiratory tract, in serum and in BAL fluid (**Figure 4C**). Quantitative RT-PCR analysis showed that the expression levels of *Il4*, a key mediator of Th2 response, and of the *Il33* gene, which is necessary for the development of allergic rhinitis, were reduced in the lungs of Mlys-IL-6 KO mice as compared to WT control mice (**Figure 4D**). Finally, we found that Th2, but not Th17, response was ablated in mice with IL-6 deficiency restricted to macrophages (**Supplementary Figure 4**). Taken together, these results suggested that macrophage-derived IL-6 plays a critical pathogenic role in the HDM-induced asthma model and that IL-6 from this cell type may contribute to the induction or amplification of Th2 inflammatory response during acute asthma.

Mice With IL-6 Deficiency in Dendritic Cells Demonstrate Attenuated Neutrophilic, but Regular Eosinophilic Response in HDM-Induced Asthma

To specifically address the role of IL-6 produced by dendritic cells in allergic airway inflammation, we used mice with tissue-restricted inactivation of IL-6 in CD11c$^+$ cells (CD11c-IL-6 KO) (23) and subjected them, together with IL-6 KO mice and littermate WT control mice, to HDM-induced asthma model (**Figure 5**). In CD11c-IL-6 KO mice, CD11c-driven *Cre* expression results in deletion of IL-6 in dendritic cells (23).

We found that ablation of IL-6 in dendritic cells reduced lymphocyte infiltration in the respiratory tract of experimental

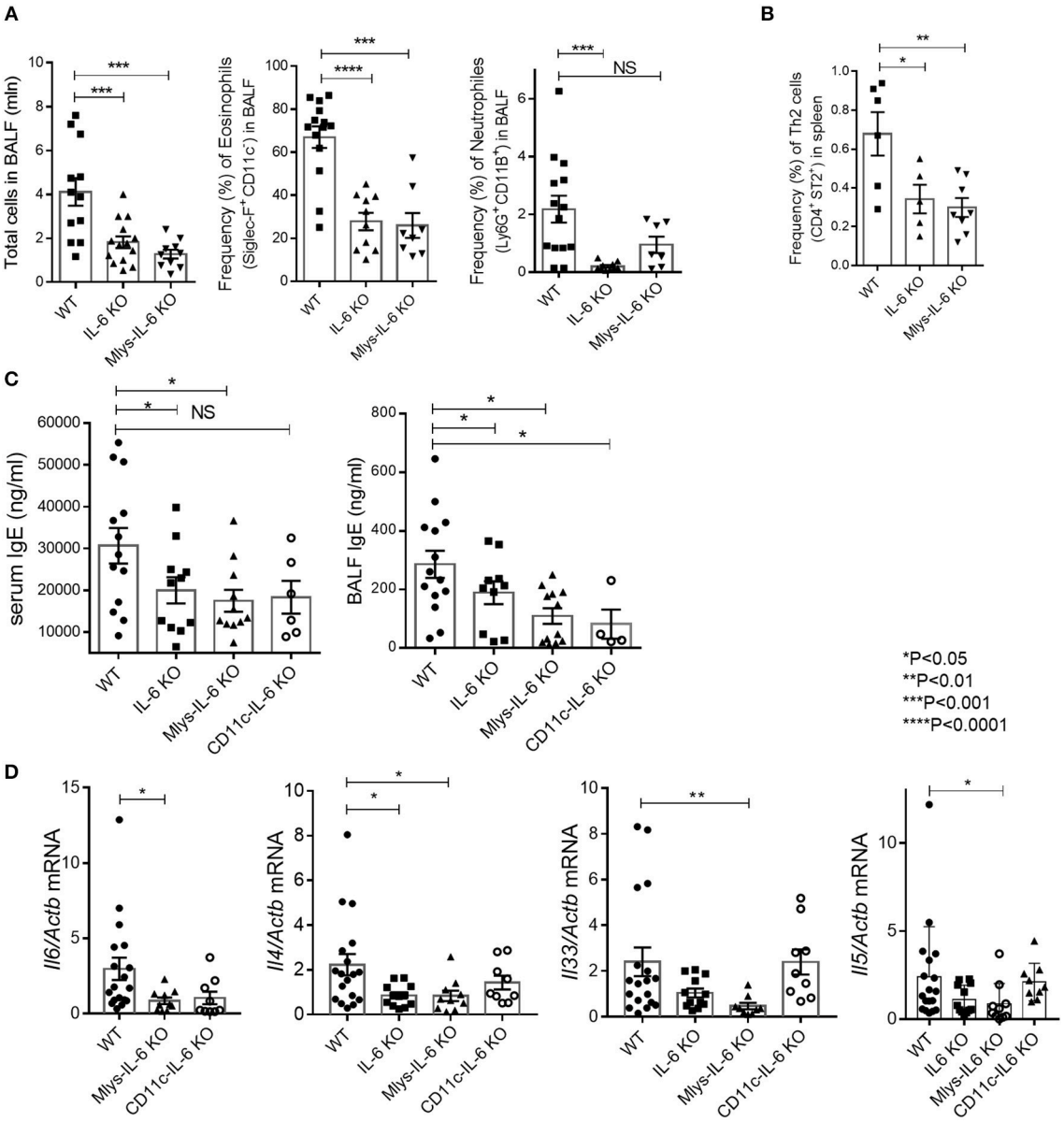

FIGURE 4 | Mlys-IL-6 KO mice demonstrate attenuated type 2 response in HDM-induced asthma model. **(A)** Total number of cells in BAL fluid and the frequency (%) of eosinophils ($CD45^+$ $SiglecF^+$ $CD11c^-$) and neutrophils ($CD45^+Ly6G^+CD11b^+$) in the BAL of WT, IL-6 KO, and Mlys-IL-6 KO mice assessed by flow cytometry. **(B)** Frequency (%) of Th2 cells ($CD45^+CD3^+CD4^+CD8^-ST2^+$) in the spleens of WT, IL-6 KO and Mlys-IL-6 KO mice assessed by flow cytometry. **(C)** Serum was collected 24 h after the last HDM challenge. The IgE levels in serum and BAL fluid were determined by ELISA. **(D)** Quantitative RT-PCR analysis of *Il6, Il4*, *Il33,* and *Il5* mRNAs in lungs 48 h after the last HDM challenge. Expression of each gene was normalized to *Actb*. Datasets were first tested for Gaussian distribution with the D'Agostino & Pearson omnibus normality test. Parametric or non-parametric One-way ANOVA with multiple comparisons tests was applied where appropriated. Data represent means ± SEM, 5–18 mice in each group. NS, not significant.

animals as compared to their littermate WT controls (**Figure 5A**). We then examined whether the deficit in IL-6 production by dendritic cells may affect the number of pathogenic cells, i.e., eosinophils, in the lungs. Surprisingly, flow cytometry analysis of bronchoalveolar lavage revealed that eosinophil accumulation in the BAL fluid did not differ from that found in WT mice (**Figure 5A**). Moreover, Th2 accumulation in the spleens was not reduced in CD11c-IL-6 KO mice, in contrast to Mlys-IL-6 KO mice (**Figures 4B**, **5B**). IgE levels in BALF were reduced both in CD11c-IL-6 KO and Mlys-IL-6 KO, compared to WT mice (**Figure 4C**). These results indicate that IL-6 from dendritic cells does not contribute to the Th2 accumulation and eosinophilic inflammatory response during asthma.

To further investigate the role of IL-6 from dendritic cells in atopy during asthma, we analyzed systemic inflammatory response by determining IgE concentration in the serum

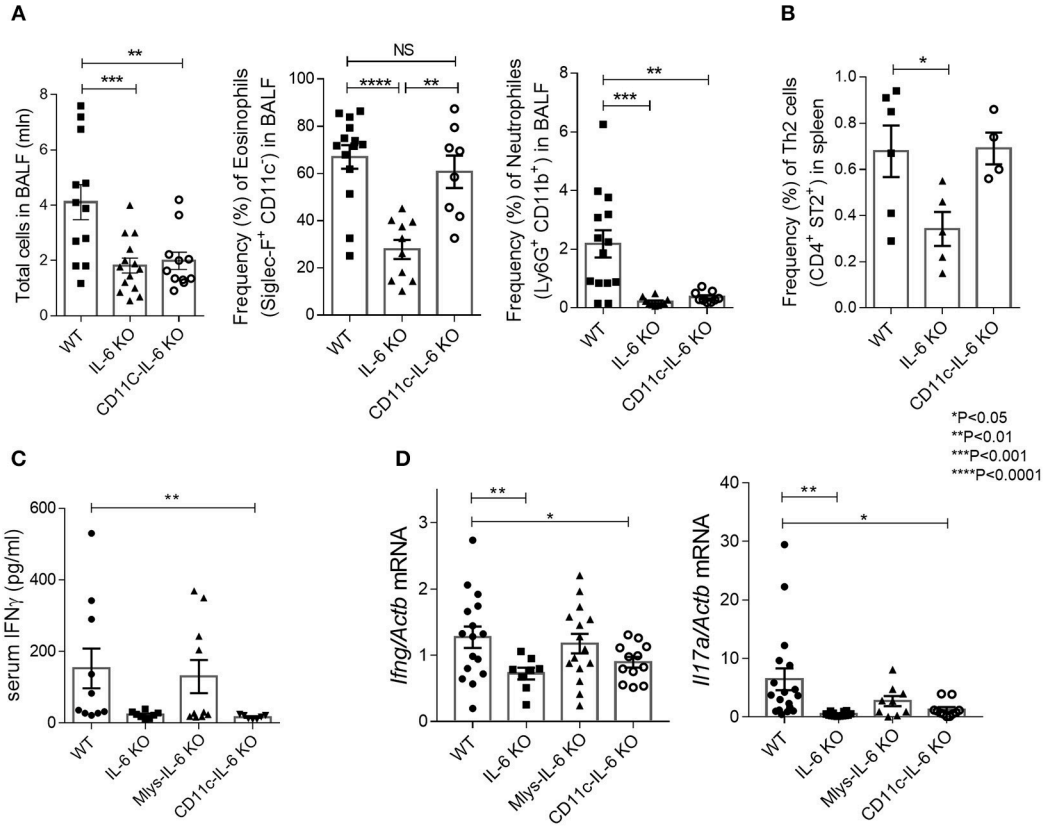

FIGURE 5 | CD11C-IL-6 KO mice demonstrate attenuated neutrophilic, but regular eosinophilic response in HDM-induced asthma model. **(A)** Total number of cells in BAL fluid and the frequency (%) of eosinophils ($CD45^+SiglecF^+CD11c^-$) and neutrophils ($CD45^+Ly6G^+CD11b^+$) in the BAL of WT, IL-6 KO, and CD11c-IL-6 KO mice assessed by flow cytometry. **(B)** Frequency (%) of Th2 cells ($CD45^+CD3^+CD4^+CD8^-ST2^+$) in the spleens of WT, IL-6 KO and CD11c-IL-6 KO mice assessed by flow cytometry. **(C)** Serum was collected 24 h after the last HDM challenge and the IgE levels in serum were determined by ELISA. **(D)** Quantitative RT-PCR analysis of *Ifng* and *Il17a* mRNAs in lungs 48 h after the last HDM challenge. Expression of each gene was normalized to *Actb*. Datasets were first tested for Gaussian distribution with the D'Agostino & Pearson omnibus normality test. Parametric or non-parametric One-way ANOVA with multiple comparisons tests were applied where appropriated. Data represent means ± SEM, 4–16 mice in each group. NS, not significant.

and BAL fluid of the experimental animals (**Figure 4C**). IgE production in CD11c-IL-6 KO mice did not differ from that in WT mice, while in mice with complete knockout of IL-6 and Mlys-IL-6 KO mice IgE production was significantly lower. To establish whether IL-6 from dendritic cells affects neutrophilic inflammatory response (**Figure 2**, right panel), we compared neutrophil infiltration in the BAL fluid of CD11c-IL-6 KO mice, as well as, of mice with complete IL-6 deficiency and WT mice after HDM administration. We found that both CD11c-IL-6 KO mice and IL-6 KO mice had attenuated number of neutrophils in BAL fluid after HDM exposure (**Figure 5A**). In contrast, Mlys-IL-6 KO mice had similar numbers of neutrophils in the BAL fluid as compared to WT mice (**Figure 4A**). Furthermore, inhibition of neutrophilic inflammation in CD11c-IL-6 KO mice was associated with a marked decrease in mRNA (**Figure 5D**) and protein (**Figure 5C**) levels of IFNγ, which is a critical regulator of respiratory neutrophilia. The expression of IL-17A, which is crucial for development of neutrophilic asthma (39), was significantly diminished in the lungs of CD11c-IL-6 KO mice as compared to WT control mice (**Figure 5D**). Finally, mice with IL-6 deficiency in dendritic cells displayed diminished Th17, but not Th2, inflammatory response (**Supplementary Figure 4**) with abrogated neutrophilic accumulation in the lungs. These observations indicate that, although IL-6 produced by dendritic cells is necessary for the development of neutrophilic inflammatory response, it does not affect eosinophilia in response to HDM administration.

DISCUSSION

Asthma is a complex heterogeneous disease, with many subtypes that differ in etiology, severity, and treatment strategies (40). Strict division of this disease into endotypes is challenging, since clearly-defined endotypes of asthma are extremely rare, and more often intermediate forms with prevalence of some modality are observed. The association between the symptoms of allergic asthma and increased expression of IL-6 in patients was documented a long time ago (41). In mice, anti-IL-6 therapy of high-dose HDM asthma is effective (24), but the contribution

of IL-6 to a more clinically relevant low-dose HDM (10 μg) asthma model was not experimentally determined. It should be noted that the inflammatory process in mouse models of HDM-induced asthma largely depends on the allergen dose and the mode of its administration. The preferred option with regard to clinical relevance is multiple administration of small doses of the allergen, rather than single injection of high dose HDM (42). High dose of HDM (more than 12.5 μg/kg) significantly increases the number of BALF lymphocytes and neutrophils compared to saline-challenged controls without the need for prior allergen sensitization. In contrast, a low dose i.n. HDM challenge (1.25 μg/kg) in allergen sensitized mice causes a significant increase in BALF of eosinophil, lymphocyte and neutrophil numbers. Thus, the low dose HDM protocol results in induction of sub-maximal levels of cellular inflammation in the BALF and is associated with an influx of eosinophils, lymphocytes and macrophages without an accompanying non-allergic cellular inflammation. In the present study, we selected an acute (2-week-long) HDM-induced asthma mouse model, driving allergen-induced inflammation without the undesirable impact from non-specific inflammatory response, which is relevant to eosinophilic endotype of severe asthma in humans.

Our results showed that ablation of IL-6 signaling as seen in IL-6 KO mice abrogated the increase in granulocyte and Th2 cell numbers in the airways (**Figure 1B**), secretion of IgE in BALF and serum (**Figure 1C**), expression of asthma-associated genes, such as *Tslp*, *Tgfb1*, *Muc5ac*, *Gob5*, *Il4* (**Figures 1D,E**), and mucus production in the lungs (**Supplementary Figure 3**) in the low-dose HDM-induced asthma. Moreover, the number of leukocytes both in BAL fluid and in lung tissue was significantly decreased in IL-6 KO mice. These findings indicate that in the context of acute asthma mouse model, IL-6 is one of the key regulatory cytokines modulating the immune response. IL-6 is known for its capability to promote the differentiation of Th2 cells, inhibit Th1 and Treg differentiation and expansion in response to allergen, activate immunoglobulin class-switching in plasma cells and enhance the differentiation of Th17 cells, which are engaged in severe asthma (11). Pharmacological blockade of IL-6 results in the reduction of airway inflammation in some asthma models (24), suggesting that this cytokine may be driving several types of responses to the allergen. However, the exact mechanism of IL-6 involvement in the pathogenesis of allergic asthma has not been determined previously, and the IL-6-producing cell types that make the most significant contribution to the development of airway hyper-reactivity and inflammation have not been established.

Although macrophages and neutrophils were considered as the predominant source of sIL-6R (43), both human and mouse $CD4^+$ T-cells can also be the source of sIL-6R upon activation (44). $CD4^+$ T-cells may, therefore, contribute to the development and progression of asthma by providing sIL-6R to cells initially non-responsive to IL-6. An important role of IL-6 in regulation of effector $CD4^+$ T-cell fate (45) was attributed to driving IL-4 production during Th2 differentiation, inhibiting Th1 differentiation and, in synergy with TGFβ, promoting Th17 cell differentiation. Thus, $CD4^+$ T cells can produce IL-6 as an autocrine regulator.

IL-6 is also produced by primary lung epithelial cells in response to a variety of different cell stress or damage signals (e.g., UV, irradiation, ROS, microbial products, viruses, or other proinflammatory cytokines). A number of studies have demonstrated overexpression of IL-6 by bronchial epithelial cells in patients with asthma, both in adults and children (46). A recent study in mouse model of asthma confirmed an important contribution of IL-6 produced by epithelial cells in the pathogenesis of aspergillus-induced asthma. It was shown that high levels of IL-6 mRNA were constitutively present in mouse primary lung epithelial cells, but not in lung resident immune cells. Moreover, direct interaction of fungal β-glucans with lung epithelial cells triggered IL-6 production by lung epitheliocytes (47). In addition to constitutive expression of IL-6 by lung epithelial cells in steady state, the exposure to allergen can further induce production of this cytokine prior to the recruitment of inflammatory cells. Thus, the presence of IL-6 in the airways of asthmatic patients may not be the result of ongoing inflammation, but rather due to the "activated state" of pulmonary epithelial cells. Finally, IL-6 production is characteristic for lung fibroblasts upon activation (48).

In the present study we demonstrate that, both in steady state conditions and following acute asthma induction with low-dose HDM, the vast majority of IL-6 positive cells are leukocytes. Moreover, the number of $IL\text{-}6^+CD45^+$ cells was significantly increased in asthmatic as compared to healthy mice. At steady state, most of the IL-6-producing cells were represented by macrophages and monocytes, whereas after the disease induction, the contribution from IL-6-producing T-cells increased (**Figure 3D**). We, thus, hypothesized that myeloid cells are the source of disease-triggering pathogenic IL-6, which stimulates the development of the inflammatory responses in the respiratory tract, while the increase in the proportion of lymphoid cells in the pool of IL-6-producing cells may be a secondary event. Additionally, taking into account that alveolar macrophages and dendritic cells may produce IL-6 with different kinetics (49), we hypothesized that the effects of IL-6 deficiency in macrophages and dendritic cells on allergic airway inflammation may differ.

Increasing evidence suggests that macrophage-derived IL-6 plays the essential role in Th2-mediated allergic response. It was previously shown that IL-6 can enhance the polarization of alternatively activated macrophages (50) in synergy with IL-4 and IL-13, the two differentiation factors for alternatively activated macrophages which also play a pivotal role in eosinophilic allergic inflammation. Interestingly, the observed effect of IL-6 on macrophage polarization was partially dependent on up-regulation of the IL4Ra chain, which is consistent with the previously published data, showing that transfer of $IL\text{-}4Ra^+$ macrophages is sufficient to enhance Th2-driven eosinophilic allergic inflammation in the lungs (51). Moreover, HDM treatment induces alternatively activated macrophage polarization and Th2-mediated eosinophilia in the lungs (52). Finally, it was recently reported that inhibition of alternatively activated macrophage polarization in HDM-induced asthma model leads to reduced eosinophilic inflammatory response and subsequent shift toward neutrophilic inflammation (53). Since macrophages constitute the predominant fraction of IL-6 producing cells in the lungs (**Figure 3D**), we evaluated the contribution of IL-6 from macrophages to the development of

allergic asthma using reverse genetics approach. In the low-dose (10 μg) HDM-induced acute asthma model, we found that cell type-restricted deletion of IL-6 in macrophages leads to significant reduction of eosinophilic inflammation in the lungs (**Figure 4A**) and to attenuation of Th2 accumulation in the periphery (**Figure 4B**). Moreover, IgE production in Mlys-IL-6 KO mice was markedly decreased as compared to WT control mice (**Figure 4C**). Our results indicate that IL-6 from macrophages promotes Th2-driven eosinophilic inflammation during HDM-induced asthma, probably, due to IL-6-mediated macrophage polarization toward the alternatively activated macrophages. Additionally, we found that Th2, but not Th17, response was ablated in mice with IL-6 deficiency restricted to macrophages (**Supplementary Figure 4**). These data provide further support for important role of macrophages in the development of eosinophilic inflammatory response to the allergen and suggests that this impact involves IL-6.

Previous studies using an adoptive transfer of IL-6 deficient dendritic cells to WT mice, indicated the role of dendritic-cell-derived IL-6 in allergic inflammation, characterized by increased Th2 response and increased eosinophilia (25). In contrast, in another study, IL-6 produced by dendritic cells was shown to inhibit Th2 inflammatory response (54). We, thus, expected that IL-6 from dendritic cells may be the main factor determining the severity of Th2 responses to HDM in allergic asthma model. However, eosinophilic, Th2-mediated responses in mice with IL-6 deficiency in dendritic cells showed only a modest decrease. Neither lung eosinophilia (**Figure 5A**), nor the accumulation of Th2 cells in the periphery (**Figure 5B**) was significantly different from that in the WT control group. Unexpectedly, we found that neutrophilic response to HDM in CD11c-IL-6 KO mice was significantly reduced (**Figure 5A**), as well as, the production of IFNγ (**Figure 5C**) and expression level of IL-17A in the lungs (**Figure 5D**). Of note, recent findings highlighted the crucial role of dendritic cell-derived IL-6 in the development of experimental encephalomyelitis through the process called trans-presentation (55) that may be a distinctive feature of this cellular source of IL-6. T cells are able to recognize IL-6 produced by dendritic cells and respond by specific signaling cascade leading to STAT activation and differentiation into Th17 subsets. This data supports our observation that mice with IL-6-deficiency in dendritic cells display decreased Th17, but not Th2 inflammation (**Supplementary Figure 4**) with abrogated neutrophilic response in the lungs. Neutrophilic asthma is characterized by increased Th1 and Th17 cell responses with release of cytokines from Th1 and Th17 cells and by neutrophil recruitment to the site of inflammation (32, 33). Moreover, severity of asthma correlates with the level of IL-17A in the lungs, sputum and BALF (33). In patients with neutrophilic asthma driven by Th1/Th17 responses the release of IL-6, IL-17, IL-8, and IFNγ is associated with more severe refractory steroid-resistant endotype. However, randomized studies of Brodalumab, a human monoclonal antibody against IL-17a, did not show significant effects in treatment of severe asthma (56). Clinical trials of Suricumab, a fully human monoclonal antibody against IL-6, are currently in progress and therapeutic efficacy of systemic IL-6 blockade for severe asthma is yet to be determined. Nevertheless, the concept of severe asthma treatment is changing from one-drug-fits-all approach to more specific endotype-dependent therapy. In addition, the idea of cell-type-specific anti-cytokine therapy is currently being evaluated in several autoimmune disease models (57, 58). Our findings suggest that IL-6 from dendritic cells may contribute to the development of neutrophilic asthma, and that IL-6 expression by dendritic cells may lead to exacerbation of allergic inflammation in the lungs. Considering the high heterogeneity of dendritic cells and macrophages in the lungs, further studies will be required to dissect the role of individual IL-6 producing myeloid cell populations in lung inflammation and airway hyper-responsiveness.

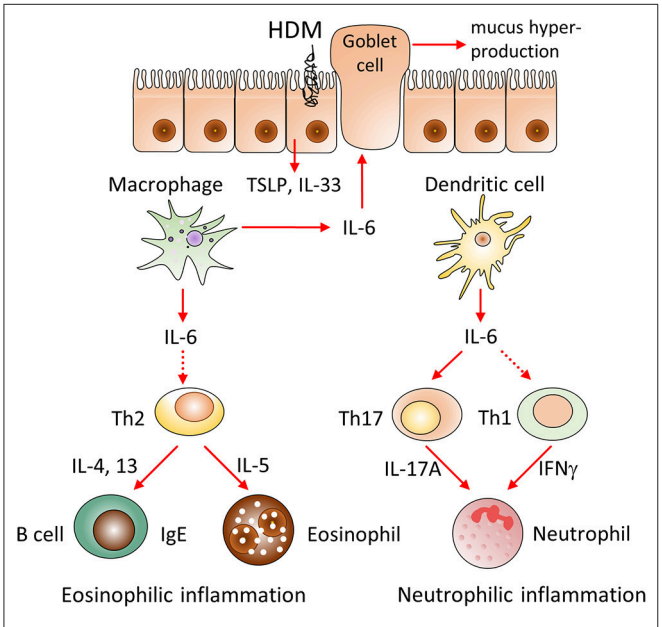

FIGURE 6 | Hypothetical model of the involvement of IL-6 from different cellular sources in the pathogenesis of HDM-induced allergic asthma. We propose that IL-6 produced by macrophages and dendritic cells distinctively promotes HDM-induced airway inflammation. IL-6 produced by macrophages contributes to type 2 allergic inflammation, including eosinophil accumulation, IgE production and mucus hypersecretion via induction of Th2 cells differentiation and production of IL-33, TSLP, IL-4, IL-13 cytokines. In contrast, IL-6 from dendritic cells induces pathogenic accumulation of neutrophils via induction of IL-17A and IFNγ, produced by Th17 and Th1 subsets. Therefore, selective targeting of IL-6 in macrophages or dendritic cells can be beneficial in corresponding eosinophilic and neutrophilic asthma endotypes.

Taken together, our results demonstrate the pathogenic role of IL-6 in clinically relevant low-dose acute HDM-induced asthma model and reveal the distinct roles of IL-6 produced by macrophages and dendritic cells in acute allergic airway inflammation. Whereas, IL-6 from macrophages contributes to type 2 allergic inflammation, IL-6 from dendritic cells is critical for induction of neutrophilic inflammation (**Figure 6**). Therefore, the approach of cell type-restricted targeting of IL-6 may be effective in the treatment of allergic asthma, especially its severe neutrophilic type.

AUTHOR CONTRIBUTIONS

EOG, EAG, ON, and RZ performed experiments. JH provided critical materials. EOG, MD, AT, and SN designed experiments, discussed results and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02718/full#supplementary-material

Supplementary Figure 1 | Asthma-associated gene expression and cytokine production in IL-6 KO and WT mice 48 h after last HDM challenge. **(A)** Quantitative RT-PCR analysis of *Muc5b, Ifng, Il5, Il1b, Il33*, and *Il10* mRNAs in lungs. Expression of each gene was normalized to *Actb*. **(B)** Serum was collected 24 h after the last HDM challenge. IFNγ levels in BALF and IL-1β levels in serum were determined by ELISA. Datasets were first tested for Gaussian distribution with the D'Agostino & Pearson omnibus normality test. Parametric or non-parametric comparison tests were applied where appropriated. Data represent means ± SEM, 5–17 mice in each group.

Supplementary Figure 2 | Gating strategy for different IL-6 producing cell populations. **(A)** Lungs from WT mice 48 h after last HDM challenge were subjected to enzymatic digestion to generate single cell suspensions of tissue samples. After that cells were stimulated with PMA and ionomycin in a humidified atmosphere containing 5% CO_2 at 37°C for 4 h. Brefeldin A was used to block protein Golgi transport. Distinct populations of leukocytes were distinguished with the following Ab panel: Fixable Viability Dye-eFluor 780, anti-CD45 (30-F11), anti-Ly6G (RB6-8C5), anti-CD103 (2E7), anti-CD11b (M1/70), anti-CD11c (N418), anti-SiglecF (1RNM44N), anti-CD4(GK1.5, eBioscience), anti-CD8(53-6.7, BioLegend), anti-ST2(RMST2-2, eBioscience), anti-CD19 (6D5, eBioscience) conjugated with FITC, PE, APC, PE-Cy7, PerCP-Cy5.5, and AmCyan. Cells were fixed and stained with anti-IL-6 PB-conjugated antibody. Mouse IgG1 Ab -PB (MOPC-21, eBioscience) was used as isotype control. **(B)** IL-6 positive cells were gated on the basis of isotype and FMO controls **(C)** Representative percentage ratio of IL-6 producing cells of different lung leukocyte populations in steady state (left) and 48 h after last HDM introduction (right) in WT mice assessed by flow cytometry. Data represent means ± SEM, 5–11mice in each group.

Supplementary Figure 3 | Histological assessment of lung inflammation on PAS-stained lung sections from WT, CD11c-IL-6 KO, MLys-Cre-IL-6 KO, and IL-6 KO mice. Red arrow denotes goblet cells in WT, CD11c-IL-6 KO, MLys-Cre-IL-6 KO lung tissue stained with Periodic acid-Schiff (PAS) staining.

Supplementary Figure 4 | Mlys-IL-6 KO mice demonstrate a decrease in frequency of Th2 cells, whereas CD11C-IL-6 KO mice show a reduction of Th17 cells in lungs in HDM-induced asthma. **(A)** Frequency (%) of activated Th2 cells ($CD45^+$ $CD3^+$ $CD4^+$ $CD8^-$ $ST2^+$ $IL\text{-}4^+$), Th1 cells ($CD45^+$ $CD3^+$ $CD4^+$ $CD8^-$ $INFg^+$ TNF^+), T reg ($CD45^+$ $CD3^+$ $CD25^+$ $FoxP3^+$ $HELIOS^+$) and Th17 cells ($CD45^+$ $CD3^+$ $CD4^+$ $CD8^-$ $IL\text{-}17A^+$ $RORyt^+$) in the lungs of WT and Mlys-IL6 KO mice assessed by flow cytometry. **(B)** Frequency (%) of activated Th2 cells ($CD45^+$ $CD3^+$ $CD4^+$ $ST2^+$ $IL\text{-}4^+$), Th1 cells ($CD45^+$ $CD3^+$ $CD4^+$ $INFg^+$ TNF^+), T reg ($CD45^+$ $CD3^+$ $CD4^+$ $FoxP3^+$ $HELIOS^+$) and Th17 cells ($CD45^+$ $CD3^+$ $CD4^+$ $IL\text{-}17A^+$ $RORyt^+$) in the lungs of WT and CD11c-IL6 KO mice assessed by flow cytometry. Datasets were first tested for Gaussian distribution with the D'Agostino & Pearson omnibus normality test and parametric comparison test (*t*-test) was applied. Data represent means ± SEM, 5–8 mice in each group. $^*P < 0.05$.

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Non-apoptotic Fas (CD95) Signaling on T Cells Regulates the Resolution of Th2-Mediated Inflammation

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Fas (CD95/APO-1) and its ligand (FasL/CD95L) promote the resolution of type 2 lung inflammation and eosinophilia. We previously found that Fas-deficiency on T cells, but not eosinophils, delayed resolution of inflammation. However, Fas can signal both cell death and have a positive signaling function that can actually activate cells. In this study, we investigated whether Fas-induced death or Fas-activated signaling pathways promote resolution of allergic lung inflammation. By increasing T cell survival through two Fas-independent pathways, using Bim-deficient T cells or Bcl-x_L overexpressing T cells, no differences in resolution of Th2-mediated inflammation was observed. Furthermore, Th2 cells were inherently resistant to Fas-mediated apoptosis and preferentially signaled through non-apoptotic pathways following FasL treatment. Utilizing Fas-mutant mice deficient in apoptotic but sufficient for non-apoptotic Fas signaling pathways, we demonstrate that non-apoptotic Fas signaling in T cells drives resolution of Th2-mediated airway inflammation. Our findings reveal a previously unknown role for non-apoptotic Fas signaling on Th2 cells in the induction of resolution of type 2 inflammation.

Keywords: allergy, Asthma, Eosinophilia, Apoptosis, Fas-FasL, Th2 cells

INTRODUCTION

Atopic asthma is supported by a type 2 immune response mediated by expansion of T helper type 2 (Th2) cells reactive against ordinarily innocuous allergens (1, 2). Th2 cells are known to promote pathologic responses associated with asthma, including eosinophilic infiltrate, mucus production, airway constriction, and antibody responses. While increased Th2 cell recruitment to the lungs drives features of allergic responses, it is possible that defective resolution of type 2 responses in asthmatics could also account for prolonged airway disease and contribute to disease phenotypes. The observation that those who suffer from intermittent or persistent asthma have elevated numbers of antigen-experienced T cells and eosinophils in sputum samples, even when samples are collected between exacerbations, supports the premise that asthma may be, in part, driven by defects in resolution of Th2 responses (3). Expanding on the known mechanisms involved in resolving established inflammation in the airways may complement current therapeutic approaches that aim to inhibit the initiation of allergic inflammation, and instead focus on developing methods to end chronic inflammation.

Fas, a member of the tumor necrosis factor (TNF) receptor family, has been broadly studied for its function during development and ability to regulate both ligand-mediated and activation-induced cell death (AICD) in a variety of cells. Asthmatic patients may have defects in Fas-mediated signaling, which could contribute to delayed resolution of inflammation. Following exposure to allergen, FasL expression was augmented in asthmatic patients (4). Low mRNA and surface expression of Fas on pulmonary T cells was associated with persistent inflammatory cell infiltrates in the airways of asthmatics, and their pulmonary lymphocytes were shown to be less sensitive to Fas-mediated apoptosis (5, 6). Similarly, asthmatic patients demonstrated an increased number of cells expressing the anti-apoptotic molecule Bcl-2 compared to normal control subjects, and the expression of Bcl-2 correlated with severity of asthma (7, 8). Together, these data suggest Fas may be important for the regulation of Th2-mediated inflammation.

Fas can initiate two primary signaling pathways: one inducing apoptosis and one promoting a non-apoptotic signaling cascade (9–11). In the initiation of apoptosis, Fas ligation causes a conformational change which allows binding of signaling molecules (including FADD, cFLIP, procaspase-8) to the intracellular C-terminal signaling death domain (12). Recruitment of these proteins along with caspase-8 forms the death-inducing signaling complex (DISC) that induces receptor internalization. Once receptors are internalized, apoptosis can be activated by direct caspase activation or indirectly through a mitochondria-mediated activation pathway. Fas-mediated non-apoptotic signaling is involved in a variety of signaling pathways independent of the death-promoting pathway (13). Fas signaling through FADD-adaptor protein triggers a MAPK signaling cascade to induce NFκB translocation, thereby augmenting cell proliferation and mobility (14–17). Further, non-apoptotic Fas signaling promotes tumor cell growth and invasion (18, 19). The mechanisms that regulate whether Fas-mediated death or non-apoptotic signaling are induced remain to be fully understood.

We previously found that Fas and FasL expression on T cells are necessary for normal kinetics of resolution of Th2-mediated airway inflammation (20, 21). Herein, we investigate the signaling pathway utilized by Fas on T cells during such inflammation. We first tested whether Fas functioned by increasing T cell survival, thereby extending inflammation. However, increasing T cell survival through Fas-independent mechanisms was not sufficient to promote a delay in resolution. Further, Th2 cells are innately resistant to Fas-mediated apoptosis (22) and preferentially signal through non-apoptotic pathways, suggesting that Fas-mediated apoptosis was not a key factor in the extended inflammatory response. Using a mouse model in which apoptotic signaling through Fas on T cells was blocked, we found that the intact non-apoptotic signaling through Fas was sufficient to promote the normal resolution of Th2-mediate airway inflammation. These data expand our understanding of Fas on T cells and identify a new paradigm for the capabilities of non-apoptotic vs. apoptotic Fas signaling during immune responses.

RESULTS

Increasing T Cell Survival Is Not Sufficient to Prolong Eosinophilic Inflammation

Our previous studies found that Fas-positive T cells promote the resolution of Th2-induced inflammation (20, 21). To test the possibility that these findings were due to changes in T cell survival, we examined whether increasing T cell survival through Fas-independent mechanisms could lead to delays in the resolution of inflammation (23). Bim is a regulatory protein important for the initiation of apoptosis in lymphocytes (24, 25). Adoptive transfer of $Bim^{-/-}$ T cells into $Rag^{-/-}$ mice ($Bim^{-/-}$>$Rag^{-/-}$) followed by sensitization and challenge resulted in a dramatic increase in the number of T cells having infiltrated into the lungs of mice at all-time points examined (**Figures 1A,B**). However, this did not correlate with changes in the numbers of eosinophils which had infiltrated into the lungs of these mice (**Figure 1C**). Similarly, histologic sections from these mice did not show any significant changes in gross pathology when examined at resolution stage of our model (**Figure 1D**). Th2 cytokine production by re-stimulated T cells from $Bim^{-/-}$>$Rag^{-/-}$ lungs was increased, but augmenting these cytokines alone was not sufficient to drive persistent eosinophilia (**Figure 1E**).

We next sought to increase T cell survival through overexpression of the anti-apoptotic protein Bcl-x_L. Similar to the $Bim^{-/-}$ T cells, $Bclx_L$ Tg T cell transfer into $Rag^{-/-}$ mice increased the numbers of T cells in the lungs at days 4 and 14 after sensitization and challenge (**Figure 2A**). $Bclx_L$ Tg T cells also failed to influence the numbers of eosinophils recruited to the lungs, nor did they change the gross histologic analysis compared to controls (**Figures 2B,C**). Together, these data suggest that increasing anti- or pro-apoptotic pathways in T cells, independent of Fas expression, do not affect the resolution of Th2 mediated responses.

Fas Preferentially Signals Through Non-apoptotic Pathways on Th2 Cells

Effector T cell subsets Th1, Th17, and Treg cells are susceptible to Fas-mediated death (26–28). However, the susceptibility of Th2 cells to Fas-mediated apoptosis remains controversial. Even when similar methodologies were used, multiple studies have conflicting conclusions in terms of how Th2 cells respond to Fas-induced apoptosis (27, 29, 30). Unlike previous studies that used antibody for Fas ligation, here we utilized a leucine zipper FasL (LzCD95L) (31) for ligation of Fas on T cells. LzCD95L mimics the membrane bound form of FasL and has been shown to be an efficient inducer of apoptosis and Fas signaling (9). We skewed Th1 and Th2 cells *in vitro*, and treated with LzCD95L to assay for the initiation of apoptosis. As expected, Th1 cells showed a dramatic sensitivity with almost 50% of total cells having DNA fragmentation as measured by a sub-G1 DNA peak (**Figure 3A**). Skewed Th2 cells were much more resistant to Fas-mediated apoptosis compared to Th1 cells, developing reduced nuclear content loss following treatment. LzCD95L induced death signaling directly through Fas, since neither Fas-deficient (LPR) nor Fas-mutant (LPRcg/wt) cells, showed

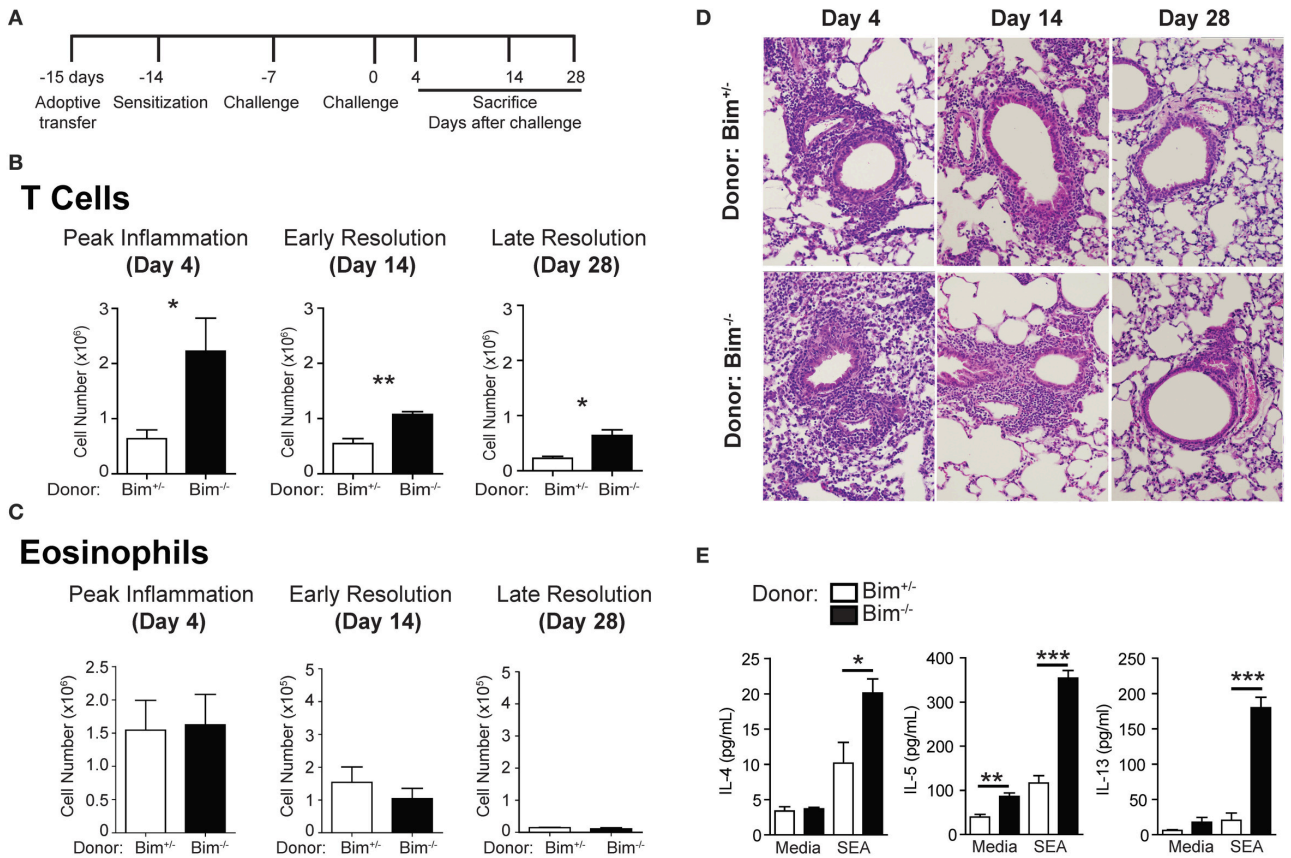

FIGURE 1 | Increasing T cell survival through Bim-deficiency is not sufficient to induced delayed resolution of inflammation. **(A)** Protocol for adoptive T cell transfer, sensitization, and challenge. *Bim*$^{-/-}$ or WT T cells were transferred into *Rag*$^{-/-}$ mice which were then sensitized and challenged. Mice were sacrificed on day 4, day 14, or day 28 after challenge and assayed for **(B)** T cell and **(C)** eosinophil infiltration into the lungs. **(D)** H&E staining of histologic sections from the lungs of sensitized and challenged mice. **(E)** T cells from the lungs at day 14 after challenge were restimulated with media or SEA and assayed for Th2-cytokine secretion. Data are representative of three independent experiments for each time point. For each independent experiment, $n \geq 5$ mice per group were used (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

sensitivity to LzCD95L treatment. These observations support the hypothesis that Th2 cells are resistance to Fas-mediated apoptosis.

We measured NFκB p65 translocation after Fas-signaling to determine whether Th2 cells can signal through Fas non-apoptotic mechanisms. *In vitro* stimulation with LzCD95L resulted in the loss of cytoplasmic NFκB p65 in Th2 cells, suggesting translocation into the nucleus following treatment (**Figure 3B**). Th1 cells expressed very little p65 and protein levels in the cytoplasm did not change following treatment. Further, LzCD95L induction of nuclear translocation of NFκB was tested by electromobility shift assay (EMSA). As has been previously reported, Th2 cells had augmented nuclear NFκB compared to Th1 cells at baseline (26). Following LzCD95L treatment, Th2 cells developed an increased amount of nuclear NFκB when compared to untreated control Th2 cells (**Figure 3C**). Together these findings suggest that Th2 cells are resistant to Fas-mediated apoptosis and preferentially signal through non-apoptotic mechanisms in response to FasL engagement.

Non-apoptotic Fas Signaling on T Cells Drives Resolution of Th2-Mediated Airway Inflammation

To address whether non-apoptotic Fas signaling in Th2 cells plays an important role in airway inflammation resolution, we utilized Fas-mutant mice with a point mutation in the death domain of Fas, LPRcg mice (32). When homozygous for the mutation, LPRcg/cg mice are deficient for apoptotic and non-apoptotic signaling similar to the Fas-deficient LPR mice. Interestingly, it has been shown that heterozygous LPRcg/wt mice maintain defects in apoptotic signaling, but are sufficient for induction of non-apoptotic Fas-signaling (33). Utilizing these mice, we asked whether non-apoptotic Fas signaling in T cells is sufficient for promoting resolution of Th2-mediated airway inflammation. T cells from littermate WT, LPRcg/wt, and LPRcg/cg mice were adoptively transferred into *Rag*$^{-/-}$ mice and then sensitized and challenged. Interestingly, CD4 T cells were either equivalent or reduced in the apoptotic-deficient mice, which is strikingly different from what was observed in

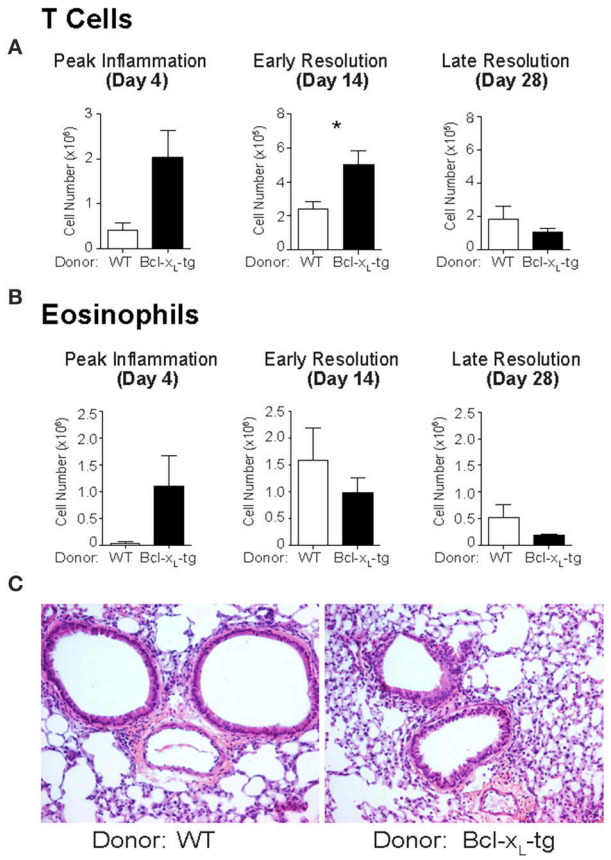

FIGURE 2 | Bcl-x_L transgenic T cells promote T cell survival but do not affect eosinophilia in the lungs following sensitization and challenge. Bcl-x_L transgenic T cells were transferred into $Rag^{-/-}$ mice then sensitized and challenged. Mice were sacrificed on day 4, day 14, or day 28 after challenge and assayed for **(A)** T cell and **(B)** eosinophil infiltration into the lungs. **(C)** H&E staining of histologic lung sections of sensitized and challenged mice at day 14. Data are representative of three independent experiments for each time point with $n \geq 5$ mice used per group for each experiment ($^*p \leq 0.05$).

the $Bim^{-/-}$ or $Bclx_L$-Tg experiments (**Figure 4A**). However, eosinophilia was augmented in LPRcg/cg>$Rag^{-/-}$ mice at both day 14 and day 28 (**Figure 4B**). In contrast, LPRcg/wt>$Rag^{-/-}$ mice showed equal or even enhanced eosinophil clearance at day 14 and day 28 compared to littermate WT>$Rag^{-/-}$ controls (**Figure 4B**). Histologic examination supported these findings (**Figure 4C**). When T cells were restimulated with SEA antigen or anti-CD3 antibody in the presence of irradiated APCs, T cells from Fas-mutant mice secreted similar levels of IL-4, IFNγ, and IL-17, but had elevated IL-5 production (**Figures 4D,E**, and data not shown). However, lung T cells did not produce sufficient cytokines for detection when isolated directly from lungs and plated in media for ELISA, suggesting that it is possible that Fas-mutant Th2 cells are not actively producing more cytokines in the lungs at day 14 following challenge. This result is also in accordance with data from **Figure 1**, supporting the hypothesis that at later stages of inflammation, Th2 cytokine levels are not regulating the chronicity of disease. Overall, these results demonstrate that non-apoptotic Fas-signaling in T cells is sufficient to promote the normal resolution of allergic airway inflammation in mice.

While Th2 cells are the primary T cell type present in the airways of sensitized and challenged mice, Treg cells have also been implicated in the regulation of inflammation in the lungs (34, 35). To test whether Tregs from Fas-mutant mice may have intrinsic defects in their ability to suppress inflammatory responses, we performed an *in vitro* suppression assay on FACS sorted Treg cells co-cultured with wild type T effector cells. We observed efficient suppression from Tregs regardless of whether they were from WT or Fas-mutant strains (**Supplemental Figure 1**). These data suggest that Treg effector function is not directly regulated through Fas-signaling pathways.

METHODS

Mice

$Rag^{-/-}$ (B6.129S7-$Rag1^{tm1Mom}$/J), $Bim^{-/-}$ (B6.129-Bcl21ll tm1.1Ast /J), and LPR (B6.MRL-Fas^{lpr}/J) were purchased from The Jackson Laboratory (Bar Harbor, Maine USA). Bcl-x_L Tg; Lck-p-Bcl-xL Tg (B6.Cg-Tg(LCKprBCL2L1)12Sjk/J) mice (36) were a gift from Dr. Jeff Rathmell (Vanderbilt University). LPRcg (MRL/MpJ-Faslprcg/Faslprcg) mice were a gift from Dr. Marcus Peter (Northwestern University), and backcrossed to C57BL/6 for 10 generations. FoxP3^{gfp} reporter mice (B6.Cg-Foxp3tm2Tch/J) were obtained from the Jackson Laboratories, and crossed to LPRcg strain. All mice were bred and housed in specific pathogen-free facilities maintained by the University of Chicago Animal Resource Center. Studies described conform to the principles set forth by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of laboratory animals in biomedical research.

Flow Cytometric Analysis

In brief, 5×10^5 cells were suspended in 50 μl of FACS buffer (1x PBS containing 0.1% sodium azide and 1% BSA), blocked with anti-CD16/32 (2.4G2), and labeled with specific antibodies. Antibodies used include anti-CD3 (145-2C11), -CD4 (GK1.5), -CD8 (53-6.7), -Ly6G (Gr1) (BD Biosciences), and -CCR3 (83101) (R&D Systems). Flow cytometric analysis was performed on an LSR Fortessa (BD Biosciences), and data were analyzed with FlowJo software (Tree Star Inc.).

S. Mansoni Allergic Inflammation Model

Inactivated *S. mansoni* eggs (5,000), a generous gift from Dr. Joel Weinstock, Tufts University, were administered to mice i.p. at day 0 and animals were challenged intratracheally on day 7 and day 14 with 5 μg of soluble eggs antigen (SEA) in 50 μL PBS (20, 37). Mice were sacrificed on days 4, day 14, and day 28 after the final i.t. challenge. At sacrifice, animals were perfused with 5 mL 1x PBS, then BAL and lungs were collected for analysis. As previously published (38), BAL was performed by lavaging 0.8 ml ice old 1x PBS into the airway via a tracheal cannula and gently aspirating the fluid. The lavage was repeated four

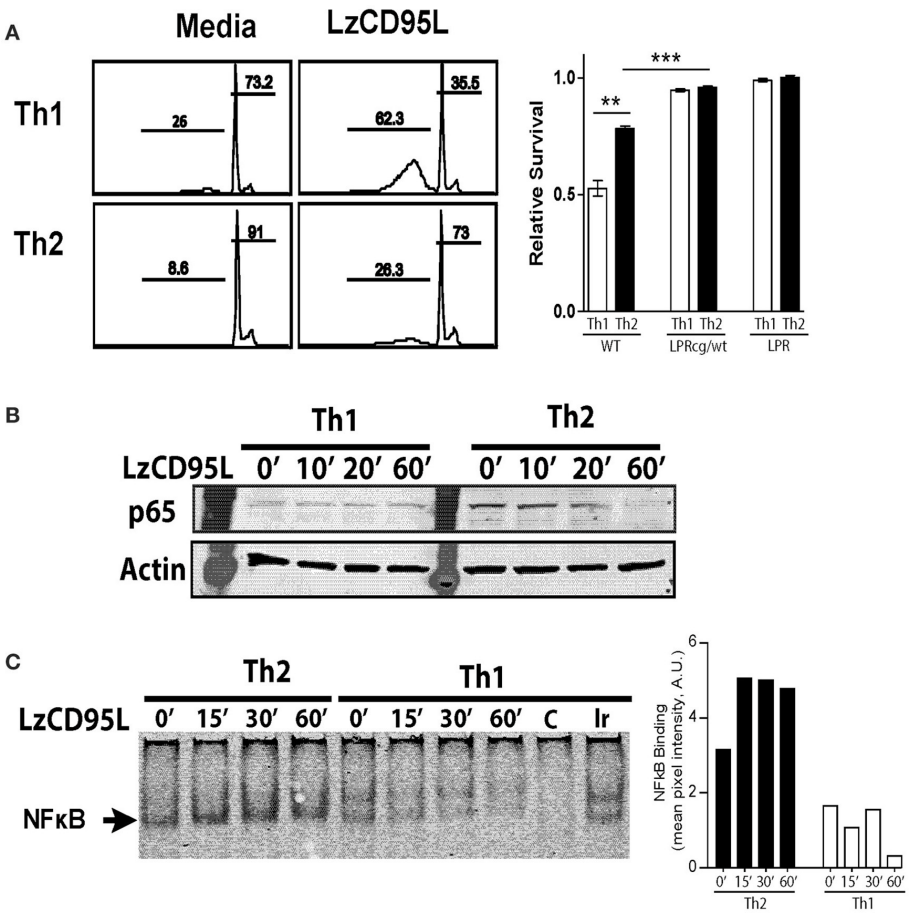

FIGURE 3 | Th2 cells preferentially signal through non-apoptotic Fas pathways following FasL treatment. *In vitro* skewed Th1 and Th2 cells from WT, LPRcg/wt, and LPR mice were treated with LzCD95L and assayed for induction of apoptosis 4 h later by propidium iodide staining. **(A)** Representative PI staining from WT Th1 and Th2 cells showing apoptotic cells in sub-G1, and relative survival rate of T cells from different mice following LzCD95L treatment. **(B)** WT Th1 and Th2 cells were treated with LzCD95L and assayed for cytoplasmic levels of p65 by western blot. **(C)** WT Th1 and Th2 cells were treated with LzCD95L and assayed for nuclear NFκB binding activity by EMSA. Densitometry measurements (right) are displayed as mean pixel intensities in arbitrary units. Data are representative data from three or more independent experiments. In panel **(A)** apoptosis assays included 5 replicated for each independent experiment (**$p \leq 0.01$, ***$p \leq 0.001$).

times, with <90% of fluid recovered. For histological analysis, lungs were fixed with formalin. Samples were paraffin embedded and cut sagitally into 5 μM sections. Sections were stained with Hematocylin and Eosin (H&E) or with Periodic Acid Schiff (PAS) by the University of Chicago Histology Core Facility.

Cytokine Secretion

Lungs from mice were isolated, cut with scissors into small pieces, and digested with 2 mg/mL Collagenase IV at 37-degrees Celsius for 45 min. Tissue was strained through nylon filters to isolate single cell suspension. T cells from lungs of mice were restimulated by co-culture irradiated (24 gray) B6 splenocytes that were loaded with antigen (1 μg/mL SEA) and were incubated at 37°C in complete medium DMEM, 10% fetal bovine serum (HyClone Characterized, Thermo Fisher, Cat# SH30396, Lot# KTH31760), 1% β-mercaptoethanol, and antibiotics. After 48 hrs, plates were directly frozen without separating supernatant from stimulated cells. Total cytokine production was measured using a Millipore Multiplex bead array following manufacturer's instructions and analyzed by a Luminex (Bio-Rad) reader.

Treg Suppression Assay

Tregs from FoxP3^{gfp} reporter mice crossed to LPRcg mutant mice were FACS sorted, then co-cultured with CFSE-labeled FACS sorted wild type FoxP3^{gfp}-negative T effector cells. Cells were plated with irradiated splenocytes and anti-CD3 (2C11) antibody to induce effector cell proliferation. CFSE dilution was measured by flow cytometry at day 4 of culture. Forty thousand of effector cells were cocultured with 0, 1,000, 2,000, 4,000, and 16,000 Treg cells to measure suppressive ability of Treg cells.

Apoptosis Assays

Apoptosis of cells was measured using the Nicoletti propidium iodide staining protocol (39). These experiments were performed

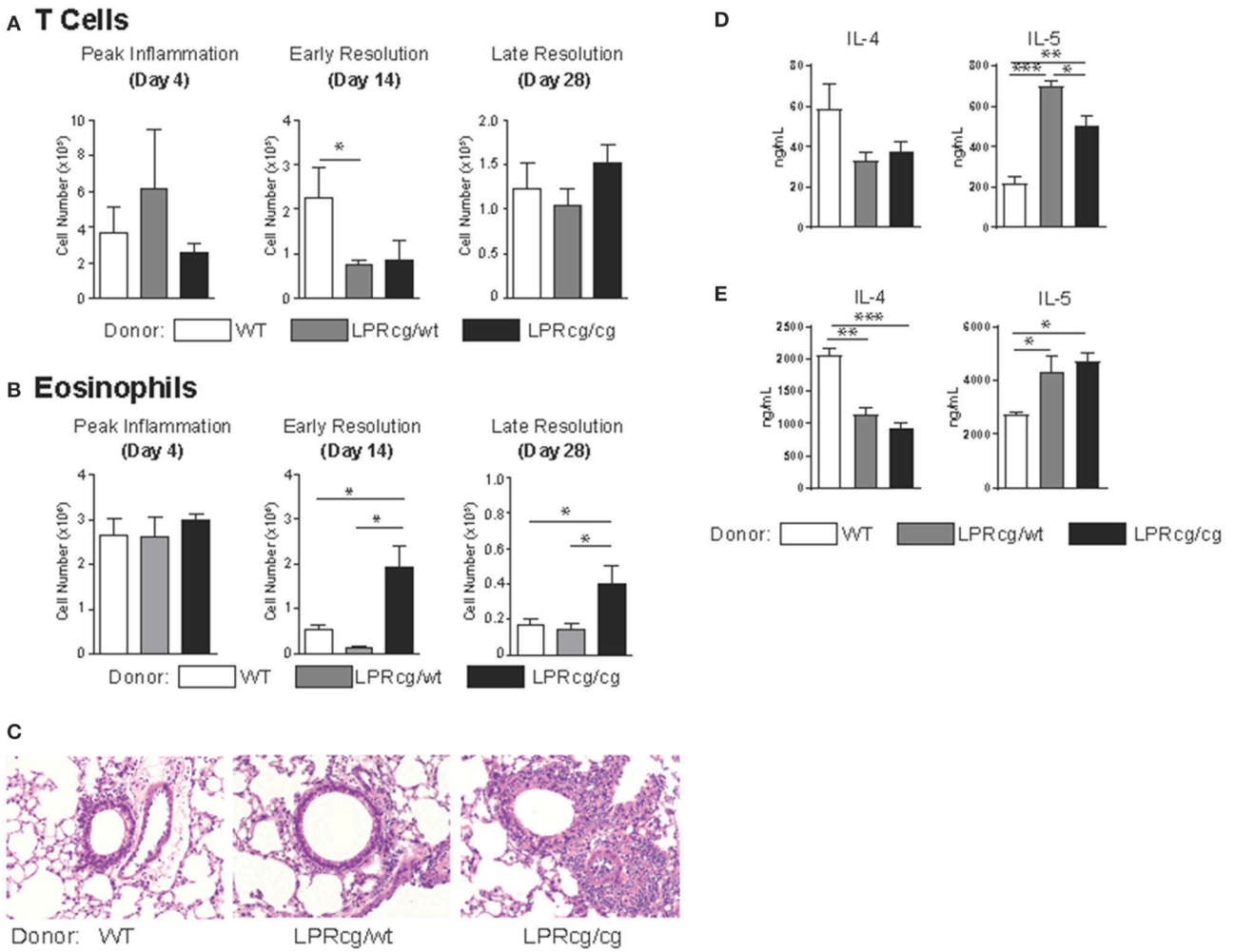

FIGURE 4 | Non-apoptotic Fas signaling in T cells is sufficient for the normal resolution of Th2-mediated inflammation. T cells from the indicated strains were adoptively transferred into *Rag*$^{-/-}$ mice which were then sensitized and challenged then assayed for numbers of **(A)** CD4 T cells and **(B)** eosinophils in the lungs at days 4, 14, and 28 after the final challenge. **(C)** H&E stains of lung sections of sensitized and challenged mice at day 14. Total lung cells were co-cultured with irradiated splenocytes with SEA antigen **(D)** or anti-CD3 antibody **(E)** for T cell cytokine production. **(A–C)** Data are representative of three independent experiments for each time point with $n \geq 5$ mice per group for each experiment. **(D,E)** data are representative of two independent experiments with *n*- of 3 or 4 mice per group (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

4 h post treatment with 200 ng/mL LzCD95L (Marcus Peter Lab, Northwestern University).

Electromobility Shift Assay (EMSA)

Nuclear extract from LzCD95L treated cells was incubated with IR-labeled NFκB oligonucleotide probe (Li-cor #829-0724) following manufacturer's instructions. Samples were run on a 4% native polyacrylamide gel at 10 V/cm for 60 min and directly imaged (Li-cor Odyssey). Mean pixel intensity of bands was performed on Image J software.

Western Blotting

Cells were lysed [25 mM HEPES (pH 7.5)], 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM NaF, 200 μM Na-orthovanadate, and protease inhibitor cocktail (Sigma Aldrich catalog #S8830, 1 tablet for 100 mL lysis buffer) and insoluble material was removed by centrifugation at 20,000 × g for 5 min. Cleared lysates were boiled in Laemmli buffer for 5 min. Samples were subjected to polyacrylamide gel electrophoresis, and analyzed by blotting with primary antibodies against p-65 (Santa Cruz), and β-actin (Sigma-Aldrich), followed by corresponding secondary antibodies conjugated with IR-dye and imaged using Li-cor Odyssey and software.

T Cell Enrichment

Nylon wool columns were incubated with PBS + 5% FBS for 1 h at 37°C to decrease background binding. Cells were incubated at 37°C on the column for 45 min and then 30 mL of 5% complete

media was passed through the column to enrich T cells. Non-adherent cells were collected and assayed for T cell purity of <90%.

Statistical Analysis

Statistics were calculated using the GraphPad (Prism 4) software with unpaired students two-tailed *T*-tests, ($^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$, ns = not significant). Error bars on all graphs represent mean ± standard error (SEM).

DISCUSSION

In this study we addressed the mechanisms by which Fas regulates the resolution of experimental asthma in mice. We identified an *in vivo* function for non-apoptotic Fas signaling in normal T cells, independent of pathways regulating cell survival. The importance of non-apoptotic Fas-signaling in other cell types such as tumor cells has been observed, but our study represents the first to investigate the role of non-apoptotic Fas signaling in maintaining immune homeostasis.

Non-apoptotic pathways have been implicated in a wide variety of cell types outside of just T lymphocytes; including fibroblasts (40) astrocytes (41), neurites (14), and a number of cancer models (11, 42–45). While others have argued that strength of signal as a key regulator of apoptotic or non-apoptotic Fas signaling, our data support the notion that some cells (particularly Th2 cells) are intrinsically programed for non-apoptotic signaling (19, 33). Further, whether these intrinsic programs are modulated by different antigen exposures (i.e., viral vs. bacterial infections) or epigenetically by chromatin remodeling have yet to be thoroughly vetted. As a recent example, T cells from patients with Human T-cell lymphotropic Virus 1 (HTLV-1) infection adopted preferential non-apoptotic Fas signaling. This non-apoptotic signaling through Fas resulted in enhanced T cell proliferation, activation, and a more rapid onset of infection (46).

In tumor cell lines, Fas mediated non-apoptotic signaling pathways through MAP kinase and NF-κB activation, have been shown to drive invasiveness (44). This non-apoptotic signaling through Fas was enhanced in cells that have a single allelic mutation of the Fas gene which completely blocked apoptotic signaling through Fas (33). This observation is analogous to the results obtained with the LPRcg/wt mouse model in our study where we observed that a single allele mutation in Fas was sufficient to inhibit death-inducing pathways, but enabled positive signals to still occur downstream of the receptor. These observations led to the conclusion that two functional Fas alleles are necessary for the induction of apoptosis, and that the presence of a mutant allele could lead to growth and cell survival, which in the case of tumors is an undesirable outcome (33, 44). Consequently, blockade of Fas signaling in a tumor line led to decreased migration and activation of JNK, NFκB, p38 pathways (42). Finally, non-apoptotic Fas signal via both ERK1/2 and p35 promotes neurite outgrowth (14). Thus, adoption of non-apoptotic signaling pathways in cells that are meant to be regulated by Fas-mediated apoptosis may lead to scenarios of disease states.

We observed that Th2 cells had cell-intrinsic resistance to Fas-mediated apoptosis, and in fact had induced NFκB activity following FasL treatment. Resistance of Th2 cells to FasL-mediated killing may be due to Th2 cells having augmented FLIP, TRAIL, and NFκB expression at baseline when compared to Th1 cells (26). Our *in vivo* data further suggested that this preference for non-apoptotic signaling represents a predisposition for Fas signaling on Th2 cells and contributes to functional outcomes in lung inflammation. However, whether cells that utilize non-apoptotic signaling are intrinsically predisposed, or whether the context of ligand exposure (such as microenvironment or ligand conformation) regulates the downstream signaling remains to be thoroughly explored. Previous studies suggested that Fas engagement of membrane-bound FasL vs. cleaved soluble-FasL had different outcomes in Fas-induced cell death and signaling (47, 48). Two animal models were developed; one expressing only soluble FasL and expressing only membrane-bound FasL. Analysis of these mice in a tumor model led to the conclusion that the nature of the FasL isoform could lead to changes in strength of signal and differential signaling outcomes in disease (19). Our data does suggest that even cells with a high degree of similarity, such as CD4 T cells subsets, have dramatically different signaling outcomes when encountering the same form of Fas ligand. In our case, using LzCD95L resulted in observations that each T cell type is skewed for preferential signaling pathways downstream of FasL-engagement. However, it is important to consider that these findings do not exclude the possibility that under some scenarios Th2 cells might undergo a shift in the balance between pro- and non-apoptotic signaling, enabling these cells to now switch to an apoptotic sensitive cell type. Unraveling these possibilities will help elucidate potential targets for intervening in diseases associated with Fas expression and function.

While our data clearly show that T cell-mediated Fas-signaling is necessary for normal resolution of Type-2 inflammation, we were not able to separate the role of Fas-signaling on Th2 cells from other T cell subpopulations. By utilizing an adoptive T cell transfer approach, we were able to create a scenario where T cells were the only cell type possessing the Fas-mutations. However, this would include CD8, as well as other CD4 T effector cells, leaving the possibility that Fas-signaling pathways on non-Th2 cells could be playing a role in Type-2 inflammation resolution. Treg cells are established regulators of inflammation in all parts of the body including lung. We show that Treg suppression was not affected in Fas-mutant mice. In addition, we demonstrate that Fas-signaling on Th2 cells induces non-apoptotic activation instead of cell death. Thus, while this study emphasizes a previously unappreciated role for Th2 cells *in vivo*, limitations exist in our ability to interpret the mechanisms as other T cell subsets may be contributing to effective resolution of inflammation.

In conclusion, using an *in vivo* model of experimental asthma, we show that non-apoptotic Fas signaling on T cells is necessary for resolution of Th2-mediated inflammation.

Resolution of inflammation was not dependent on regulatory mechanisms associated with T cell apoptotic pathways or T cell numbers present in the airways. Translating these findings into humans for targeting of Fas pathways in patients may present a variety of challenges and limitations. For instance, as Fas is expressed in most hematopoietic and stromal cells, specific targeting of Fas to the cells of interest may be impossible. Moreover, broadly targeting Fas or FasL pathways by antibodies or recombinant proteins could have unpredictable outcomes: inducing apoptosis in some cells and activation in other cell types, as well as causing organ damage like liver injury (49). However, based on our results, optimization of drug targeting of Th2 cells specifically appear to be a potential mechanism to drive the resolution of Type-2 inflammation. Expanding on our understanding discrete function of Fas on Th2 effector T cells may allow for unique targeting in allergic diseases, specifically asthma and potentially other responses involving Th2 cells.

AUTHOR CONTRIBUTIONS

AS and MP conceived and supervised the project. JW, CF, and AS wrote the manuscript. JW, CF, KB, CR, FV, and JT performed and analyzed experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02521/full#supplementary-material

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Allergen Exposure in Lymphopenic Fas-Deficient Mice Results in Persistent Eosinophilia Due to Defects in Resolution of Inflammation

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Asthma is characterized by chronic airway type-2 inflammation and eosinophilia, yet the mechanisms involved in chronic, non-resolving inflammation remain poorly defined. Previously, our group has found that when Rag-deficient mice were reconstituted with Fas-deficient B6 LPR T cells and sensitized and challenged, the mice developed a prolonged type-2-mediated airway inflammation that continued for more than 6 weeks after the last antigen exposure. Surprisingly, no defect in resolution was found when intact B6 LPR mice or T cell specific Fas-conditional knockout mice were sensitized and challenged. We hypothesize that the homeostatic proliferation induced by adoptive transfer of T cells into Rag-deficient mice may be an important mechanism involved in the lack of resolution. To investigate the role of homeostatic proliferation, we induced lymphopenia in the T cell-specific Fas-conditional knockout mice by non-lethal irradiation and sensitized them when T cells began to repopulate. Interestingly, we found that defective Fas signaling on T cells plus antigen exposure during homeostatic proliferation was sufficient to induce prolonged eosinophilic airway inflammation. In conclusion, our data show that the combination of transient lymphopenia, abnormal Fas-signaling, and antigen exposure leads to the development of a prolonged airway eosinophilic inflammatory phase in our mouse model of experimental asthma.

Keywords: Th1/Th2 cells, eosinophils, apoptosis, lung, inflammation, asthma, lymphopenia

INTRODUCTION

Asthma is a heterogeneous airway disease characterized by variety of clinical phenotypes. One of the most common phenotypes exhibited by patients, atopic asthma, is characterized by the presence of T helper type 2 (Th2) cells and the persistence of airway inflammation. Why asthmatic patients develop chronic inflammatory responses in their airways and lungs remains an unresolved question in pulmonary medicine. Determining the mechanisms involved in the perpetuation of respiratory inflammatory events will allow for a better understanding of the chronic nature of atopic disease.

Although apoptosis may provide a mechanism for the removal of activated T-cells in healthy individuals, studies have suggested that this process may be delayed in the airways of asthmatics (1). First, it has been found that the percentage of apoptotic lymphocytes in induced sputum was significantly decreased in patients with asthma compared with healthy controls (2). Second, asthmatics demonstrated increased numbers of cells positive for the anti-apoptotic molecule Bcl-2 compared to normal control subjects, and the expression of Bcl-2 correlated with severity of asthma (3, 4). Third, low expression of Fas mRNA and surface Fas receptor on pulmonary $CD3^+$ T cells has been associated with persistence of inflammatory cellular infiltrates in asthmatic airways (5), and peripheral blood leukocytes from asthmatics have been shown to be less sensitive to Fas-mediated apoptosis (6). Finally, murine studies suggest that the persistence of eosinophils during Th2 airway inflammation is due to defects in apoptosis in these cells (7–10). Together, these studies suggest that understanding the role of apoptosis pathways may be a valuable avenue of study for atopic asthma.

Allergen-specific Th2 cells can be induced in mice, and they promote airway inflammation characterized by eosinophilia, goblet cell hyperplasia, and airway constriction. However, the animal models generally do not lead to prolonged inflammation unless there is either continued antigen-exposure for long periods of time or when there are specific genetic alterations in the mice. For instance, transgenic mice that over-express IL-5 (11), IL-9 (12), IL-11 (13), or IL-13 (11), or mice that are deficient for T-bet, all develop a spontaneous chronic Th2-type lung inflammatory disease that presents with eosinophilic inflammation, collagen deposition in the airways, and airway hyper-reactivity to methacholine challenge (14). These animal models of airway inflammation are spontaneous and do not involve any antigen exposure. In our previous studies, we investigated the role of a cell surface death receptor Fas (CD95), and its ligand (FasL) in the resolution of airway inflammation. Using adoptive transfer of either B6 or B6.LPR T cells into $Rag^{-/-}$ mice, we found that Fas-deficiency on T cells led to a delay in resolution and the development of a prolonged inflammatory response. Mice that received wild type T cells resolved their acute allergen-induced inflammation within 2 weeks following the last challenge, while mice that received Fas-deficient T cells developed a prolonged inflammatory phase that lasted at least 4 weeks longer (15). In a follow-up study using adoptive transfer models, we also demonstrated that FasL-deficiency (GLD) on T cells led to prolonged airway inflammation (16). Thus, our murine model of prolonged Th2-mediated airway inflammation is unusual since it develops due to a failure to resolve an acute response, and not due to chronic allergen challenges or genetic manipulation.

Using a T cell-specific conditional deletion of Fas, we were able to address whether homeostatic proliferation was involved in the prolonged inflammatory phase. Interestingly, we found that these mice failed to induce the prolonged airway inflammation and eosinophilia after sensitization and challenge. Inducing homeostatic proliferation in these conditional T cell specific Fas-deleted mice restored the delayed resolution. These findings show a new potential mechanism involved in eosinophilic airway inflammation that could be involved in human asthma as well.

MATERIALS AND METHODS

Animals

C57BL/6 (B6) mice were purchased from The Division of Cancer Treatment at the National Cancer Institute (Frederick, MD). B6.129S7-$Rag1^{tm1Mom}$ ($Rag^{-/-}$) and B6.MRL-$Tnfrsf6^{LPR}$ (B6.LPR) mice were purchased from The Jackson Laboratory. $Fas^{fl/fl}$ lck-cre mice were a kind gift from Dr. Alexander V. Chervonsky (17). All animals were bred and housed within a specific pathogen-free barrier facility maintained by the University of Chicago Animal Resources Center. The studies reported here conform to the principles outlined by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research. All animal procedures and housing were approved by the University of Chicago Animal Resources Center and the Institutional Animal Care and Use Committee.

Antibodies and Flow Cytometry

Anti-mouse CD3 (clone 17A2; BD Biosciences, San Diego, CA), anti-mouse CCR3 (clone 83101.111; R&D Systems, Minneapolis, MN), and anti-mouse Ly6G (GR1, BD Biosciences) antibody were used for flow of bronchial alveolar lavage (BAL) T cells ($CD3^+$ side scatter low) and eosinophils ($CCR3^+Ly6G^-$ and side scatter high). Data was acquired on an LSR-II (Becton-Dickinson, San Jose, CA), and analyzed using FlowJo software (Treestar).

S. mansoni Sensitization and Challenge

These methods were previously described (15, 18). Briefly, at day-14, mice were immunized by intraperitoneal (i.p.) injection of inactivated *S. mansoni* eggs. At days-7 and 0, the mice were challenged with 5 μg of soluble egg antigen (SEA) by intranasal and intratracheal aspiration, respectively. The mice were sacrificed at 4, 14, 21, or 28 days after the last challenge. B6.LPR mice were used at 5–7 weeks of age in order to ensure that they had not yet developed lymphoproliferative disease. For some experiments, the mice were irradiated with 6 Gy, 6 days prior to the *S. mansoni* sensitization.

BAL Analysis

Bronchioalveolar lavage (BAL) was performed by delivering ∼0.8 ml of cold PBS into the cannulated trachea and gently aspirating the fluid. The lavage was repeated a total of four times, and a total volume of 2.5–3 ml BAL was collected. The percentage of cell types found within BAL fluid was determined by flow cytometric analysis with cell type–specific markers.

Adoptive Transfer

B6 and B6.LPR T cells were harvested from lymph nodes of donor mice and enriched by non-adherence to a nylon wool column. 10^7 cells were adoptively transferred intravenously into each recipient. The purity, as determined by flow cytometry, was between 90 and 95% $CD3^+$ T cells.

Analysis of Lung Histological Changes

Lungs were removed from mice after completion of BAL and fixed by immersion into 4% paraformaldehyde. Lobes were

sectioned sagittally, embedded in paraffin, cut into 5 μm sections, and stained with H&E for analysis.

Detection of Th1 and Th2 Cytokines

T cells were incubated at a concentration of 4×10^6 cells/ml in a 48-well plate which was pre-coated with αCD3 antibody (145-2C11). Supernatants were harvested after 48 h in culture. Cytokine production was measured using a Millipore Multiplex bead array following manufacturer's instructions and analyzed by Luminex (Bio-Rad) reader.

Statistical Analysis

Graph generation and statistical analysis were performed using Prism software (version 5.0; GraphPad). Differences in total cells and eosinophils in the BAL fluid and in lung histological scoring were determined by using an unpaired Student's two-tailed *t*-test. If the *F*-test differed significantly, Mann-Whitney tests were used. Error bars represent SEM. Statistical significance was claimed whenever $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

RESULTS

A Prolonged Inflammatory Phase Is Not Observed in B6.LPR Mice

Using a murine model of Th2-mediated airway inflammation (15, 18), we have previously demonstrated an airway disease with several important similarities to allergic asthma. This protocol (**Figure 1A**) involves sensitizing mice with inactive *S. mansoni* eggs by intraperitoneal injection (i.p) and challenging through intratracheal administration with soluble eggs antigen (SEA) antigen. This protocol leads to a robust Th2 response, consisting of 70–75% of eosinophils, 10–15% of T cells, and 10–15% macrophages, at the peak of inflammation on day 4 after the last challenge. By 14 days after the last challenge (early resolution phase), the airway inflammation is almost fully resolved in WT B6 mice.

Consistent with our previous publication (15), we find that when T cells from B6.LPR (Fas-deficient) mice are transferred into a $Rag^{-/-}$ host, then sensitized and challenged, the animals were unable to resolve their airway inflammatory response at similar rates to $Rag^{-/-}$ animals receiving control B6 T cell transfers (**Figure 1B**). Elevated T cell and eosinophil numbers were found at 14 and 21 days post challenge (**Figure 1B**), as well as elevated overall inflammation (**Figure 1C**). Our previous study had shown similar responses at the peak of inflammation suggesting that the prolonged inflammation observed at later time points was not simply a more robust acute phase response, but a defect in resolution associated with Fas-deficiency on the T cells (15). It remained untested whether B6.LPR animals themselves would show similar defects in resolution of allergic airway inflammation. Following sensitization and challenge (**Figure 1D**), B6.LPR mice responded similarly at day 14 post challenge to the T cell adoptive transfer model, with elevated BAL T cells and eosinophils (**Figure 1E**). Surprisingly, unlike the adoptive transfer model (15), we found that B6.LPR mice had resolved their airway inflammation by day 21 after challenge (**Figures 1E,F**). These data suggest that Fas-deficiency is not sufficient to induce the prolonged inflammatory phase.

Conditional Deletion of Fas on T Cells Does Not Affect the Resolution of Inflammation

To address the possibility that deletion of Fas on T cells specifically could regulate the resolution of airway inflammation, we obtained Fas-conditional knockout mice ($Fas^{fl/fl}$) and bred them to the T cell-specific Lck-cre mice. $Fas^{fl/fl}$ lck-cre mice were sensitized and challenged following protocol 2 (**Figure 1D**) and assessed for inflammatory cell infiltrates in the airways. $Fas^{fl/fl}$ lck-cre mice developed normal numbers of airway eosinophilia, lymphocyte infiltration, as well as vascular and peribronchial inflammation at day 4 after challenge as compared to littermate controls ($Fas^{fl/fl}$) (data not shown). At 14 days after challenge, the $Fas^{fl/fl}$ lck-cre mice showed elevated airway inflammation and tissue inflammation compared to littermate controls (**Figure 2**). Thus, $Fas^{fl/fl}$ lck-cre mice have a similar delay in the resolution of airway inflammation as observed in B6.LPR and B6.LPR>$Rag^{-/-}$ mice at the early resolution phase. However, the $Fas^{fl/fl}$ lck-cre mice showed complete resolution of inflammation by day 21 after sensitization and challenge. These data suggest that while T cell-specific Fas signaling is required for effective resolution of inflammation during the early phase (day 14), the lack of Fas signaling on T cells specifically is not sufficient for the prolonged inflammation seen in the T cell transfer models.

Antigen Exposure During Lymphopenia in T Cell-Specific Fas-Conditional Knockouts Leads to Development of Persistent Eosinophilia

By adoptively transferring T cells into lymphopenic recipients such as $Rag^{-/-}$, T cells will undergo homeostatic proliferation to fill the open niche (19). This lymphopenia-induced proliferation is also well-documented in humans. Homeostatic proliferation of T cells occurs in humans during immune system development, as a mechanism to maintain T cell memory, and after transient lymphopenia during certain viral infections (20, 21). The role of lymphopenia has not been investigated as a factor in asthma pathology. Since we observed the development of prolonged airway inflammation only in a model in which Fas-deficient T cells are transferred into a lymphopenic host, we sought to test whether induction of lymphopenia in the intact $Fas^{fl/fl}$ *lck-cre* or B6.LPR mice would induce the prolonged eosinophilia as previously found in the adoptive transfer model.

To address this hypothesis, we induced transient lymphopenia by sub-lethal irradiation (6 Gy) in B6, Fas $^{fl/fl}$, and $Fas^{fl/fl}$ lck-cre mouse strains. In both $Fas^{fl/fl}$ lck-cre and control $Fas^{fl/fl}$ mice, we found that splenic lymphocytes were decreased 97% 24 h after irradiation, but after 6 days the numbers of lymphocytes began to recover and were almost completely normal by 21 days post irradiation (**Figure 3**). Thus, we sensitized $Fas^{fl/fl}$lck-cre mice and control $Fas^{fl/fl}$ littermates at 6 days after irradiation when homeostatic proliferation is occurring and challenged them as described in the protocol (**Figure 4A**). Sensitized and

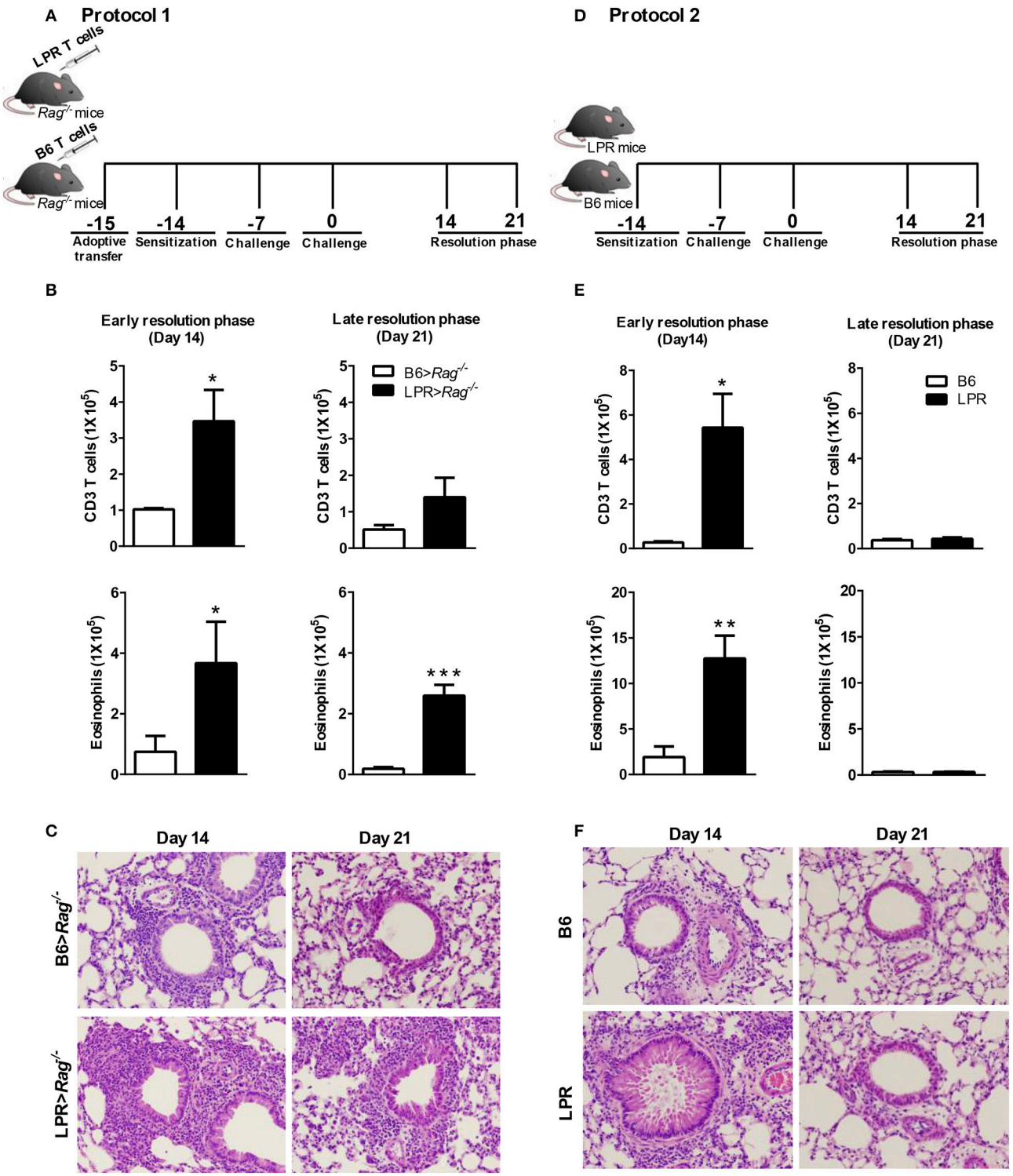

FIGURE 1 | Fas signaling is required for normal resolution of eosinophilic airway inflammation. B6 and B6.LPR T cells were adoptively transferred into Rag$^{-/-}$ mice 1 day before sensitization (noted as B6>Rag$^{-/-}$ and LPR>Rag$^{-/-}$ mice, respectively). The reconstituted mice were sacrificed on days 14 or 21 after the final challenge, and the BAL was analyzed **(A,B)**. B6 and B6.LPR mice were also sensitized and sacrificed on days 14 or 21 after the final challenge, and the BAL was analyzed **(D,E)**. Representative H&E stained sections of lungs at days 14 and 21 **(C,F)** Lung tissues from B6>Rag$^{-/-}$, LPR>Rag$^{-/-}$, B6, and LPR mice were fixed in 4% paraformaldehyde and embedded in paraffin. Approximately five mice per group per time point were analyzed. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. Error bars represent SEM.

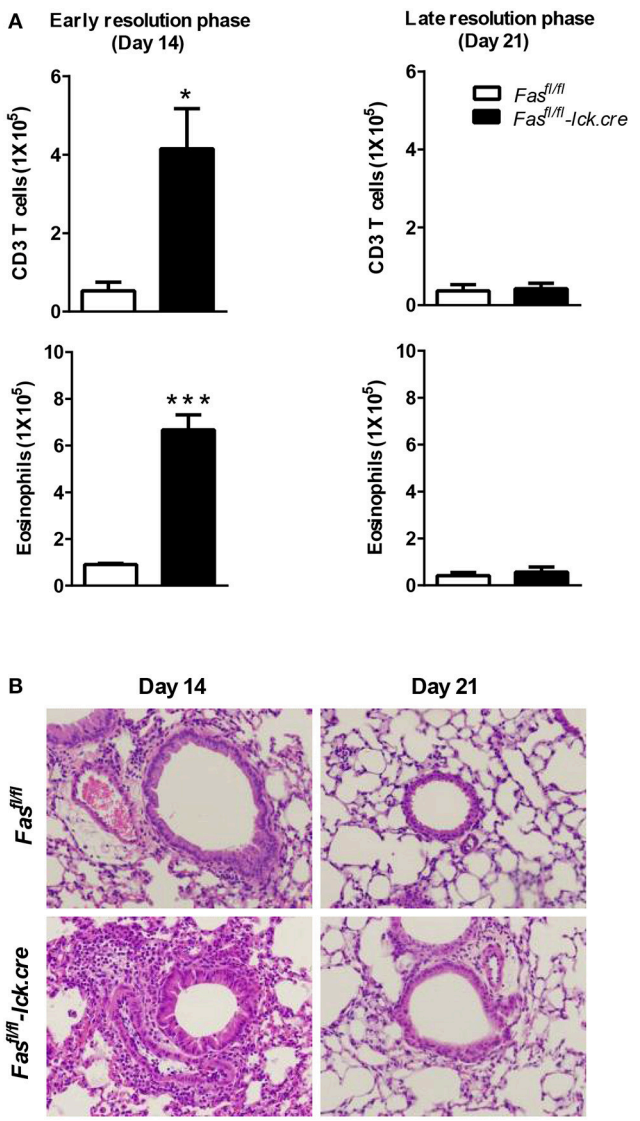

FIGURE 2 | T cell-specific Fas signaling is sufficient to resolve early eosinophilic inflammation. $Fas^{fl/fl}$ lck-cre and $Fas^{fl/fl}$ (control mice) were sensitized and sacrificed on days 14 and 21 after the final challenge and the BAL was analyzed **(A)**. Representative H&E stained sections of lungs at days 14 and 21 **(B)**. Lung tissues from mice were fixed in 4% paraformaldehyde and embedded in paraffin. Approximately five mice per group per time point were analyzed. $^*P < 0.05$. $^{***}P < 0.001$. Error bars represent SEM.

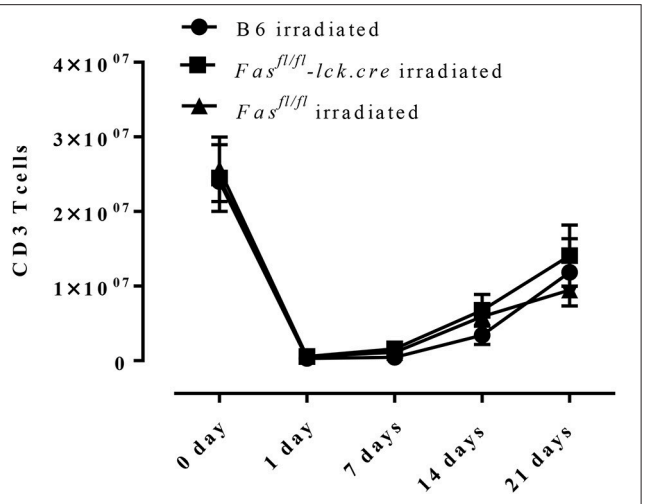

FIGURE 3 | T cell recovery after irradiation-induced lymphopenia. B6 mice, $Fas^{fl/fl}$ lck-cre, and $Fas^{fl/fl}$ mice were irradiated with 6 Gy, CD3 T cells in the spleen were counted and analyzed by flow cytometry. Approximately 3–4 mice per group per time point (5-time points total) were analyzed.

challenged lymphopenic $Fas^{fl/fl}$ lck-cre mice developed normal levels of airway inflammation at day 4 after challenge whether or not they were irradiated (data not shown). However, the irradiated $Fas^{fl/fl}$ lck-cre mice showed increased airway levels of T cells and eosinophils at days 14 and 21 compared to $Fas^{fl/fl}$lck-cre mice that were not irradiated (**Figures 4A,B**). This was also readily observable in the cellular infiltration around airways and vessels in H&E sections (**Figure 4C**). Thus, Fas deficiency on T cells is sufficient to induce prolonged airway inflammation when the antigen exposure occurs during time periods when the T cells are undergoing homeostatic proliferation.

To determine the nature of the T cell response at 21 days that is inducing the prolonged inflammation, lung T cells were restimulated *in vitro* and cytokine production of IFN-γ, IL-10, IL-17, IL-4, IL-5, and IL-13 were measured. Interestingly, the level of IFN-γ production by irradiated $Fas^{fl/fl}$lck-cre T cells was significantly less compared to non-irradiated T cells at day 21 (**Figure 4D**). These data are consistent with our previous findings that the failure of T cells to produce IFN-γ in the LPR>$Rag^{-/-}$ mice plays an important role in the resolution of Th2 airway inflammation (15). However, levels of IL-4 and IL-17 were higher in irradiated $Fas^{fl/fl}$lck-cre cells than T cells from non-irradiated $Fas^{fl/fl}$lck-cre mice, consistent with increased levels of inflammation (**Figure 4D**). There were no differences in levels of IL-10 **(Supplementary Figure 1)**, IL-13 and IL-5 between groups (data not shown). Finally, even at day 28 after the last challenge, we found that $Fas^{fl/fl}$lck-cre irradiated animals had persistent eosinophilia and **decreased IFN-γ production by T cells** (**Figure 4E**). Cytokine production by T cells was also evaluated at this time point, and the irradiated $Fas^{fl/fl}$lck-cre animals showed a reduction in IFN-γ levels with an increase in IL-13 (data not shown). Together, these data support a conclusion that T cells undergoing homeostatic proliferation rely on Fas for regulation of inflammatory responses.

LPR Mice Develop Prolonged Type 2 Inflammation When the Sensitization Occurs While T Cells Are Undergoing Homeostatic Proliferation

Our data showed that B6.LPR mice do not sustain prolonged inflammation by day 21 after our sensitization and challenge protocol (**Figure 1E**). Similar to the irradiation protocol just

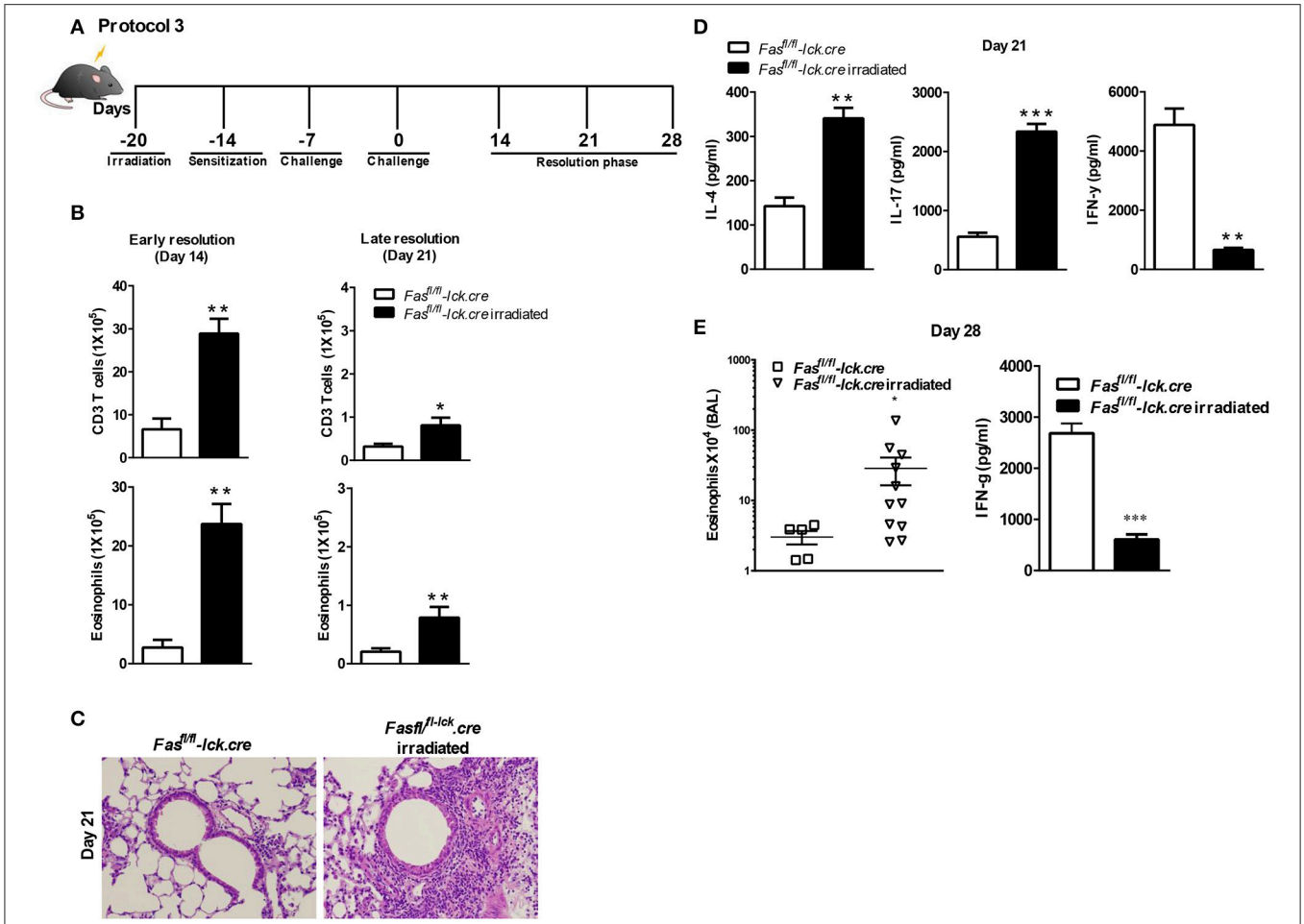

FIGURE 4 | Antigen exposure during lymphopenia in T cell-specific Fas conditional knockouts leads to development of prolonged eosinophilic airway inflammation. *Fas*$^{fl/fl}$ *Ick-cre and Fas*$^{fl/fl}$ *Ick* (control mice) were irradiated (or not treated) with 6 Gy, 6 days prior to a *S. mansoni* sensitization, and sacrificed on day 14 or day 21 after the final challenge. The BAL was analyzed **(A** and **B)**. Representative H&E stained sections of lungs at day 21 **(C)**. Lung tissues from mice were fixed in 4% paraformaldehyde and embedded in paraffin **(C)**. Lung T cells from *Fas*$^{fl/fl}$ *Ick-cre and Fas*$^{fl/fl}$ irradiated mice at day 21 were re-stimulated with anti-CD3 and measured by a Bioplex system as described in Material and Methods for IL-4, IL-17, and IFN-γ **(D)**. At day 28 post challenge, the eosinophil number in the BAL was evaluated **(E)** and IFN-γ by lung T cells . Approximately five-eleven mice per group per time point were analyzed. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$ Error bars represents SEM.

described for the Fas$^{fl/fl}$lck-cre mice, we sensitized B6.LPR mice and control mice (B6) at 6 days after irradiation, when homeostatic proliferation is occurring, and challenged them as described in protocol 3 (**Figure 4**). Irradiated B6.LPR mice had increased airway levels of total cells (data not shown) and eosinophils (**Figure 5**), while the number of T cells did not change significantly during the late resolution phase when compared irradiated B6 mice. Further, we observed an increased infiltrate of cells around the airways and vessels in the B6. LPR irradiated mice compared to irradiated B6 mice (**Figure 5B**).

To determine the nature of the T cell response that was induced during the prolonged inflammation, lung T cell cytokine production from restimulated cells was measured for IFN-γ, IL-10 IL-17, IL-4, IL-5, and IL-13. Interestingly, the levels of IFN-γ production were significantly less in the B6.LPR irradiated mice compared to non-irradiated B6.LPR mice (**Figure 5C**). We also found a significant difference in IL-4 between B6.LPR non-irradiated mice and B6.LPR irradiated mice at 21 days after the last challenge. B6.LPR irradiated mice produced significantly more IL-4 (**Figure 5C**) but the same levels of IL-17 (data not shown). These findings again suggest that IFN-γ production attenuates airway inflammation in a murine model of asthma, and these data are consistent with our previous findings that the failure of B6.LPR T cells to produce IFN-γ in adoptive transfer model plays an important role in the ability to resolve Th2-medicated inflammation.

DISCUSSION

Many advances have been made in our understanding of factors involved in type 2 airway inflammation onset, but

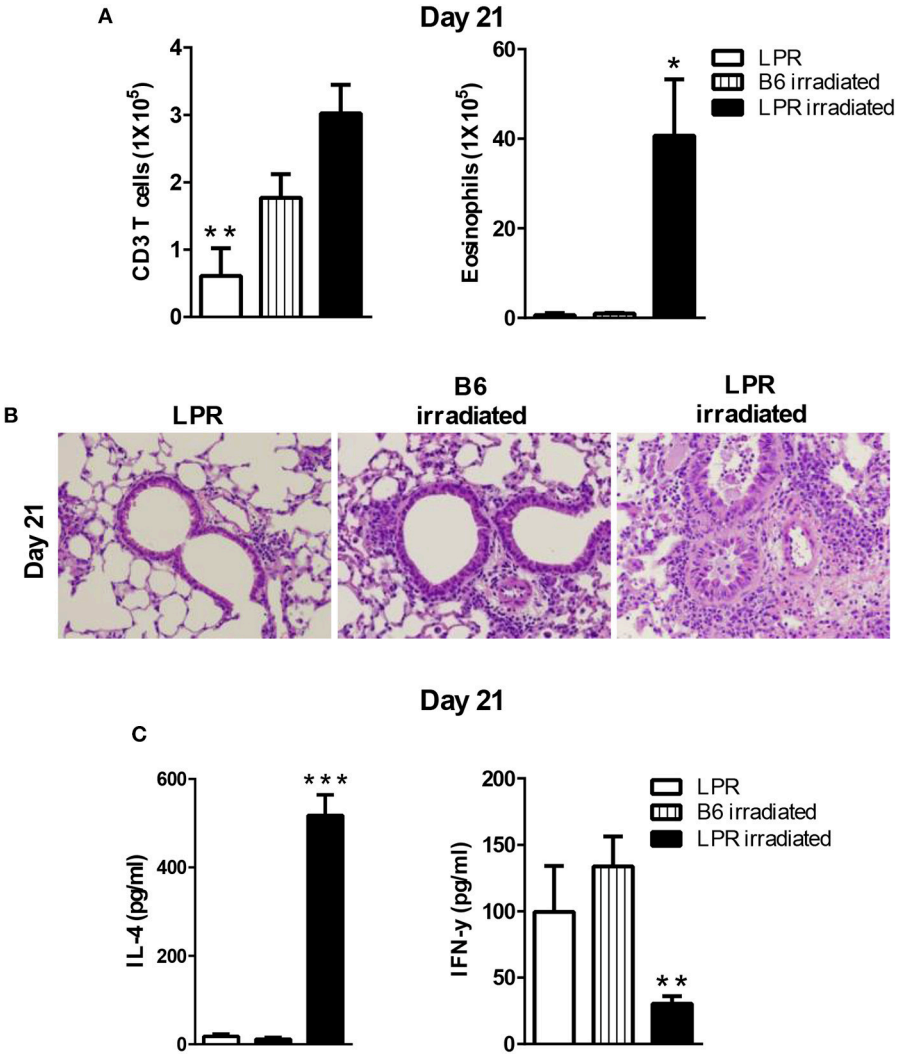

FIGURE 5 | LPR mice are able to develop prolonged inflammation when the sensitization occurs during lymphopenia. B6.LPR and B6 and B6.LPR irradiated mice (6 Gy, 6 days prior to the *S. mansoni* sensitization) were sacrificed on days 14 or 21 after the final challenge and the BAL was analyzed **(A)**. Representative H&E stained sections of lungs at day 21 **(B)**. Lung tissues from mice were fixed in 4% paraformaldehyde and embedded in paraffin. **(C)** Lung T cells from B6.LPR and B6.LPR irradiated mice at day 21 were re-stimulated with anti-CD3 and measured by a Bioplex system as described in Material and Methods for IL-4 and IFN-γ . Approximately, five mice per group per time point were analyzed. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}p < 0.001$. Error bars represent SEM.

little is known about the mechanisms involved in the resolution process. Studies conducted in mice to investigate mechanisms associated with chronicity of eosinophilic inflammation have been carried out by exposing mice to continuous allergen challenges. However, the persistent lung inflammation observed in asthmatic patients cannot be explained by continuous allergen exposure (22). Thus, the objective of this investigation was to determine the possible mechanisms involved in the development of prolonged airway inflammation without continued exposure to allergen (15). The novelty of our study is that the combination of three factors, defective Fas-signaling on T cells, allergen exposure, and homeostatic proliferation, are all necessary to develop chronic Th2 inflammation (**Figure 6**). Thus, asthmatic patients may not only have hyperactivity of the Th2 immune response but may also have defects in their ability to resolve inflammation.

Lymphopenia is commonly associated with many viral infections including respiratory syncytial virus (RSV), influenza, measles, rubella, and parvovirus, West Nile virus and rhinovirus (RV) (23–27). Viral respiratory infections can have a profound effect on many aspects of asthma including its initiation, exacerbation, and severity (28–30). Interestingly, RSV and RV infections in children have been found to be an important risk factor for later development of asthma (31–35). Mechanisms linking RV infection and asthma development involve allergen exposure and host factors such as polymorphisms. Our data in a mouse model shows that homeostatic proliferation plays an important role in the development of persistent inflammation and eosinophilia. Thus, it is possible that lymphopenia is a part of

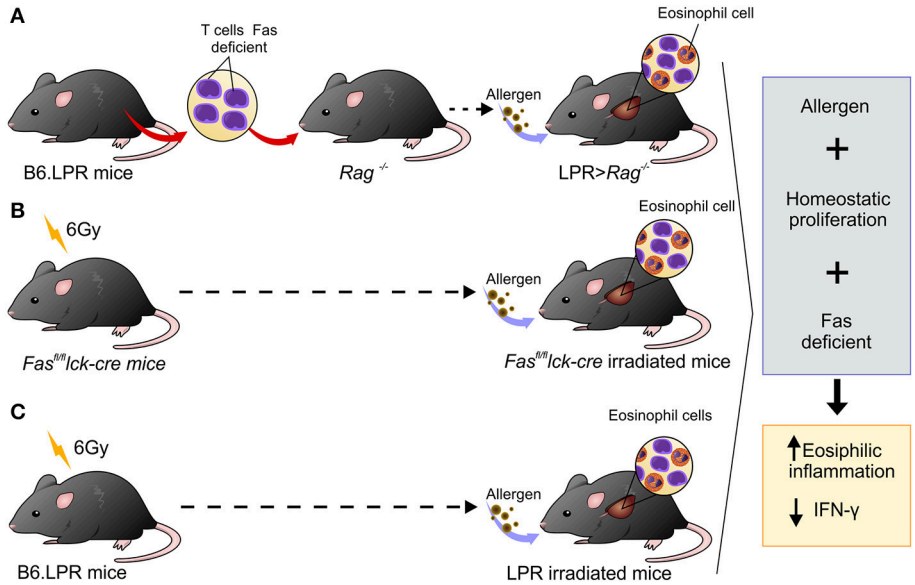

FIGURE 6 | Schematic representation showing three different experimental designs leading to persistent eosinophilia in a mouse model. **(A)** B6.LPR T cells were adoptively transferred into *Rag*$^{-/-}$ mice, **(B)** *Fas*$^{fl/fl}$ *lck-cre* irradiated with 6 Gy and **(C)** B6.LPR irradiated with 6 Gy. All three situations involve transient lymphopenia, abnormal Fas-signaling, and antigen exposure resulting in decreasing levels of IFN-γ in the lung.

the mechanism that associates RSV and other viruses with asthma risk.

Besides viral infection, it is also important to note that elevated rates of homeostatic proliferation are associated with some autoinflammatory syndromes, such as rheumatoid arthritis and systemic lupus erythematosus (10, 36). Chemotherapy and bone marrow transplantation can be associated with autoimmune manifestations (37). Although studies that correlate chemotherapy with asthma induction or severity are rare, unexplained asthma symptoms in patients receiving Tamoxifen for the treatment of breast cancer have been described (38).

Our data demonstrate that expansion of T cells in Lpr>*Rag*$^{-/-}$ mice is not only due to homeostatic proliferation, but also requires specific antigen stimulation (data not shown). However, we have yet to determine whether polyclonal stimuli could replace homeostatic proliferation and play a role in the prolonged eosinophilia found in our model. Recently, it has been shown that recurrent T cell homeostatic proliferation results in global gene expression changes, including the progressive upregulation of FasL, granzyme B, and programmed cell death protein 1 (PD-1) (39). Considering that PD-1 is expressed during chronic T cell activation and recurrent T cell homeostatic proliferation, and considering that PD-1 expression on T cells inhibits IFN-γ production (40), it is possible that PD-1 may play a role on our model.

There is a possibility that a neutralizing Fas antibody treatment reproduces the same results observed in our model, considering that neutralizing Fas antibody may limit the expansion of antigen-specific T cells. Further, it has previously been reported that the administration of neutralizing antibody to FasL (clone MFL4) in Balb/c mice induce increased and persistent eosinophilia (10). However, in this case, the acute prolonged eosinophilia was resolved by day 10 after challenge, not during a chronic inflammatory phase as observed in our model. Further, this study used Balb/c and not C57BL/6 mice, and a different model of airway inflammation. Nevertheless, this paper and our data both suggest that Fas functions to dampen the acute and persistent lung inflammatory response characteristic of asthma.

The prolonged inflammatory phase observed in our experiments is correlated with decrease levels of IFN-γ production and increases in Th2 cytokines from lung T cells. After recovery from viral infections, reduced IFN-γ expression in PBMCs and airway cells has been observed and associated with both increased asthma risk and asthma severity (41, 42). Thus, it is important to note that the factors observed in our mouse model that led to prolonged Th2 inflammation, lymphopenia and decreased IFN-γ production, can also be observed during viral infections. IFN-γ promotes Fas-mediated apoptosis of allergen-activated T lymphocytes in the airways of atopic asthmatic patients (43). Other studies have shown that IFN-γ has a potent local and systemic effects through its actions on the airway epithelium in mice (44). Also, studies of inhaled IFN-γ in humans show that mild asthmatics that inhaled IFN-γ for 3 weeks exhibited a reduction in airway eosinophils (45). Further, IFN-γ has been shown to have a critical role in triggering T cell apoptosis via the up-regulation of Fas on the surface of activated CD4+ T cells (46). Finally, it has been shown that FasL activation exerts an inhibitory effect on IL-5, IL-9, and GM-CSF during the allergic airway responses in the BAL fluid of mice (10).

We find that IFN-γ production by T cells is reduced when either the *Fas*$^{fl/fl}$*lck-cre* or LPR mice develop persistent airway inflammation past 21 days. We have previously transferred IFN-γ$^{-/-}$ T cells into Rag$^{-/-}$, using same approach as used in **Figure 1A** with LPR T cells in to the Rag$^{-/-}$and our previous study of FasL-deficient (GLD) T cells into the Rag$^{-/-}$(15). All these mice (IFN-γ$^{-/-}$0 > *Rag*$^{-/-}$, LPR>*Rag*$^{-/-}$, and GLD>*Rag*$^{-/-}$) develop chronic eosinophilic inflammation (15, 16). Since IFN-γ production by T cells is greatly reduced in the absence of Fas expression, these data suggest that IFN-γ plays an important role in the resolution of eosinophilic lung inflammation in our model. It is possible that there is an impaired apoptosis of Ag-specific Th2 cells in the lungs of these mice due to the decrease in IFN-γ induced apoptosis. The association between Fas and IFN-γ signaling leading to the resolution of eosinophilic airway inflammation needs to be carefully dissected to fully understand their relative contributions to allergic disease models.

Here, we focus on uncovering the role of homeostasis proliferation in the development of persistent inflammation observed in LPR> *Rag*$^{-/-}$ mice. However, we did not assess other asthma characteristics such as airway hyper responsiveness (AHR). Our group has previously shown that Fas-deficient T cells, homeostatic proliferation and antigen (using LPR>*Rag*$^{-/-}$ mice) have both persistent eosinophilia and airway hyper responsiveness to methacholine at day 42 (15). Although it is known that the pathways leading from eosinophilic inflammation may not be the same ones that cause changes in lung function (47, 48), data from our group show that both occur together in this chronic inflammation model.

In addition to IFN-γ, the sustained lung eosinophil inflammation observed in our model is also associated with a prolonged capacity of lung T cells to maintain IL-4 production. Mice deficient in the IL-4 gene are defective in their development and maintenance of Th2 cells, as well as their ability to attenuate allergic airway inflammation in murine models (44, 49). Besides IL-4, Fas$^{fl/fl}$lck-cre irradiated mice had increased production of IL-17. These findings are in line with the literature that demonstrates that IFN-γ can inhibit Th17 differentiation (45). Interestingly, it has been shown that IFN-γ facilitates antigen-induced apoptosis of Th17 (50). Thus, in the future could be interesting to evaluate the role of IL-17 in the persistent inflammatory phase of our model.

There is increasing evidence that persistent and non-resolution of allergic inflammation pathways may be related to the poor functioning of pro-resolvins lipid mediators (such as lipoxins, resolvins, protectins, and maresins) (51). These mediators aren't immunosuppressive, but instead support the resolution of ongoing immune responses. For instance, Resolvin E1 (RvE1) is a potent mediator that promotes resolution of inflammatory airway responses. Interestingly, RvE1 regulates the production of IL-17 and IFN-γ by T cells (51). Thus, the role of resolvins in our persistent eosinophilic inflammation models needs to be investigate as RvE1 may be involved in the dysregulation of IFN-γ production by Fas-deficient T cells.

In summary, we have demonstrated that the combination of transient lymphopenia, abnormal Fas-signaling, and antigen exposure leads to the development of a prolonged airway inflammatory phase in our mouse model of experimental asthma. This phase is correlated with decrease levels of IFN-γ in the lung and increases in Th2 cytokines. Taken together, our findings suggest that in humans, transient lymphopenia, as well as features of the host such as genetic polymorphisms in Fas, are likely to significantly influence immune responses in the airways and lungs.

AUTHOR CONTRIBUTIONS

AS, CF, and JW conceived and designed the experiments. CF, JW, CR, JT, and KB performed the experiments. AS, CF, and JW analyzed the data. AS contributed reagents, materials, analysis tools. AS, CF, and JW wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02395/full#supplementary-material

Supplementary Figure 1 | IL-10 did not differ between groups, *Fas*$^{fl/fl}$ *lck-cre* and *Fas*$^{fl/fl}$ *lck* irradiated at 21 days after last challenge. Lung T cells from *Fas*$^{fl/fl}$ *lck-cre* and *Fas*$^{fl/fl}$ *lck* (control mice) irradiated mice at day 21 after the final challenge were -re-stimulated with anti-CD3 measured by Bioplex system as describe in Material and Methods for IL-10. Approximately 4–5 mice per group were analyzed. Error bars represents SEM.

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MINI REVIEW
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Involvement and Possible Role of Eosinophils in Asthma Exacerbation

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Eosinophils are involved in the development of asthma exacerbation. Recent studies have suggested that sputum and blood eosinophil counts are important factors for predicting asthma exacerbation. In severe eosinophilic asthma, anti-interleukin (IL)-5 monoclonal antibody decreases blood eosinophil count and asthma exacerbation frequency. However, even in the absence of IL-5, eosinophilic airway inflammation can be sufficiently maintained by the T helper (Th) 2 network, which comprises a cascade of vascular cell adhesion molecule-1/CC chemokines/eosinophil growth factors, including granulocyte-macrophage colony-stimulating factor (GM-CSF). Periostin, an extracellular matrix protein and a biomarker of the Th2 immune response in asthma, directly activates eosinophils *in vitro*. A major cause of asthma exacerbation is viral infection, especially rhinovirus (RV) infection. The expression of intercellular adhesion molecule (ICAM)-1, a cellular receptor for the majority of RVs, on epithelial cells is increased after RV infection, and adhesion of eosinophils to ICAM-1 can upregulate the functions of eosinophils. The expressions of cysteinyl leukotrienes (cysLTs) and CXCL10 are upregulated in virus-induced asthma. CysLTs can directly provoke eosinophilic infiltration *in vivo* and activate eosinophils *in vitro*. Furthermore, eosinophils express the CXC chemokine receptor 3, and CXCL10 activates eosinophils *in vitro*. Both eosinophils and neutrophils contribute to the development of severe asthma or asthma exacerbation. IL-8, which is an important chemoattractant for neutrophils, is upregulated in some cases of severe asthma. Lipopolysaccharide (LPS), which induces IL-8 from epithelial cells, is also increased in the lower airways of corticosteroid-resistant asthma. IL-8 or LPS-stimulated neutrophils increase the transbasement membrane migration of eosinophils, even in the absence of chemoattractants for eosinophils. Therefore, eosinophils are likely to contribute to the development of asthma exacerbation through several mechanisms, including activation by Th2 cytokines, such as IL-5 or GM-CSF or by virus infection-related proteins, such as CXCL10, and interaction with other cells, such as neutrophils.

Keywords: bronchial asthma, eosinophils, neutrophils, periostin, rhinovirus

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INTRODUCTION

Bronchial asthma is a chronic disease with airway hyperresponsiveness (AHR), reversible airflow limitation, and airway inflammation (1, 2). Asthma is recognized as a heterogeneous disease that has different phenotypes with distinct clinical characteristics, or different endotypes with distinct functional or pathophysiological mechanisms including eosinophilic asthma or non-eosinophilic

asthma (3, 4). Recent studies have suggested that eosinophils play important roles in the development of asthma exacerbation (5–7). Therefore, suppressing eosinophilic inflammation and distinguishing eosinophilic from non-eosinophilic asthma may be useful for the treatment or prevention of asthma exacerbation. In the present review, the involvement and possible role of eosinophils in asthma exacerbation is discussed.

ETHICS STATEMENT

Our studies in this review were approved by the Institutional Review Board of Saitama Medical University Hospital, and written informed consent was obtained from the patients.

ROLE OF EOSINOPHILIC INFLAMMATION IN ASTHMA

Eosinophils, which tend to accumulate at sites of allergic inflammation, contribute to the development of bronchial asthma. They release a number of mediators, including specific granule proteins, such as major basic protein (MBP), radical oxygen species, cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-8, and lipid mediators, such as cysteinyl leukotrienes (cysLTs) (8, 9). However, previous studies investigating the effectiveness of anti-IL-5 monoclonal antibody (mAb) treatment for asthmatics have suggested that eosinophils may only play a small role (10, 11). For example, it has been reported that anti-IL-5 mAb reduces the sputum or blood eosinophil count, but has no effect on histamine-induced AHR or allergen-induced late asthmatic response (10), which suggests that eosinophils do not play a role in the development of AHR or allergen-induced airflow obstruction.

By contrast, the role of eosinophils in the development of airway remodeling has been established at a relatively early phase (12). Eosinophil-deficient mice are reportedly protected from peribronchiolar collagen deposition (13). Eosinophils produce transforming growth factor (TGF)-β (14), which may contribute to airway fibrosis. Additionally, eosinophils can produce cysLTs (15) and be a major cellular source of cysLTs in the airways of individuals with seasonal allergic asthma or aspirin-exacerbated respiratory disease (16, 17), which also contribute to airway remodeling. Anti-IL-5 mAb suppresses airway remodeling (reduction of tenascin, lumican, and procollagen III) as well as airway eosinophils expressing mRNA for TGF-β1 and concentrations of TGF-β1 in the bronchoalveolar lavage (BAL) fluid of asthmatics (18).

As for the role of eosinophils in asthma exacerbation, recent studies have reported that sputum and blood eosinophil counts are important factors for predicting asthma exacerbation (5–7). Treatment for normalizing sputum eosinophil counts can help prevent asthma exacerbation (5), and blood eosinophil counts are associated with exacerbation frequency (6, 7). Furthermore, in severe asthmatic patients with sustained blood or sputum eosinophilia, anti-IL-5 treatment decreases both blood eosinophil counts and asthma exacerbation frequency (19–21). Eosinophil-derived granule products, such as MBP mediate epithelial cell damage, thereby, inducing AHR (22, 23). In accordance with these findings, anti-IL-5 mAb is now prescribed for patients with severe eosinophil-dominant asthma in the clinical setting.

However, even with anti-IL-5 mAb, eosinophils can still accumulate and activate in the airways of asthmatics. One study reported that anti-IL-5 mAb may be insufficient to inhibit eosinophil activation in the airway (24). To accumulate in asthmatic airways, circulating eosinophils needs to adhere to vascular endothelial cells and then migrate over cells, which are regulated primarily by cytokines or chemokines induced by a number of cells, such as T helper (Th) 2 cells (**Figure 1**) (25). The crucial step for selective eosinophil recruitment is likely the adhesion of eosinophils with endothelial cells through the α4 integrin/vascular cell adhesion molecule (VCAM)-1 (25–27). The expression of VCAM-1 in endothelial cells is upregulated by IL-4 and IL-13, after which, blood eosinophils adhere spontaneously to VCAM-1 (28, 29). The interaction of eosinophils with VCAM-1 induces eosinophil superoxide anion (O_2^-) generation and degranulation, and therefore may be the first step of eosinophil activation (28–30).

After adhering to the endothelial cells, CC chemokines, such as eotaxin and regulated upon activation, normal T-cell expressed and secreted (RANTES) effectively induce eosinophil transmigration over endothelial cells expressing VCAM-1 (**Figure 1**) (25, 31). A number of studies have reported an increase in CC chemokines in the airways of asthmatic patients. The airway expression of eotaxin and its receptor, CCR3, are elevated in atopic asthmatics compared with normal controls (32). In patients with acute eosinophilic pneumonia, monocyte chemotactic protein (MCP)-4, which is also a CCR3 ligand, is involved in the development of eosinophil transendothelial migration (33); however, the role of MCP-4 in asthma has yet to be fully clarified.

GM-CSF play an important role in eosinophil activation after migration process, even without IL-5 (**Figure 1**). GM-CSF induces eosinophil O_2^- generation and the release of specific granule proteins *in vitro* when incubated with VCAM-1 or intercellular cell adhesion molecule (ICAM)-1 (29). Furthermore, GM-CSF, but not IL-5, activates eosinophils of airways after segmental allergen challenge (34, 35). These findings suggest that in the absence of IL-5, the Th2 network, which includes a cascade of VCAM-1/CC chemokines/GM-CSF, is likely the primary pathway for maintaining eosinophilic infiltration and activation in asthma (25).

Moreover, cysLTs may be involved in the eosinophil accumulation in the airways of asthma. Inhalation of LTE_4 stimulates the accumulation of eosinophils in asthmatic airways (36). LTD_4 upregulates the β2 integrin expression of human eosinophils and increases eosinophil adhesiveness to ICAM-1 *in vitro* (37). Furthermore, β2 integrin-enhanced adhesion increases the effector functions of eosinophils. Therefore, cysLT-induced β2 integrin activation may be a key process in regard to cell activation in asthmatics (25, 29). In addition, LTD_4 induces eosinophil transendothelial migration, O_2^- generation, and the release of specific granule proteins primarily through β2 integrin

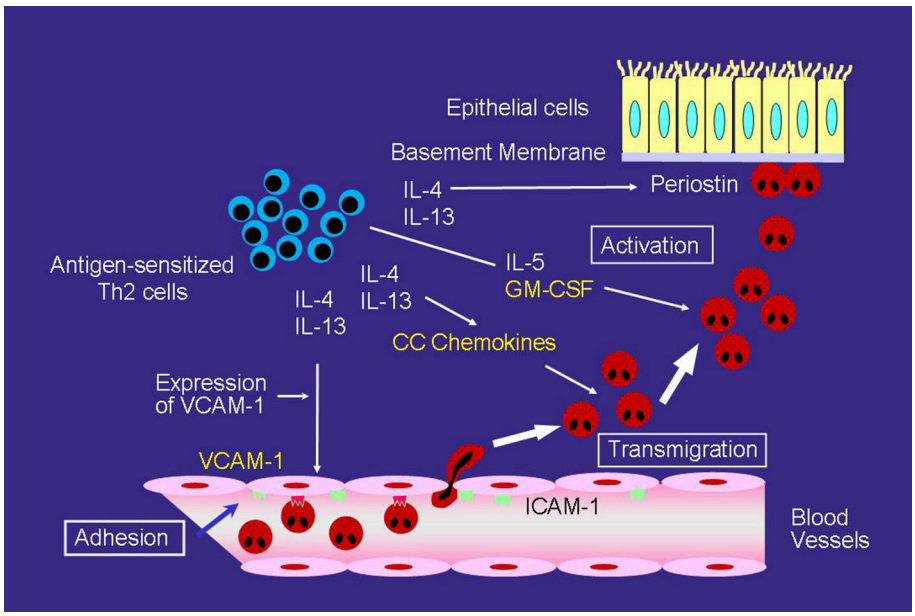

FIGURE 1 | Mechanisms of eosinophilic airway inflammation in bronchial asthma. IL-5 plays an important role in the development of eosinophilic airway inflammation. However, in the absence of IL-5, the Th2 network, which includes a cascade of VCAM-1/CC chemokines/GM-CSF, can maintain eosinophilic infiltration and activation. Yellow text indicates a cascade of VCAM-1/CC chemokines/GM-CSF.

and the cysLT1 receptor (38). Furthermore, leukotriene receptor antagonist (LTRA) suppresses eosinophil airway inflammation *in vivo* (39–41). These findings suggest that cysLTs, along with the Th2 network, contribute to the development and maintenance of airway eosinophilic inflammation in asthma.

Periostin is an extracellular matrix protein that is highly expressed in the airways of asthmatics in response to Th2 cytokines, such as IL-13 (42), and is a biomarker of Th2-mediated immune responses in bronchial asthma (43, 44). Periostin also functions as a matricellular protein (42) that binds to cellular receptors and activates cells, including eosinophils. Periostin directly induces eosinophil adhesion, O_2^- generation, and degranulation through the αMβ2 integrin *in vitro* (45).

INTERACTIONS OF VIRAL INFECTION AND EOSINOPHILS IN THE DEVELOPMENT OF ASTHMA EXACERBATION

Viral infection, especially rhinovirus (RV) infection, is a major cause of asthma exacerbation. In some community-based studies, viral infections have been identified in 80–85% of cases involving asthma exacerbation, and RV was found to be involved in about 65% of the patients in whom the causative virus was identified (46, 47). RVs have tremendous diversity (48). In addition to about 100 classical serotypes of the RV species A (RV-A) and B, over 60 types of RV-C were recently discovered by molecular techniques (48). Recent clinical data suggests that RV-C (49, 50) or RV-C and RV-A (51, 52) can induce more severe illness or asthma exacerbations, compared with other RVs, such as RV-B.

The numbers of not only neutrophils, but also eosinophils, increase in asthmatic airways during or after a viral infection (53–55). Experimental RV infection induces increased recruitment of eosinophils into the airway after segmental allergen challenge in allergic rhinitis patients, but not in non-allergic volunteers (53). Viral infection increases the eosinophil count in the airway epithelium of patients with allergic asthma (55), and high levels of eosinophilic cationic protein are observed in the sputum of asthmatic patients with viral infection (54). Therefore, eosinophils are indeed recruited to and activated in asthmatic airways during or after a viral infection.

Recent studies have suggested that the presence of eosinophil inflammation may be a risk factor for virus-related asthma exacerbation (56, 57). High fractional exhaled nitric oxide and sputum eosinophils are associated with an increased risk of future virus-induced exacerbations (57). Epithelial cells are damaged by eosinophil-derived granule products, such as MBP (23), and this increases the susceptibility to RV infection (**Figure 2**) (58). Furthermore, eosinophils can suppress the RV-induced expression of interferons (IFNs), anti-viral cytokines, including IFN-λ from epithelial cells, likely through the production of TGF-β, resulting in an increased quantity of RV (**Figure 2**) (56). Therefore, reducing the eosinophil count could be a reasonable strategy for suppressing virus-induced asthma exacerbation.

ICAM-1 is a cellular receptor for the majority of RV-A (major) and all of RV-B (59), and RV infection increases ICAM-1 expression on epithelial cells (60). ICAM-1 is also an adhesion molecule, and adhesion of eosinophils to ICAM-1 can activate the functions of eosinophils (28, 29). Therefore, eosinophil adhesion to epithelial cells via ICAM-1 may activate eosinophils during

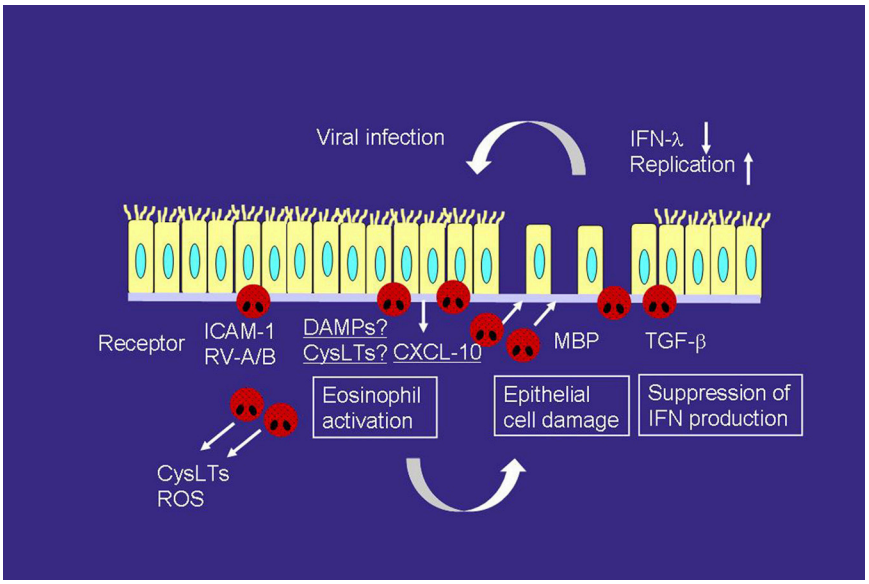

FIGURE 2 | Interactions of viral infection and eosinophils in the development of asthma exacerbation. RV infection releases a variety of mediators, including CXCL-10 and cysLTs, which can directly activate eosinophils and induce asthma exacerbation, from airway epithelial cells. RV infection increases the expression of ICAM-1, which also activates eosinophils. On the other hand, activated eosinophils release MBP, which induces epithelial cell damage. Eosinophils also produce TGF-β, which can suppress the expression of IFN-λ, anti-viral cytokines, on epithelial cells. Therefore, eosinophilic airway inflammation can increase susceptibility to RV infection. ROS, reactive oxygen species; TGF, transforming growth factor.

RV-induced asthma exacerbation (**Figure 2**). Cadherin-related family member 3 (CDHR3) is a recently found receptor for RV-C (61). In this context, a coding single nucleotide polymorphism (SNP) in CDHR3 has been shown to be related to the severe exacerbation of childhood asthma (62). Moreover, this SNP has been reported to increase the expression of the CDHR3 protein on the cell surface (61, 62), resulting in increased RV-C binding and progeny yields (61). Because the cadherin family members are involved in cell adhesion, eosinophil adhesion to CDHR3 may activate eosinophil functions in a manner similar to that as ICAM-1.

CXCL10 may also play a role in the virus-induced asthma exacerbation (**Figure 2**). RV infection produces CXCL10 from bronchial epithelial cells *in vitro* and *in vivo* (63). Specifically, concentrations of serum CXCL10 are elevated in virus or RV-induced asthma; correlations are reported between higher levels of CXCL10 and disease severity, including airflow limitation (63). CXCL10 induces eosinophil adhesion, O_2^- generation, eosinophil-derived neurotoxin release, and cytokine production through CXCR3, expressed on eosinophils, *in vitro* (64). As for other CXCR3 ligands, CXCL9 is involved in severe asthma (65) and produced from epithelial cells by RV infection (66). CXCL9 induces eosinophil adhesion, O_2^- generation, and eosinophil-derived neurotoxin release *in vitro* (64), whereas it inhibits eosinophil chemotaxis through a CCR3-dependent mechanism (67, 68).

CysLTs are upregulated in the asthmatic airways during virus or RV-induced exacerbation (69, 70). Respiratory syncytial virus (RSV) induces LTC_4 synthase expression on bronchial epithelial cells (71). Therefore, cysLTs are likely to be involved in virus-induced eosinophilic inflammation (**Figure 2**), and LTRA may be useful for virus-induced asthma treatment. The LTRA montelukast suppresses the respiratory symptoms of RSV bronchiolitis (72) as well as the frequency of virus-induced asthma exacerbation (73). Moreover, montelukast inhibits eosinophil adhesion induced by CXCL10 and ICAM-1 *in vitro* (74), both are virus-infection-related proteins.

Innate immune responses play roles in the development of eosinophilic airway inflammation; this process involves type 2 innate lymphoid cells (ILC2) as well as epithelial cell-related cytokines including IL-33, IL-25, and thymic stromal lymphopoietin, (75, 76). The ILC2 stimulated by these cytokines produce IL-5 and IL-13 and induce eosinophilic inflammation. In fact, ILC2 are upregulated in severe asthmatics (77). Recent studies have suggested that innate immune responses contribute to virus-induced asthma exacerbation. For example, IL-33-dependent type 2 inflammation plays an important role in RV-induced asthma exacerbation *in vivo* (78).

During viral infections, damage-associated molecular pattern molecules (DAMPs) can be released by stressed or damaged cells, and function as endogenous danger signals (79). Damaged epithelial cells are capable of inducing eosinophilic migration, *specific granule protein release*, and cytokine production, likely via the release of DAMPs (80). Uric acid (UA) or adenosine triphosphate (ATP), an important DAMP, activates eosinophil functions *in vitro* (81, 82); however, the role of DAMPs in the development of asthma exacerbation remains unclear.

INTERACTIONS OF NEUTROPHILS AND EOSINOPHILS IN THE DEVELOPMENT OF SEVERE ASTHMA OR ASTHMA EXACERBATION

Both neutrophilic and eosinophilic inflammation may play roles in severe asthma [(83–85)]. Neutrophilic inflammation has been shown to be involved in the pathogenesis of asthma exacerbation (86), which occurs frequently in severe asthma. The European Network for Understanding Mechanisms of Severe Asthma (ENFUMOSA) study suggested that compared with patients with mild-to-moderate asthma, those with severe asthma have both a greater sputum neutrophil count and an increased release of eosinophil-derived mediators (84). IL-8 plays an important role in the accumulation of neutrophils in inflammation sites, and IL-8 expression is upregulated in the airways of severe asthmatic patients (87, 88). In addition, we reported that neutrophils that had migrated to IL-8 induce the transbasement membrane migration of eosinophils *in vitro*, even without eosinophil chemoattractants (89); this neutrophil-induced eosinophil migration is suppressed by LTB_4 antagonist or platelet-activating factor (PAF) antagonist. LTB_4 and PAF are potent chemotactic factors for eosinophils (90, 91); therefore, IL-8-stimulated neutrophils can lead to eosinophil accumulation in asthmatic airways through LTB_4 or PAF (92).

Lipopolysaccharide (LPS) may play a role in inducing IL-8 or neutrophilic inflammation in the airway of severe asthmatics. In the BAL fluid of asthmatic children, LPS levels correlate with airway neutrophils or IL-8 (93). Furthermore, the BAL fluid LPS and genes associated with LPS signaling activation are higher in corticosteroid-resistant asthma (94). Furthermore, a positive correlation is observed between IL-8 mRNA expression in BAL cells and the amount of LPS in BAL fluid (94). Several studies have suggested that Gram-negative bacteria or house dust plays a role in the LPS upregulation in the airways of severe asthmatics. We previously reported that LPS-stimulated neutrophils induce the transbasement membrane migration of eosinophils *in vitro* (95).

IL-17 is another candidate for the upregulation of IL-8 expression (96). Sputum IL-17 concentration correlates with the clinical severity of asthma (97), and the airway expression of IL-17 is increased in severe asthmatics only (98). Furthermore, a correlation between the number of bronchial cells that produce IL-17 and the number of bronchial neutrophils and frequency of asthma exacerbation has been reported (86). In addition, we reported that the dopamine D1-like receptor antagonist attenuates the Th17-mediated immune response and neutrophilic airway inflammation in mice (99) this could be reasonable strategy for controlling neutrophilic airway inflammation in severe asthma or asthma exacerbation.

The role of neutrophil extracellular traps (NETs) in the pathogenesis of asthma exacerbation has recently been highlighted. In a mouse model, RV infection triggered a double-stranded DNA (dsDNA) release that was associated with the formation of NETs; this is known as NETosis (100). Furthermore, in humans, a significant correlation is identified between the release of host dsDNA after RV infection and the exacerbation of type-2 allergic inflammation (100).

ROLE OF MAST CELLS AND PROSTAGLANDIN (PG) D_2 IN EOSINOPHILIC INFLAMMATION OF ASTHMA

Mast cells also play roles in the development of severe asthma. Mast cell numbers and PGD_2 concentrations are increased in the lower airway of patients with severe asthma (101, 102). Mast cells are major cellular sources of PGD_2, and D prostanoid (DP) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) are receptors for PGD_2 (103). Recently, the role of CRTH2 in the pathogenesis of asthma has been highlighted. CRTH2 is expressed on Th2 cells, ILC2, eosinophils, and basophils (103). PGD_2 induces chemotaxis in eosinophils through CRTH2 (104), and CRTH2 antagonist suppresses eosinophil chemotaxis and respiratory burst (105). CRTH2 antagonists are already being developed (103, 106), and clinical trial data suggest that CRTH2 antagonists may target eosinophilic asthma (103, 107).

CONCLUSION

Eosinophils are likely to contribute to the development of asthma exacerbation. This process can involve cytokines, such as IL-5 or GM-CSF, chemokines, such as CCR3 ligands, matricellular proteins, a danger signal, and other cells, such as neutrophils or mast cells.

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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HFA-BDP Metered-Dose Inhaler Exhaled Through the Nose Improves Eosinophilic Chronic Rhinosinusitis With Bronchial Asthma: A Blinded, Placebo-Controlled Study

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Background: Eosinophilic chronic rhinosinusitis (ECRS) is a subtype of chronic rhinosinusitis with nasal polyps in Japanese. ECRS highly associated with asthma is a refractory eosinophilic airway inflammation and requires comprehensive care as part of the united airway concept. We recently reported a series of ECRS patients with asthma treated with fine-particle inhaled corticosteroid (ICS) exhalation through the nose (ETN).

Objective: To evaluate fine-particle ICS ETN treatment as a potential therapeutic option in ECRS with asthma.

Methods: Twenty-three patients with severe ECRS under refractory to intranasal corticosteroid treatment were randomized in a double-blind fashion to receive either HFA-134a-beclomethasone dipropionate (HFA-BDP) metered-dose inhaler (MDI) ETN ($n = 11$) or placebo MDI ETN ($n = 12$) for 4 weeks. Changes in nasal polyp score, computed tomographic (CT) score, smell test, and quality of life (QOL) score from baseline were assessed. Fractionated exhaled nitric oxide (FENO) was measured as a marker of eosinophilic airway inflammation. Response to corticosteroids was evaluated before and after treatment. Additionally, deposition of fine-particles was visualized using a particle deposition model. To examine the role of eosinophils on airway inflammation, BEAS-2B human bronchial epithelial cells were co-incubated with purified eosinophils to determine corticosteroid sensitivity.

Results: HFA-BDP MDI ETN treatment improved all assessed clinical endpoints and corticosteroid sensitivity without any deterioration in pulmonary function. FENO and blood eosinophil number were reduced by HFA-BDP MDI ETN treatment. The visualization study suggested that ETN at expiratory flow rates of 10–30 L/min led to fine particle deposition in the middle meatus, including the sinus ostia. Co-incubation of eosinophils with BEAS-2B cells induced corticosteroid resistance.

Conclusions: Additional HFA-BDP MDI ETN treatment was beneficial in patients with ECRS and should be considered as a potential therapeutic option for eosinophilic airway inflammation such as ECRS with asthma. (UMIN-CTR: R000019325) (http://www.umin.ac.jp/ctr/index.htm).

Keywords: airway medicine, asthma, eosinophilic chronic rhinosinusitis, exhalation through the nose, inhaled corticosteroid, united airway

INTRODUCTION

Eosinophilic chronic rhinosinusitis (ECRS) characterized by ethmoid-predominant sinusitis with eosinophilic inflammation is a refractory airway disease categorized as a subgroup of chronic rhinosinusitis with nasal polyps (1, 2). With an increasing incidence, ECRS has been officially designated as intractable disease in Japan. Approximately half of the patients with ECRS have bronchial asthma (3), which is a risk factor of relapse after endoscopic sinus surgery (4). Furthermore, in those with severe ECRS, asthma is significantly associated with ECRS in more than 80% of cases (4, 5). Conversely, a recent report suggests that more than 50% of patients with severe asthma also had ECRS (6). Thus, ECRS should be recognized as an eosinophilic airway inflammation extending to the lower airway and treated concurrently with asthma based on the united airway concept. We recently reported on the efficacy of "airway medicine" for ECRS with asthma (7–10), where "airway medicine" refers to comprehensive care of the upper and lower airway using fine-particle inhaled corticosteroids (ICS). Fine particles travel toward the upper airway during inhalation through the mouth followed by exhalation through the nose (ETN) (7), suggesting that these fine particles could be delivered not only to the lower airway but also to the inflammatory sites in the upper airway, such as the middle meatus to which the ethmoid sinus opens. However, these reports included retrospective evaluation as a case series, and the ETN methods were inconsistent. In some cases, knowledge of the effectual flow conditions for ETN treatment improved sinusitis without any changes in treatment. Therefore, we conducted a blinded, placebo-controlled study with a consistent ETN method to confirm the beneficial effect of fine-particle ICS ETN treatment for ECRS with asthma.

MATERIALS AND METHODS

Subjects

The target population for this study was geared for patients with severe ECRS refractory to intranasal corticosteroid treatment who were referred to our department for surgical treatment. All subjects had bronchial asthma. ECRS and bronchial asthma were diagnosed according to the Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis (4) and the Global Initiative for Asthma guidelines (11), respectively. Twenty-three patients were randomized in a double-blinded fashion to receive either 800 μg of HFA-BDP metered-dose inhaler (MDI) ETN ($n = 11$) or the same volume of placebo MDI ETN ($n = 12$) for 4 weeks, in addition to current therapy, which was not changed. All subjects had already stopped treatment with intranasal corticosteroids at the point of entry into the study. At the start of treatment, the method of MDI ETN was explained to all subjects as follows. Fine particles released from MDI were inhaled orally for 3 s using a valved holding chamber; the subjects then held their breath for 3 s and performed ETN for 3 s. The method of MDI ETN was based on a preliminary study in which the flow conditions of the ETN treatment was evaluated (**Supplementary Material**). We confirmed the subjects' technique and flow conditions under ETN treatment using a spirometer (CHESTGRAPH HI-105, Chest M.I., Tokyo, Japan) with a facemask (LiteTouch VHC Mask, Philips, Amsterdam, Netherlands). Patients covered their mouth and noses with the facemask (**Supplementary Figure 1A**), inhaled through the mouth, and exhaled through the nose in the manner they would when using normal ETN treatment. The effectual flow-volume curve (**Supplementary Figure 1B**) was obtained from each patient.

Changes from baseline in nasal polyp score (12), computed tomographic (CT) score defined by the Lund-Mackay scale (13), smell test using odor stick identification test for Japanese (OSIT-J) (14), and quality of life (QOL) score [Sino-nasal outcome test-22 (SNOT-22); The Washington University in St. Louis, Missouri (15) and Asthma Control Test (ACT) (16)] were assessed. Fractionated exhaled nitric oxide (FENO) was measured as a marker of eosinophilic airway inflammation. Response to corticosteroids was evaluated before (visit 1) and after treatment (visit 2). This study was approved by the local ethics committee of Kansai Medical University (approval number: KANIRIN1502) and registered in the University Hospital Medical Information Network in Japan (UMIN-CTR: R000019325).

Cell Preparation

Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque PLUS® (GE Healthcare, Uppsala, Sweden). Eosinophils (purity > 98%) were isolated from the peripheral blood of healthy volunteers with mild eosinophilia (∼4–8% of total white blood cells) by negative selection using a MACS system with Eosinophil Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The human bronchial epithelial cell line BEAS-2B was obtained from the European Collection of Authenticated Cell Culture (Salisbury, UK).

Quantitative RT-PCR

Total RNA extraction and reverse transcription were performed using a PureLink RNA Micro kit (Invitrogen, Carlsbad, CA) and a PrimeScript RT MasterMix (Perfect Real Time;

Abbreviations: ECRS, eosinophilic chronic rhinosinusitis; ESS, endoscopic sinus surgery; ETN, exhalation through the nose; ICS, inhaled corticosteroid.

Takara Bio, Shiga, Japan). Gene transcript levels of FK506-binding protein 51 (*FKBP51*), *CXCL8*, protein phosphatase 2 catalytic subunit alpha isozyme (*PPP2CA*), and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were quantified by real-time PCR using a Rotor-Gene SYBR Green PCR kit (Qiagen, Hilden, Germany) on a Rotor-Gene Q HRM (Corbett Research, Cambridge, UK). Amplification primers (5′-3′) were: *FKBP51* (NM_004117), forward (F)—CAG CTG CTC ATG AAC GAG TTT G, reverse (R)—GCT TTA TTG GCC TCT TCC TTG G; *CXCL8* (NM_ 000584.3), forward (F)—ACT GAG AGT GAT TGA GAG TGG AC, reverse (R)—AAC CCT CTG CAC CCA GTT TTC; *PPP2CA* (NM_ 002715), forward (F)—CGC CAT TAC AGA GAG CCG AG, reverse (R)—TAC TTC TGG CGG CTG GTT GAG; and *GAPDH* (NM_002046), F—TTC ACC ACC ATG GAG AAG GC, R—AGG AGG CAT TGC TGA TGA TCT.

Corticosteroid Sensitivity

PBMCs were treated with dexamethasone for 45 min, followed by TNFα (10 ng/ml) stimulation overnight. The ability of dexamethasone to inhibit TNFα-induced CXCL8 release was determined in cell medium by sandwich ELISA according to the manufacturer's instructions (R&D Systems). IC_{50} of dexamethasone on CXCL8 production (Dex-IC_{50}), calculated using Prism® 6.0 statistical software (GraphPad, San Diego, CA), was used as a marker for corticosteroid sensitivity. In addition, BEAS-2B cells were co-incubated with purified eosinophils overnight. After removal of eosinophils, BEAS-2B cells were treated with dexamethasone (10^{-7} M) for 45 min, followed by co-stimulation with TNFα (10 ng/ml) for 4 h. The ability of dexamethasone to enhance *FKBP51* and inhibit TNFα-induced *CXCL8* levels were evaluated by RT-PCR.

In-cell Western Assay

PBMCs fixed with 4% formaldehyde for 20 min were permeabilized and blocked. Cells were incubated with primary antibodies (rabbit polyclonal antibody to phospho-glucocorticoid receptor (GR) Ser^{226}; Abcam, Cambridge, UK and the mouse monoclonal antibody to GR; Santa Cruz Biotechnology, Dallas, TX) and the fluorescently-labeled secondary antibodies (IRDye 800CW goat anti-rabbit and IRDye 680RD goat anti-mouse; LI-COR Bioscience, Lincoln, NE). Ratio of fluorescence intensity of phospho-GR Ser^{226} to that of GR was analyzed by Odyssey infrared imaging system (LI-COR) according to the manufacturer's instructions.

Cell Survival

Viability of purified eosinophils was evaluated using double staining with annexin V and 7-Amino-Actinomycin D (7-AAD) (BD Pharmingen, Franklin Lakes, NJ). In some experiments, eosinophils were incubated with of IL-5 (1 ng/ml). Caspase-3 activity in purified eosinophils was assayed with a Caspase-3 assay kit (BioVision, Milpitas, CA) according to the manufacturer's instructions.

Evaluation of Deposited Particles

Deposited particles were visualized in a particle deposition model using a human nasal cavity cast. Briefly, fine particles (JIS Test Powders 1, No. 11; The Association of Powder Process Industry and Engineering, Japan) with an average diameter of 2.13 μm placed in the pharyngeal side were suctioned from the external naris site under constant pressure to mimic the ETN process. In addition, in contrast to the above-mentioned method, fine particles placed in the external naris were suctioned from the pharyngeal side. Particle deposition was evaluated under direct vision.

Statistical Analysis

Comparisons of two datasets were performed using the Mann–Whitney *U*-test, Wilcoxon matched-pairs signed rank test, or Fisher's exact test as appropriate. Other data were analyzed by analysis of variance with *post-hoc* Bonferroni test adjusted for multiple comparisons. Differences were considered statistically significant if p was <0.05. Descriptive statistics were expressed as means ± standard deviation (**Figure 1** and **Table 1**) or means ± standard error of the mean (**Figure 5**).

RESULTS

Patient Characteristics

Baseline characteristics of the study patients are summarized in **Table 1**. There were no differences in any of the characteristics including the condition and severity of ECRS with asthma between the groups. Other medication use was similar between the two groups.

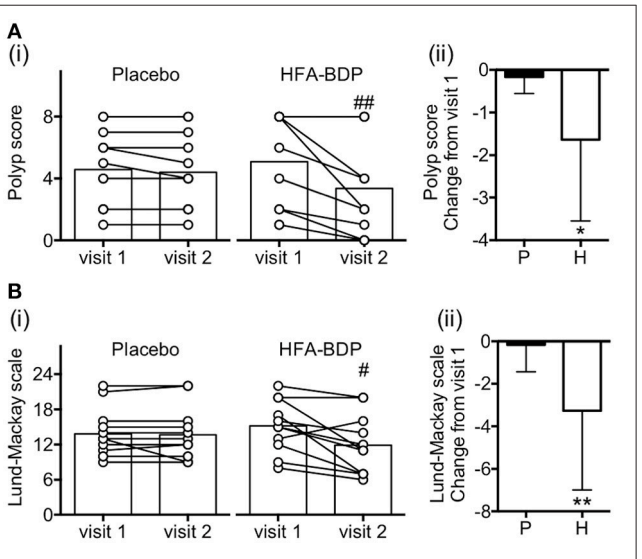

FIGURE 1 | Effect of HFA-BDP MDI ETN on nasal polyp score and sinus CT findings. Nasal polyp score **(A)** and sinus CT score **(B)** were evaluated before (visit 1) and 4 weeks after treatment (visit 2). Individual values and means of 12 patients in the placebo group (P; **A** [i]) and 11 patients in the HFA-BDP group (H; **B** [i]) are shown. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (vs. visit 1). Changes from visit 1 to visit 2 were also compared between two groups (**A** [ii] and **B** [ii]). Data represent mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$ (vs. placebo).

TABLE 1 | Baseline characteristics of eosinophilic chronic rhinosinusitis patients with bronchial asthma.

	Placebo (n = 12)	HFA-BDP (n = 11)
Age	50.1 ± 11.3	53.4 ± 14.3
Gender (M/F)	7/5	3/8
JESREC score	14.8 ± 1.6	15.6 ± 1.6
Severity of asthma (mild/moderate/severe)	8/2/2	5/4/2
NSAIDs intolerance	1	2
Smoking history (never/ex-smoker)	7/5	10/1
ESS history (Y/N)	4/8	2/9
Total IgE (IU/ml)	233 ± 254	280 ± 219
Eosinophils (/μL) [%]	469 ± 110 [7.6 ± 2.0]	581 ± 173 [9.5 ± 3.2]
FENO (ppb)	63.6 ± 61.5	77.6 ± 51.6
LMS (total/ethmoid)	13.8 ± 4.2/5.3 ± 1.6	15.2 ± 4.5/5.7 ± 1.5
Polyp score	4.6 ± 2.5	5.1 ± 3.1
FEV_1 %pred.	84.0 ± 20.0	88.7 ± 17.9
FEF_{25-75} %pred.	49.1 ± 20.2	57.5 ± 24.9
FVC %pred.	99.5 ± 17.4	100.6 ± 14.4
ACT	23.0 ± 3.1	22.7 ± 3.1
SNOT-22	41.2 ± 21.8	33.7 ± 18.3
OSIT-J	3.1 ± 4.3	3.9 ± 4.5
TREATMENT		
ICS (μg)	150 ± 207	250 ± 314
LABA	5	4
LTRA	3	4
Anti-histamine	3	4
Theophylline	2	3
Macrolide	2	1

ACT, Asthma Control Test; ESS, endoscopic sinus surgery; FENO, fractionated exhaled nitrogen oxide; FEV_1, forced expiratory volume in 1 s; FEF_{25-75}, forced expiratory flow between 25% and 75% of vital capacity; FVC, forced vital capacity; ICS, inhaled corticosteroid (equivalent doses of fluticasone propionate); JESREC, Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis; LABA, long-acting β_2-agonist; LAMA, long-acting muscarinic antagonist; LMS, Lund-Mackay scale; LTRA, leukotriene receptor antagonist; SNOT-22, Sino-Nasal Outcome Test-22. Values are number of subjects and mean ± standard deviation.

HFA-BDP MDI ETN Reduces Nasal Polyps and Sinus Opacification

After 4 weeks of treatment, the nasal polyp score was significantly improved in 8 of the 11 patients receiving HFA-BDP, compared with two of twelve patients receiving placebo (**Figure 1A**). Sinus CT findings evaluated by Lund-Mackay scale was also improved after HFA-BDP treatment compared with placebo (**Figure 1B**). Conversely, there was a significant improvement in the smell test in both groups (data not shown).

HFA-BDP MDI ETN Improves Clinical Markers of Airway Inflammation Concomitant With Subjective Evaluation

HFA-BDP MDI ETN had a beneficial effect on FENO, a clinical marker of airway eosinophilic inflammation and blood eosinophils, as well as on the findings associated with sinusitis (**Figures 2A,B**). In addition, reflecting the improvement in clinical findings, subjective evaluation by SNOT-22 and ACT improved significantly in the HFA-BDP group (**Figures 2C,D**). Regarding pulmonary function, there were no significant improvements in the indicators of airway obstruction such as %FEV1 and $\%FEF_{25-75}$ (data not shown).

HFA-BDP MDI ETN Leads to Restoration of Corticosteroid Sensitivity

The beneficial effect of HFA-BDP MDI ETN was further confirmed by a significant reduction in Dex-IC_{50} from 346.2 ± 443.6 nM before treatment to 45.9 ± 93.0 nM after treatment (**Figure 3A**). Supporting these findings, HFA-BDP MDI ETN reduced the increase in phosphorylation of GR at Ser^{226}, a biomarker of GR inactivation (17, 18) (**Figure 3B**).

Potential of Fine Particle Deposition in Paranasal Sinus Ostium

In the particle deposition model using a human nasal cavity cast, we found that fine particles flowing from the pharynx to the external naris at flow rates of 10–30 L/min were deposited in the middle meatus where the sinus ostia are located, although the deposition was less in the epipharynx area (**Figure 4A**). Conversely, fine particles flowing from the external naris to the pharynx at the same flow rates were deposited mainly in the nasal vestibule and epipharynx and not in the middle meatus (**Figure 4B**).

Coexistence of Eosinophils Reduces Corticosteroid Sensitivity

Survival of eosinophil was prolonged when the cells were co-incubated with BEAS-2B cells, with a reduction in caspase 3 activity (**Figures 5A,B**). More importantly, co-existence of eosinophils significantly reduced the ability of dexamethasone to enhance *FKBP51* mRNA expression and inhibit TNFα-induced *CXCL8* mRNA expressions in BEAS-2B cells (**Figures 5C,D**). In line with the observed corticosteroid insensitivity, coexistence of eosinophils also reduced the mRNA expression of *PPP2CA*, a serine/threonine phosphatase PP2A catalytic subunit that plays an important role in the regulation of its complexes and activity (**Figure 5E**) (19).

DISCUSSION

The current study revealed that additional HFA-BDP MDI ETN treatment was beneficial in ECRS patients with bronchial asthma. A potential explanation is that fine-particle ICSs could be delivered by ETN not only to the lower airway but also to the upper airway sites where inflammation exists, such as the middle meatus to which the ethmoid sinus opens, supporting our findings in earlier studies (7, 9).

Although the efficacy of intranasal corticosteroids for ECRS patients was previously indicated (20, 21), their effect has been considered as incomplete and transient (22). We previously attempted to simulate fine-particles deposition using computed fluid dynamics (CFD) analysis in a three-dimensional

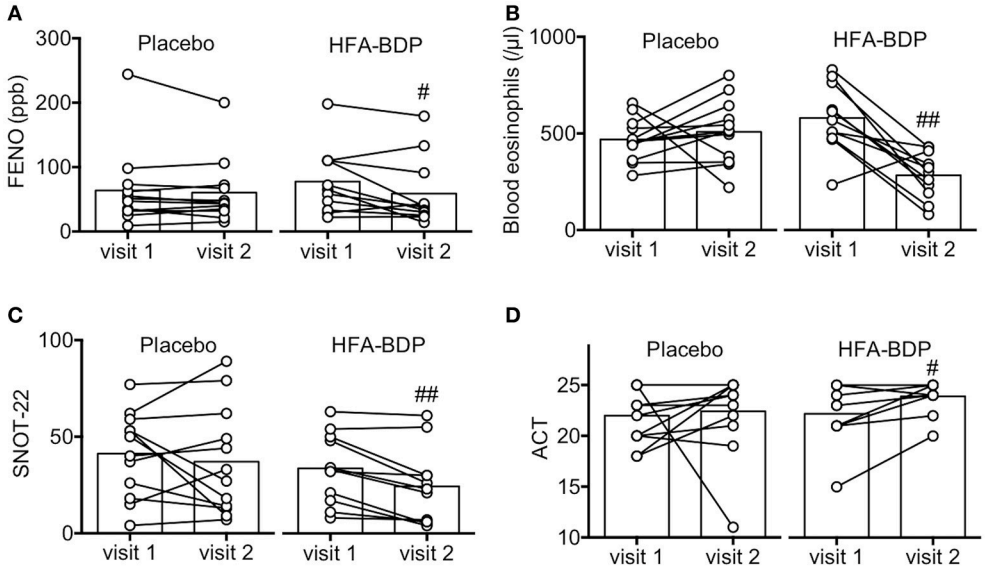

FIGURE 2 | Effect of HFA-BDP MDI ETN on eosinophilic airway inflammation. Fractionated exhaled nitric oxide (FENO) **(A)** and blood eosinophil count **(B)** were measured as markers of eosinophilic inflammation before (visit 1) and 4 weeks after treatment (visit 2). Sino-nasal outcome test-22 (SNOT-22) **(C)** and asthma control test (ACT) **(D)** were also evaluated as QOL questionnaire. Individual values and means of 12 patients in the placebo group and 11 patients in the HFA-BDP group are shown. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (vs. visit 1).

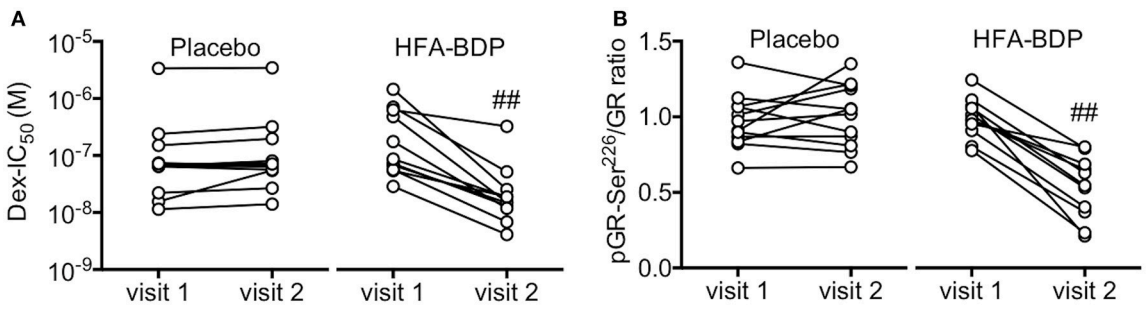

FIGURE 3 | Effect of HFA-BDP MDI ETN on corticosteroid sensitivity. IC_{50} values for dexamethasone on TNFα-induced CXCL8 production (Dex-IC_{50}) **(A)** and phosphorylation levels of GR-Ser^{226} **(B)** in PBMCs were measured before (visit 1) and 4 weeks after treatment (visit 2) as markers of corticosteroid sensitivity and GR inactivation, respectively. Individual values of 12 patients in the placebo group and 11 patients in the HFA-BDP group are shown. $^{\#\#}P < 0.01$ (vs. visit 1).

anatomically accurate and subject-specific model reconstructed from CT data (9, 23): CFD analysis with flow rates of 30 L/min from the pharynx to the external nares revealed that fine particles (1 μm) were deposited in the nasal cavity and reached the ethmoid sinus area to a certain extent, with ∼10% of the fine particles deposited in the upper airway, even in the model without endoscopic sinus surgery (ESS) (**Supplementary Table 2** and **Supplementary Video 1**). Further, we confirmed that the fine particles released by HFA-BDP MDI at least partially (30–50%) flowed out through the external nares (**Supplementary Figure 2** and **Supplementary Video 2**) and the possibility that fine particles might be deposited in the nasal cavity at flow rates between 15 and 30 L/min. Conversely, CFD analysis with flow from the external nares to the pharynx showed only 3% deposition on the ethmoid sinus area. This finding is supported by a previous report by Hyo et al. who reported that only 3% of the small particles in the μm range reached the paranasal sinuses (24) and by our particle deposition model with a human nasal cavity cast in the current study (**Figure 4**). This may account for clinical effect of fine-particle ICS ETN for severe sinusitis which is refractory to intranasal corticosteroid treatment (7, 9).

After a 4-week double-blinded period, 2 of the 12 patients in the placebo group dropped out of this study. One patient had an exacerbation of asthma, and the other patient quit follow-up at our hospital due to deterioration of ECRS. The remaining 21 patients received HFA-BDP MDI ETN treatment after the end of the double-blinded period and were followed for at least 1 year. Although all patients were referred to our department for surgical treatment, HFA-BDP MDI ETN treatment provided

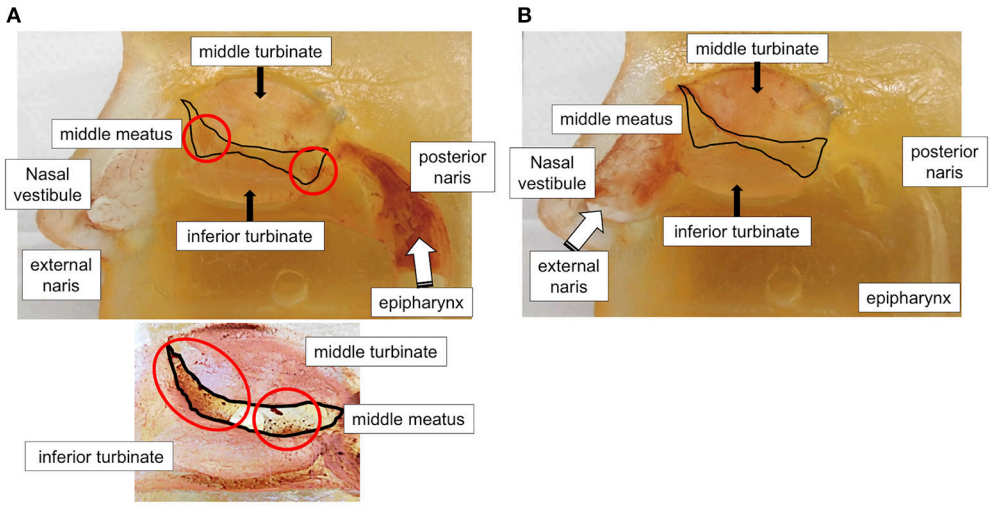

FIGURE 4 | Evaluation of fine particle deposition in the nasal cavity. Visualization of fine particles in a particle deposition model using a human nasal cavity cast. Flow of fine particles from the pharynx to the external naris **(A)** and from the external naris to the pharynx **(B)** were evaluated. Lower panel in **(A)** shows the magnified view from another angle. White arrows, block circles, and brown dots and patches in red circles indicate flow direction, middle meatus area, and deposited particles, respectively.

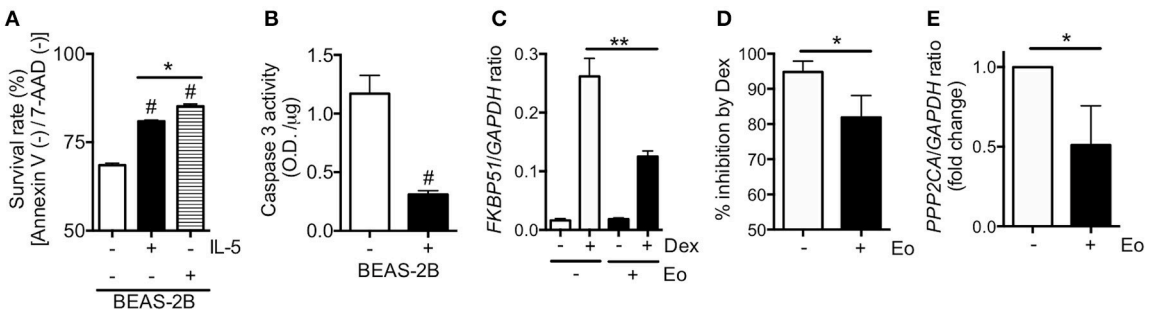

FIGURE 5 | Impact of eosinophil co-incubation on corticosteroid sensitivity. Purified eosinophils were incubated with or without BEAS-2B cells overnight. Eosinophil viability **(A)** and caspase 3 activity in eosinophils **(B)** were evaluated. BEAS-2B cells were incubated with or without purified eosinophils overnight. The ability of dexamethasone (Dex, 10^{-7} M) to enhance *FKBP5* **(C)** and inhibit TNFα-induced *CXCL8* **(D)** mRNA levels were evaluated. *PPP2CA* mRNA levels **(E)** were also measured. Data represent means of three **(A,B)** or four **(C–E)** experiments ± standard error of the mean. $^{\#}P < 0.05$ (vs. non-treatment control), $^{*}P < 0.05$, $^{**}P < 0.01$ (as shown between two groups).

prolonged, good control in 10 of the 21 patients with no need for surgery. Thus, fine-particle ICS ETN treatment provided long-term benefit in severe ECRS patients with asthma. A recent large-scale study in Japan reported that ∼60% of patients with severe ECRS experienced recurrence within 3 years after ESS (4). However, at our department, comprehensive care with a fine-particle ICS MDI ETN treatment reduced the recurrence rate up to 30% (data not shown).

Individuals with refractory eosinophilic airway inflammation might exhibit corticosteroid resistance, as indicated in a previous report (25). We also found that the response to corticosteroids was reduced in ECRS patients with asthma (9). Further, as shown in **Figure 5**, co-incubation of eosinophils with bronchial epithelial cells prolongs eosinophil survival and reduces corticosteroid sensitivity, concomitant with PP2A impairment. PP2A can regulate corticosteroid response by dephosphorylation of GR at Ser^{226} (17). Taken together, these findings suggest that inhibition of eosinophilic airway inflammation by HFA-BDP MDI ETN treatment could reduce phosphorylation levels of GR at Ser^{226}, resulting in the restoration of corticosteroid sensitivity (**Figure 3B**). Conversely, we should consider that the effects of ETN treatment with fine-particle ICS alone, including HFA-BDP, depend on the severity of disease. For some patients with severe ECRS and asthma, ICS/LABAs are required to restore corticosteroid sensitivity (9, 26).

The current study evaluated the additional effect of HFA-BDP MDI ETN treatment which might be a potential therapeutic option for eosinophilic airway inflammation such as ECRS with asthma. Although this randomized, placebo-controlled study was

conducted in a double-blind fashion, the single-center design with a small sample size is a major limitation. A multicenter clinical trial with large sample size is recommended to confirm the utility of this novel option for airway medicine based on the concept of united airway.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Ministry of Health Labour and Welfare. The protocol was approved by the medical ethics committee of Kansai Medical University. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

YK, TY, MA, HY, and KT were involved in the conception and design of this study. YK and MA enrolled patients. YK, HT, TY, and AK conceived the experiments. YK, HT, TY, and AK were involved in analysis and interpretation of the data. YK, HI, HY, and KT were involved in drafting the manuscript for important intellectual content. All authors contributed to revisions and approved the final draft.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02192/full#supplementary-material

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ORIGINAL RESEARCH
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Eotaxin-3 as a Plasma Biomarker for Mucosal Eosinophil Infiltration in Chronic Rhinosinusitis

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Objective: Chronic rhinosinusitis with nasal polyps exhibits marked eosinophilic infiltration and its mucosal eosinophilia is associated with more severe symptoms. The Japanese epidemiological survey of refractory eosinophilic chronic rhinosinusitis found that patients with nasal polyps required multiple surgeries when there were higher infiltrating eosinophils in the mucosa. In order to identify plasma biomarkers for local eosinophil infiltration in rhinosinusitis for surgery, we examined the levels of molecules in the plasma of patients and compared the number of infiltrating eosinophils in the nasal mucosa.

Materials and Methods: Mucosal tissues from 97 patients with chronic rhinosinusitis (CRS) were obtained from the nasal polyps during surgery. Tissues were immediately fixed and sections were stained with hematoxylin-eosin. The number of eosinophils in the mucosa was counted at HPF (x 400). Blood samples were obtained and the plasma was stored at −80°C. We measured the plasma cytokine and chemokine levels using multiple assay systems according to the manufacturers' protocols. The tissues were divided into high- and low-eosinophil mucosal infiltration group for recurrence after endoscopic sinus surgery (ESS). We also observed chemokine secretion from nasal fibroblasts.

Results: The plasma level of eotaxin-3/ CC chemokine ligand 26 (CCL26) was significantly higher in the high-eosinophil mucosal infiltration group ($p < 0.005$). The number of infiltrating eosinophils in the mucosa was significantly higher in the group with the higher eotaxin-3 level ($p < 0.001$), but there was no significant difference in the blood eosinophil numbers among two groups. A significant positive correlation was found between the mucosal eosinophil count and the plasma levels of eotaxin-3 ($p < 0.005$). The levels of interleukin 33 (IL-33) ($p < 0.001$) and thymic stromal-derived lymphopoietin (TSLP) ($p < 0.005$) were significantly higher in the high-level eotaxin-3 group. IL-13 strongly induced the secretion of eotaxin-3 from human nasal fibroblasts ($p < 0.05$).

Conclusion: This is the first report suggesting eotaxin-3 as a plasma biomarker for mucosal eosinophil infiltration. Furthermore, the level of eotaxin-3 was found to be closely related to IL-33 and TSLP levels which indicate respiratory diseases.

Keywords: eotaxin-3, plasma biomarker, eosinophil, nasal polyp, rhinosinusitis

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INTRODUCTION

Chronic rhinosinusitis demonstrates marked heterogeneity, both at the molecular pathophysiological level and at the clinical phenotype level. This disease is divided into 2 subgroups, chronic rhinosinusitis with nasal polyps (CRSwNP) and chronic rhinosinusitis without nasal polyps (CRSsNP) (1). Nasal polyps have negative effects on many aspects of the quality of life (physical health, general health, social functioning, sleep, mental health, and workplace absenteeism) due to nasal obstruction, olfactory disturbance, rhinorrhea, and symptoms caused by lower airway involvement (2, 3). In patients 80–90% of the nasal polyps are characterized by prominent eosinophilia (4). The clinical classification of CRSwNP according to the degree of eosinophilic infiltration in nasal polyps is controversial (5).

Eosinophilic chronic rhinosinusitis (ECRS) is an emerging classification of CRS, and is thought to more accurately reflect the underlying pathophysiology. There is a wide variation among reports, and no consensus currently exists regarding the cut off for the diagnosis of ECRS. Mucosal eosinophilia is defined as >10 eosinophils per high-power field (HPF) according to histopathology profiling and remodeling changes (6–8). On the other hand, mucosal eosinophilia defined as ≥70, >100, or >120 mucosal eosinophils/HPF (9–11) is associated with poorer outcomes after endoscopic sinus surgery (ESS). A study conducted by 15 institutions participating in the Japanese epidemiological survey of refractory eosinophilic chronic rhinosinusitis revealed that the cut-off value of 70 mucosal eosinophils/HPF led to the most significant difference ($P < 0.001$) in the risk of recurrence in 1,716 patients treated by ESS (12).

T helper 1 (T_H1) cells in patients with CRSsNP and T_H2 cells in patients with CRSwNP are dominant (13, 14). In nasal polyps, immunoreactivity of the chemokine ligand (CCL) eotaxin subfamily comprising eotaxin-1 (CCL11), eotaxin-2 (CCL24), and eotaxin-3 (CCL26) was noted (15). Staphylococcus aureus enterotoxin B stimulation of dispersed nasal polyp cells induced significant interleukin 17A (IL-17A) synthesis (16). Thymic stromal-derived lymphopoietin (TSLP) was significantly increased in eosinophilic CRSwNP, and the expression of IL-33 was enhanced in epithelial cells in both eosinophilic and non-eosinophilic CRSwNP compared with controls (17).

The nasal mucosal eosinophilic status provides prognostic information about disease severity and outcome of CRS including surgeries. In this study, we examined different molecules in order to identify a plasma biomarker for mucosal eosinophil infiltration in CRS patients with low- and high-risk requiring multiple surgeries, as well as the correlation between the nasal tissue eosinophil count and cytokine levels. The patients could be divided into two groups according to the molecular levels. Furthermore, the patients were divided into two groups according to the plasma levels, and mucosal eosinophils, blood eosinophils, and levels of other cytokines were evaluated. We also observed chemokine secretion from nasal polyp-derived fibroblasts.

Abbreviations: HPF, high-power field; CRSwNP, chronic rhinosinusitis with nasal polyps; CCL, chemokine ligand; CRSsNP, chronic rhinosinusitis without nasal polyps; ECRS, Eosinophilic chronic rhinosinusitis; ESS, endoscopic sinus surgery; IL, interleukin; ROC, receiver operating characteristic; AUC, area under the curve; TSLP, Thymic stromal-derived lymphopoietin; T_H2, T helper 2; ELISA, enzyme-linked immunosorbent assay; ILC2, innate lymphoid cells; BAFF, B cell activating factor; tPA, tissue plasminogen activator.

MATERIALS AND METHODS

Subjects

We assessed patients with CRS treated by ESS. The diagnosis of sinus disease was based on patient history, clinical examination, and nasal endoscopy according to the guidelines of the European Position Paper on Rhinosinusitis and Nasal Polyps (18). Our study excluded patients who received systemic or topical corticosteroids before surgery, patients whose information on systemic or topical corticosteroids was unknown, patients who were followed up for <28 days after surgery, patients whose white blood cell count was 10,000/μl or more, and patients from whom there was no pathological specimen. Preoperative demographics and medical history including sex, age, age of onset, reaction to drugs, smoking history, complications, and drug allergies, were obtained for each patient. Blood samples were taken to perform complete blood counts. This study was approved by the ethics committee of each institution including the general public through the Division of Otorhinolaryngology, Head & Neck Surgery, University of Fukui. Nasal polyps were obtained from patients with CRS.

Histological Analysis

Mucosal tissues from patients with CRS were obtained from the nasal polyps or polypoid lesions of the ethmoid cavity during surgery. Tissues were immediately fixed in 10% formalin, embedded in paraffin, and cut into thin sections. Sections were stained with hematoxylin–eosin. The number of eosinophils in the mucosa was counted in HPF in the three densest areas with cellular infiltrate beneath the epithelial surface, and the mean number of eosinophils was calculated. Histological examinations were performed by three expert doctors blinded to the clinical data.

Human Nasal Polyp-Derived Fibroblast Cell Culture

Nasal polyp was obtained from patients with CRS during nasal surgery. Nasal specimens were cultured in 10 cm dishes containing RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated FCS (Gibco, Grand Island, NY), 0.29 mg/ml glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C in 5 % CO_2 and humidified air. Nasal fragments were removed and the first passage was performed. After a period of 3–4 weeks, nasal mucosa-derived fibroblast cell lines were established. The cells were used at passage numbers 3–5. Epithelial cells were confirmed not to be contaminated by immunohistochemical examination using cytokeratin and vimentin markers. The cells were cultured in the presence of IL-13 for appropriate periods, then culture supernatants were harvested and stored at −80°C.

Enzyme-Linked Immunosorbent Assay (ELISA)

Blood samples were obtained and centrifuged immediately to prevent the degradation of complement components, and the plasma was stored at −80°C within 1 h of blood collection. We measured the plasma levels of cytokines and the culture supernatants. We conducted multiple the assay system using Multiplex Assays (Millipore, Billerica, MA) according to the manufacturers' protocols.

Statistical Analysis

The non-parametric Mann-Whitney test was performed to evaluate differences. Correlation analysis was carried out using Spearman's rank correlation coefficient. We used a ROC (receiver operating characteristic) curve to calculate the area under the curve, and the closest point to top-left of ROC was determined as the optimal cut-off point. A p-value of <0.05 was considered statistically significant.

RESULTS

The Cytokine Levels in Low- and High-Eosinophil Mucosal Infiltration

A total of 67 males and 30 females aged 19 to 73 years old were included in the study. In the 37 patients that could be observed more than 3 years after surgeries, the ROC (receiver operating characteristic) curve for nasal mucosal eosinophil counts was used to discriminate the patients with recurrence from those without recurrence. Area under the curve (AUC) was 0.7533 ($p = 0.014$) (**Figure 1**). The optimal cut-off point of mucosal eosinophil counts was 55.0 infiltrating eosinophils/HPF to differentiate the subjects with recurrence from those without recurrence (with 64.0% sensitivity and 83.3% specificity). The high-mucosal eosinophil infiltration group was defined as those having 55.0 or more infiltrating eosinophils/HPF in the mucosa. All others were in the low-risk group. Subject characteristics are presented in **Table 1**. In the low- ($n = 54$) and high- eosinophil mucosal infiltration ($n = 43$), the age (mean ± SE) was 52.0 ± 1.9 and 49.5 ± 2.2 years old, the mucosal eosinophil count (/HPF) was 26.2 ± 2.2 and 134.2 ± 14.1 ($p < 0.0001$), the blood eosinophils (%) were 5.0 ± 0.6 and 7.9 ± 0.7 ($p < 0.0005$), the blood neutrophils (%) was 57.6 ± 1.3 and 54.4 ± 1.6, respectively. We next assessed the levels of eotaxin-1, eotaxin-2, eotaxin-3, IL-4, IL-5, IL-10, IL-12, IL-13, IL-21, IL-33, TSLP, TNFα, INFγ, IP-10, and MCP-1 in both groups. As shown in **Table 2**, the level of eotaxin-3 (84.3 ± 5.3) in the low-risk group was significantly lower than that (1122.6 ± 15.8) in the high-risk group ($p < 0.005$). There was no significant difference in the levels of other molecules including eotaxin-1, etaxin-2, or other cytokines, between the two groups in this study.

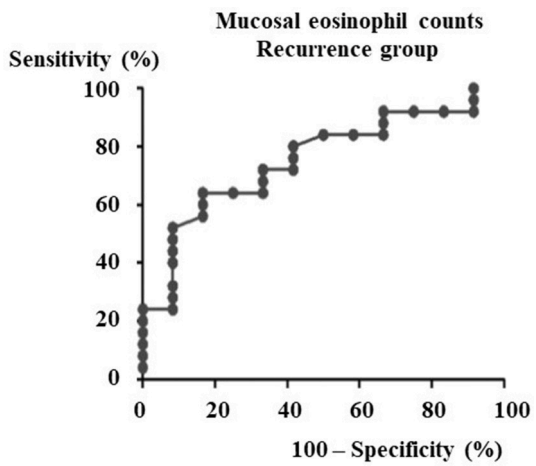

FIGURE 1 | ROC curve for recurrence group after ESS to determine the cut-off point of mucosal eosinophils number. From the data of the mucosal eosinophils number diagnostic test by ROC curve against the recurrence group after ESS (Area under the curve = 0.7533, $p = 0.014$), the cut-off level of mucosal eosinophils number is 55.0 pg/ml.

Correlation Between Nasal Mucosal Eosinophil Count and the Plasma Cytokine Levels

A plasma biomarker should be a significant relationship to mucosal eosinophil counts in CRS. The correlation coefficients (γ) and p-value for comparison between mucosal eosinophil counts and the levels of eotaxin-1, eotaxin-2, eotaxin-3, IL-4, IL-5, IL-10, IL-12, IL-13, IL-21, IL-33, TSLP, TNFα, INFγ, IP-10, and MCP-1 were examined using the Spearman's rank correlation coefficient. As shown in **Table 3**, a significant positive correlation was found between the mucosal eosinophil count and the plasma level of eotaxin-3 ($p < 0.005$). We found no correlation between the mucosal eosinophil count and the levels of eotaxin-1, eotaxin-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-21, IL-33, TSLP, TNFα, INFγ, IP-10, or MCP-1.

TABLE 1 | Demographics by eosinophil mucosal infiltration status for chronic rhinosinusitis groups.

	Low-eosinophil mucosal infiltration ($n = 54$)	High-eosinophil mucosal infiltration ($n = 43$)
Age (y) (Mean ± SE)	52.0 ± 1.9	49.5 ± 2.2
Male, no./total (%)	36/54 (66.7)	31143 (72.1)
Mucosal eosinophil counts (/HPF)	26.2 ± 2.2	134.2 ± 14.1***
Blood eosinophil (%)	5.0 ± 0.6	7.9 ± 0.7**
Blood neutrophil (%) (Mean ± SE)	57.6 ± 1.3	54.4 + 1.6
Recurrence (%)	11.1% (2/18)	52.6% (10/19)*

*High-eosinophil mucosal infiltration group is defined when the numbers of infiltrating eosinophils are 55.0 or more than 55.0 / high-power field in the mucosa. The other is low-group. P-values for comparison between low- and high- eosinophil mucosal infiltration groups. The recunence rate is shown in the patients that could be observed more than 3 years after surgeries (*p < 0.01, **p < 0.0005, ***p < 0.0001, ESS, endoscopic sinus surgery; HPF, per high-powered field).*

TABLE 2 | The plasma cytokine levels of low- and high-eosinophil mucosal infiltration groups.

	Low-eosinophil mucosa l infiltration ($n = 54$) (Mean ± SE)	High-eosinophil mucosal infiltration ($n = 43$) (Mean ± SE)	*p*-value
Eotaxin-1 (ng/ml)	1.3 ± 0.1	1.1 ± 0.1	0.369
Eotaxin-2 (ng/ml)	7.0 ± 0.4	6.0 ± 0.4	0.091
Eotaxin-3 (pg/ml)**	84.3 ± 5.3	122.6 ± 15.8	0.002**
IL-4 (pg/ml)	164.8 ± 20.6	194.0 ± 33.4	0.974
IL-5 (pg/ml)	132.9 ± 108.7	30.6 ± 10.9	0.918
IL-10 (pg/ml)	156.8 ± 118.5	139.1 ± 107.3	0.318
IL-12 (pg/ml)	56.4 ± 30.3	22.6 ± 5.0	0.305
IL-13 (pg/ml)	138.5 ± 109.2	52.0 ± 34.1	0.139
IL-21 (pg/ml)	26.8 ± 2.4	28.0 ± 2.8	0.476
IL-33 (pg/ml)	57.6 ± 18.4	165.2 ± 55.4	0.768
TSLP (pg/ml)	57.5 ± 8.5	82.6 ± 17.3	0.510
TNFa (pg/ml)	59.4 ± 6.5	51.2 ± 4.1	0.633
INFy (pg/ml)	26.2 ± 7.2	26.5 ± 3.2	0.933
IP-10 (pg/ml)	843.6 ± 118.3	716.8 ± 89.3	0.645
MCP-1 (ng/ml)	5.3 ± 0.4	5.2 ± 0.4	0.965

*p-values for comparison between low- and high- eosinophil mucosal infiltration groups (**$p < 0.005$).*

TABLE 3 | Correlation between nasal mucosal eosinophil counts and the plasma cytokine levels.

	γ	*P*-value
Eotaxin-1	−0.113	0.267
Eotaxin-2	−0.172	0.091
Eotaxin-3	0.330	0.001**
IL-4	−0.065	0.525
IL-5	−0.007	0.944
IL-10	−0.038	0.708
IL-12	−0.148	0.147
IL-13	−0.186	0.069
IL-21	0.148	0.148
IL-33	0.041	0.691
TSLP	0.091	0.372
TNFα	−0.149	0.148
INFγ	0.111	0.273
IP-10	−0.073	0.474
MCP-1	0.029	0.778

*Correlation coefficients (γ) and P-value for comparison between mucosal eosinophil counts and the plasma cytokine levels were examined using the Speannan's conelation coefficient by rank. (**$p < 0.005$).*

Tissue Eosinophil Infiltration and Blood Eosinophils Between High- and Low-Level Eotaxin-3 Groups

The ROC curve for plasma eotaxin-3 levels was used to discriminate high-eosinophil mucosal infiltration group after ESS from the other group. The optimal cut-off point of plasma eotaxin-3 level was 78.8 pg/ml (with 61.4% sensitivity and 75.5% specificity) (**Figure 2A**). In the patients that could be observed more than 3 years after surgeries, the recurrence rate of low-level eotaxin-3 group (13.3 %) was significantly lower than that of high-level eotaxin-3 group (45.5 %) ($p < 0.05$) (**Figure 2B**).

As shown in **Figure 3**, we divided the enrolled patients into two groups according to the plasma level of eotaxin-3. In low-level eotaxin-3 group ($n = 42$), the level of eotaxin-3 was 78.8 pg/ml or lower, whereas in the high-level eotaxin-3 group ($n = 55$), the level was higher than 78.8 pg/ml. The nasal mucosal eosinophil count (mean ± SE = 47.5 ± 7.9 / HPF) was significantly lower in low-level eotaxin-3 group than in high-level eotaxin-3 group (mean ± SE = 94.4 ± 12.9/HPF) ($p < 0.001$) (A). On the other hand, there was no significant difference in the percentage (B) or the number (C) of blood eosinophil between the two groups (low-level eotaxin-3 group: mean ± SE = 5.6 ± 0.7%, 369.4 ± 42.4/ml) (high-level eotaxin-3 group: mean ± SE = 6.8 ± 0.6 %, 433.6 ± 36.2/ml).

Cytokine Levels Among High- and Low-Level Eotaxin-3 Groups

We also compared the plasma levels of other cytokines between two groups according to the level of eotaxin-3 as in **Figure 3**. **Figure 4A** shows that the level of IL-33 (mean ± SE = 175.0 ± 44.4 pg/ml) was significantly higher in high-level eotaxin-3 group than that (mean ± SE = 65.6 ± 17.0 pg/ml) in low-level eotaxin-3 group ($p < 0.001$). The level of TSLP (mean ± SE = 84.8 ± 14.5 pg/ml) was also significantly higher in high-level eotaxin-3 group than those (mean ± SE = 47.4 ± 7.9 pg/ml) in low-level eotaxin-3 group ($p < 0.005$) (**Figure 4B**).

IL-13-Induced Eotaxin-3 Protein Secretion in Human Nasal Polyp Fibroblasts

TSLP and IL-33 are closely related to innate lymphoid cells (ILCs) and T_H2 cells, inducing IL-4, IL-5, and IL-13 production. Since the expression of eotaxin-3 in human nasal fibroblast is quite unknown, we established fibroblast lines from small pieces of human nasal polyps respectively, from 8 individuals and then examined eotaxin-3 protein secretion in fibroblasts stimulated by IL-13 for 48 h. As shown in **Figure 5**, IL-13 significantly enhanced the secretion of eotaxin-3 in human nasal fibroblasts ($p < 0.05$), while we could not detect any increase of eotaxin-1 or eotaxin-2. The eotaxin-3 production in the presence of IL-13 was strongly induced more than 240 times higher than in the absence of IL-13.

DISCUSSION

In this study, we found a significant positive correlation between the plasma level of eotaxin-3 and the mucosal eosinophil count. The level of eotaxin-3 in high-eosinophil mucosal infiltration group was significantly higher than that in low- group ($p < 0.005$). The nasal mucosal eosinophil counts, plasma IL-33 levels, and TSLP level were significantly higher in high-level eotaxin-3 group than those in low-level eotaxin-3 group. IL-13 strongly induced the secretion of eotaxin-3 from human nasal fibroblasts. Based on research data, TSLP and IL-33 that

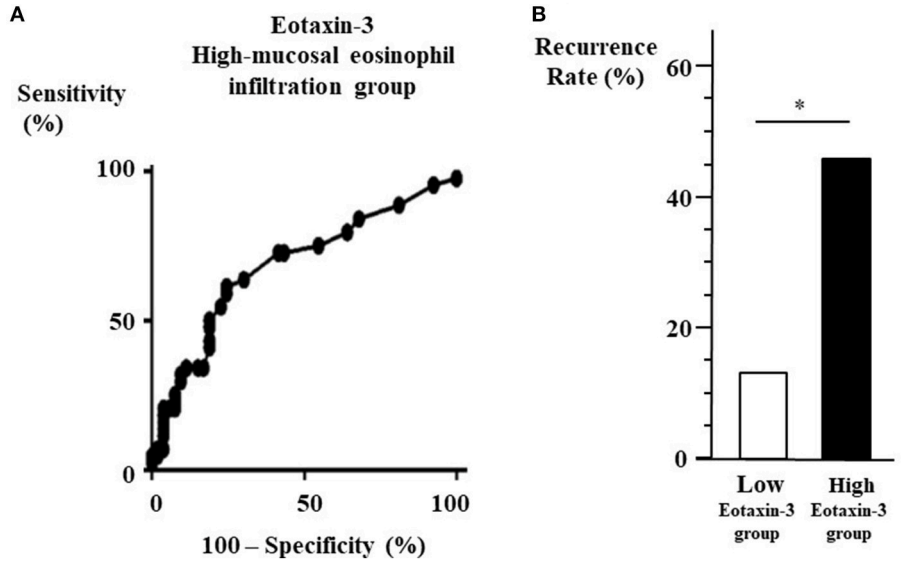

FIGURE 2 | (A) ROC curve for high-eosinophil mucosal infiltration group to determine the cut-off point of plasma eotaxin-3 level**.** From the data of the eotaxin-3 diagnostic test by ROC curve against the high-eosinophil mucosal infiltration group after ESS (Area under the curve = 0.6829, $p = 0.002$), the cut-off level of eotaxin-3 is 78.8 pg/ml. **(B)** Recurrence rate between high- and low-level eotaxin-3 groups. The 37 patients that could be observed more than 3 years after surgeries were divided into two groups according to the plasma level of eotaxin-3. The recurrence rate in high-level eotaxin-3 group was higher than that in the other group (*$p < 0.05$).

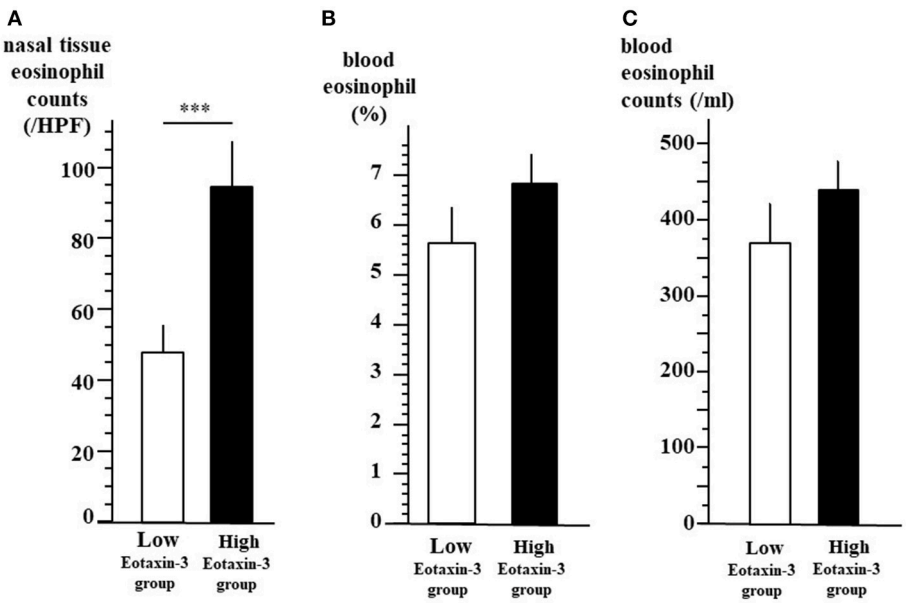

FIGURE 3 | Tissue eosinophil infiltration and blood eosinophils between high- and low-level eotaxin-3 groups**.** All the 97 patients were divided into two groups according to the plasma level of eotaxin-3. In low-level eotaxin-3 group, the level of eotaxin-3 was 78.8 pg/ml or lower. In the high-level eotaxin-3 group, the level of eotaxin-3 were higher than 78.8 pg/ml. The mean ± SE of the nasal mucosal eosinophil count per HPF **(A)**, the percentage **(B)**, and the number **(C)** of blood eosinophil are indicated by open (low-level eotaxin-3 group) and closed (high-level eotaxin-3 group) bars and lines (***$p < 0.001$).

are secreted from epithelial cells and can induce type 2 ILCs and T_H2 cells, and then the levels of IL-4, IL-5, and IL-13 increase. IL-13 induces eotaxin-3 production from fibroblasts and epithelial cells. Eotaxin-3 induced the tissue infiltration of eosinophils (**Figure 6A**). Thus, eotaxin-3 could function in pathology of mucosal eosinophil infiltration in CRSwNP. Bronchial epithelial cell injury leads to production of type 2 alarmins such as IL-33 and TSLP, also in asthma. Type 2 ILCs

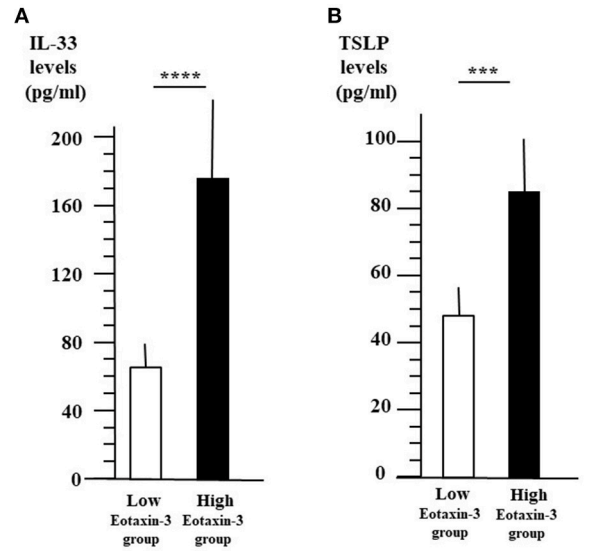

FIGURE 4 | IL-33 and TSLP levels between high- and low-level eotaxin-3 groups. The patients were divided into two groups according to the plasma level of eotaxin-3 as in **Figure 2**. The mean ± SE of plasma IL-33 **(A)** and TSLP levels **(B)** are indicated with the open (low-level eotaxin-3 group) and closed (high-level eotaxin-3 group) bars and lines (****$p < 0.001$, ***$p < 0.005$).

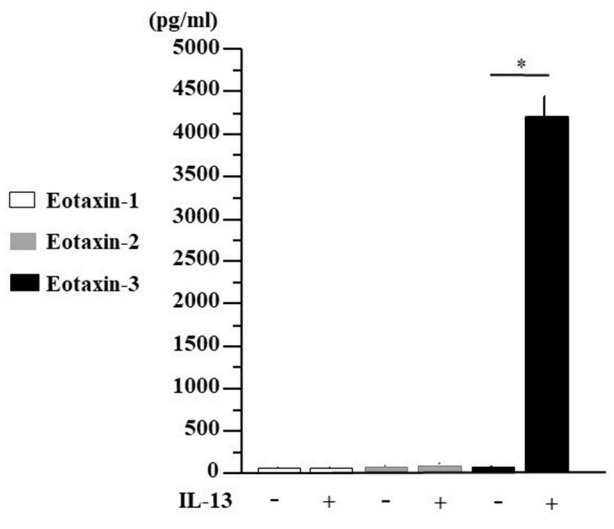

FIGURE 5 | IL-13-induced eotaxin-3 protein secretion in human nasal fibroblasts. Nasal polyp was obtained from patients during surgery and fibroblast lines were established. Human nasal fibroblasts were stimulated with IL-13 (10 ng/ml) for 48 h. Supernatants were harvested for the analysis for eotaxin-1, eotaxin-2, and eotaxin-3 production by ELISA. The results are expressed as the mean ± SE ($n = 8$) *$p < 0.05$.

produce, in response to TSLP or IL-33, large amounts of the T_H2 cytokines IL-5, IL-13 and, to a lesser extent, IL-4 (19, 20). Eotaxin-3 is the most efficient eotaxin to induce the migration or transmigration of eosinophils (21). The hematoxylin–eosin staining shows eosinophils infiltrate also in the tissue of CRS (**Figure 6B**).

Eosinophil-predominant disorders are closely related to allergic diseases, such as asthma, allergic rhinitis, atopic dermatitis, eosinophilic esophagitis. The eotaxin-3 expression was found optimal from the data set to define type 2 inflammation based on airway mucosal IL-13-driven gene expression and how this related to clinically accessible biomarkers for patients with mild to severe asthma and non-atopic healthy control subjects (22). Allergic subjects have a significant immediate response of nasal symptoms as well as a significant increase significantly raising levels of eotaxin-3 after nasal allergen challenge (23). The gene expression level for eotaxin-3 was higher in skin changes of atopic dermatitis patients (24). The serum eotaxin-3 level was significantly higher in patients with urticaria than in the healthy controls (25). The pathogenesis of eosinophilic esophagitis depends on local epithelial immune activation with production of eotaxin-3 and TSLP (26). On the microarray, 1,999 genes were differentially expressed between eosinophilic gastritis in children and the controls ($p < 0.05$), including significant upregulation of eotaxin-3 (27).

Precision medicine (28) is medical treatment that precisely classifies the patient's condition to select a suitable treatment method, personal medical care, and tailor-made medical treatment. Moreover, for CRS or allergic diseases, biomarkers, genetic factors, relationships with environmental factors, and phenotypes are often separated for therapy (29, 30). Defining endotypes can help clinicians predict disease prognosis, and select subjects suitable for a specific therapy for CRS (31). CRS is caused by dysregulated immunological responses inducing various mediators and inflammatory cells, including innate lymphoid cells, TSLP, and IL-33, which are mainly secreted by epithelial cells in response to external stimuli. They then act on type 2 ILCs and Th2 cells, inducing IL-4, IL-5, and IL-13. In this study, IL-33 and TSLP levels were significantly higher in the high-level eotaxin-3 group than those in the low-level eotaxin-3 group. We noted the significant positive correlation between the plasma levels of eotaxin-3 and IL-33 ($p < 0.001$) or TSLP ($p < 0.005$).

T_H2 cytokines (IL-4 or IL-13) play important roles in CRS. IL-4 or IL-13 induce TSLP production by nasal fibroblasts (32). Local IgE and B cell activating factor (BAFF, BLyS) production are also signature characteristics of nasal polyps (33). IL-4 induces class switch recombination to IgE with the CD40 ligand, BAFF, and BLyS (34). IL-4 and IL-13 also down-regulate tissue plasminogen activator (t-PA) in epithelial cells while t-PA activates fibrinolysis cascades involve in fibrin deposition and dissolution (35). IL-13 stimulation of human nasal epithelial cells dominantly induced eotaxin-3 expression (36). We also found IL-13 significantly increased eotaxin-3 production by human nasal fibroblasts. The strong induction of eotaxin-3 from nasal tissue, high levels of eotaxin-3 in nasal polyp (36), and its expression of endothelial cells (15) could keep the plasma high levels of eotaxin-3 in the patients with ECRS. The nasal mucosal eosinophil count was significantly 2 times higher in plasma high-level eotaxin-3 group than in low-level eotaxin-3 group. The blood eosinophil count was 1.2 times higher in high-level eotaxin-3 group than in low-level eotaxin-3 group while there was no significant difference in the percentage or the number

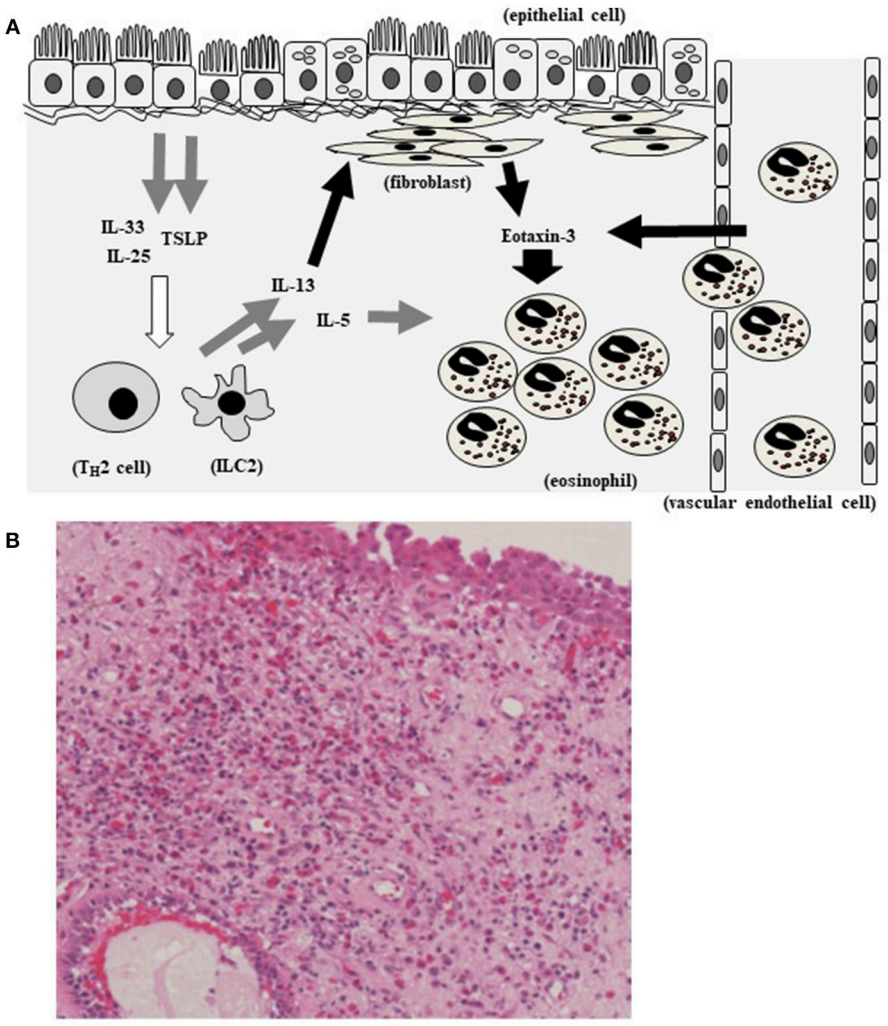

FIGURE 6 | Hypothetical model showing eotaxin-3 and eosinophil infiltration in CRS. TSLP and IL-33 that are secreted by epithelial cells and act on type 2 ILCs and T_H2 cells, inducing IL-5 and IL-13. IL-13 induces eotaxin-3 production from fibroblasts and epithelial cells. Eotaxin-3 induced the tissue infiltration of eosinophils **(A)**. Eosinophils infiltrate in the tissue of CRS (The section is stained with hematoxylin–eosin) **(B)**.

of blood eosinophil between the two groups. Increased eotaxin-3 levels in tissue could induce mucosal infiltration of eosinophils more strongly compared with the increase of blood eosinophils. And, the recurrence rate of high-level eotaxin-3 group was significantly 3 times higher than that of low-level eotaxin-3 group for 3 years after surgeries ($p < 0.05$). Dupilumab is a fully human monoclonal antibody against the IL-4 receptor α subunit, which inhibits signaling of IL-4 and IL-13. Of note, during this trial, the serum eotaxin-3 levels decreased and then nasal polyp scores began to decrease. On the other hand, the blood eosinophil count did little change in the group using dupilumab (37).

Human monoclonal antibodies are available or under clinical trials in allergic disease or refractory CRS. T_H2 cytokines such as IL-4, IL-5, IL-13, epithelial-derived cytokines such as TSLP, IL-33, and acquired immunity markers including IgE could be candidates for molecular targets. In the randomized, double-blind, placebo-controlled study on the effects of humanized monoclonal anti-IgE antibody, omalizumab on NP and comorbid asthma (38), omalizumab reduced the total nasal endoscopic polyp score and demonstrated significant benefits for nasal and respiratory symptoms, such as nasal congestion, anterior rhinorrhea, loss of smelling sense, wheezing, and dyspnea. On the contrary, there was no reduction in inflammatory mediators in the treated group. Eosinophils, mast cells, and ILC2s mainly produce IL-5. The anti-IL-5 mAb, reslizumab reduced the polyp size, blood eosinophilic counts, and ECP concentration in nasal secretions (39). Responders had higher IL-5 levels in nasal secretions compared with non-responders. In the clinical trial of the anti-IL-5 mAb, mepolizumab, there was significant improvement in the nasal polyposis severity, VAS score, endoscopic nasal polyp score, and all individual VAS symptom scores in the mepolizumab compared with placebo

groups (40). However, it remains unclear which biomarker can be used to select good responders to anti-IL-5 treatment. Dupilumab can inhibit signaling of IL-4 and IL-13 central to T_H2-cell–mediated inflammation. Its clinical efficacy for T_H2-cell–mediated diseases of asthma and atopic dermatitis has been confirmed and it also improved the nasal symptoms in perennial allergic rhinitis and comorbid asthma (41). A randomized clinical trial demonstrated a potential role of dupilumab in NP by significantly decreasing the nasal polyp score ($p < 0.001$) and improving in olfaction and CT scores ($p < 0.001$), as well as other clinical outcomes such as nasal symptoms and quality of life (37). Significant improvements with dupilumab were also observed for the 22-item sinonasal symptoms ($p < 0.001$). The serum eotaxin-3 levels decreased within 2 weeks, nasal polyp scores began to decrease at 4 weeks, and then sinonasal symptoms improved gradually. The serum levels of total IgE also decreased thereafter.

As CRS involves several mechanisms with high heterogeneity and different therapeutic responses, biomarkers and endotyping (42, 43) help to determine the optimal primary therapeutic modality, select good responder to a specific treatment, and predict treatment outcomes. In this study, the significant positive correlation between the plasma levels of eotaxin-3 and the mucosal eosinophil count support the ability of dupilumab to reduce nasal mucosal eosinophil infiltration against little change of blood eosinophil counts. Dupilumab can reduce the blood eotaxin-3 levels that are significantly higher in the high-risk group for recurrence after surgery. Also proton pump inhibitors can decrease eotaxin-3 expression in patients with CRSwNP (36). Eotaxin-3 levels could be one of the suitable plasma biological parameters for the efficacy of dupilumab or proton pump inhibitors in CRS. This may be a breakthrough in the treatment of recalcitrant CRS. Further studies including multivariate analysis are needed to investigate the potential use of monoclonal antibodies as adjunct therapy or with other medications or surgery.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Declaration of Helsinki 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 ethics committee of each institution including the general public through the Division of Otorhinolaryngology, Head and Neck Surgery, University of Fukui.

AUTHOR CONTRIBUTIONS

TY, HS, and YM: conception and design; TY, HS, YM, SU, TT, MS, YKat, TN, SF, YKaw, and SS: analysis and interpretation; TY, HS, and YM: drafting the manuscript for important intellectual content.

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MINI REVIEW
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An Approach to the Evaluation of Persistent Hypereosinophilia in Pediatric Patients

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Hypereosinophilia (HE) is currently defined by a peripheral blood absolute eosinophil count (AEC) of ≥1,500 cells/microL. Although mild blood eosinophilia (AEC 500–1,500 cells/microL) is observed relatively frequently within the pediatric population, persistent HE is uncommon and should prompt additional clinical evaluation. While the clinical manifestations and underlying etiologies of HE in adults have been well-characterized, there is a paucity of data on HE in children. Limited evidence suggests that many similarities between adult and pediatric HE likely exist, but some important differences remain between these populations. The evaluation of HE in children can be challenging given the broad differential diagnosis, which includes primary hematologic disorders and secondary eosinophilia in which the increased eosinophil levels are propagated by disease states that promote eosinophil production and survival. On the basis of the underlying etiology, clinical manifestations can range from benign, self-resolving elevations in the AEC to life-threatening disorders with the potential for significant end-organ damage. Given the broad differential diagnosis of HE, it remains essential to systematically approach the evaluation of unexplained HE in children. This review will discuss the differential diagnosis for pediatric HE, highlighting etiologies that are more prevalent within the pediatric population. Additionally, a summary of the epidemiology of pediatric HE will be presented, with focus on some of the differences that exist between pediatric and adult HE. Finally, a directed approach to the diagnostic evaluation of children with HE will be discussed.

Keywords: children, eosinophil, hypereosinophilia, differential diagnosis, epidemiology, diagnostic testing

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INTRODUCTION

Eosinophils are terminally differentiated granulocytes with important roles in innate immune function, tissue remodeling and repair, and disease pathogenesis (1). The eosinophil level in the peripheral circulation is tightly regulated, with eosinophils representing only a small minority (typically <5–6%) of the circulating leukocyte population. Although commonly assessed in the peripheral blood, eosinophils are primarily tissue-dwelling cells (2). Under normal homeostatic conditions, the vast majority of eosinophils leave the circulation and migrate into specific tissues where they can reside for several weeks. The gastrointestinal tract serves as the largest tissue reservoir for eosinophils, where these cells are normally present in the mucosal tissue from the stomach to the large intestine (3, 4). Eosinophilia, denoted by an increased absolute eosinophil

count (AEC) in the blood, can be driven by a number of important disease states in children and reflects the balance between eosinophil production in the bone marrow, trafficking from the bone marrow into the tissues and eosinophil apoptosis. Transient eosinophilia is observed relatively frequently in the pediatric population and is generally clinically insignificant. However, patients with chronic (persistent) eosinophilia can have a spectrum of clinical consequences, ranging from relatively benign disorders to disease states associated with significant end-organ dysfunction and potentially life-threatening sequelae. Defining the underlying pathology propagating eosinophilia is an essential first step in the management of pediatric hypereosoinophilia (HE) in order to tailor an appropriate treatment strategy. In this review, we will discuss the differential diagnosis for HE in children, focusing on etiologies that are more prevalent in the pediatric population and epidemiologic differences between children and adults. Additionally, we will present a directed approach to the diagnostic evaluation of pediatric HE, highlighting some important red flags that should prompt medical providers to pursue more intensive evaluation.

TERMINOLOGY

The AEC represents the frequency of circulating eosinophils in the peripheral blood (in cells per microliter [cells/microL]). Eosinophil levels in the peripheral blood vary by age, with higher upper threshold limits seen in infants and toddlers compared to adolescents and adults (5–7). However, for most children >2 years of age, an AEC value of >700 cells/microL is considered abnormally elevated. The severity of eosinophilia has been arbitrarily classified into mild (AEC from the upper limit of normal to 1,500 cells/microL), moderate (AEC 1,500–5,000 cells/microL) and severe (AEC>5,000 cells/microL) (4, 8). Eosinophilia can be transient, episodic or persistent (chronic). The term HE has been reserved for patients with moderate-to-severe persistent blood eosinophilia, defined as a blood AEC ≥1,500 cells/microL obtained on at least 2 separate occasions (interval ≥1 month) or marked tissue eosinophilia (4). Tissue HE can be defined as a percentage of eosinophils that exceeds 20% of all nucleated cells in the bone marrow or tissue infiltration that is deemed extensive by a pathologist (4). In situations where eosinophils are not directly observed within the tissue, the histologic evidence of extracellular deposition of eosinophil-derived granule proteins (ex. major basic protein, eosinophil peroxidase or eosinophil-derived neurotoxin) in the tissue can also be used as surrogate markers of tissue eosinophilia (9). Finally, the term hypereosinophilic syndrome (HES) is an umbrella term describing a heterogeneous group of disorders that are characterized by HE and evidence of end-organ damage or dysfunction directly attributable to tissue eosinophilia (10). From a practical standpoint, it remains important to recognize clinically that end-organ manifestations of eosinophilia may not manifest when the HE is first noted, with some individuals developing signs of organ dysfunction years after the HE initially presents (11).

CAUSES OF HYPEREOSINOPHILIA IN CHILDREN

The underlying differential diagnoses for HE in children is similar to that of adults, with some notable exceptions. Pediatric HE can be associated with a variety of underlying etiologies that can be separated pragmatically into two main categories (primary and secondary HE) based on the underlying mechanisms driving the eosinophil expansion (**Figure 1.**)

Primary (Clonal) HE

Primary HE results from abnormalities within the bone marrow compartment that propagate the expansion of an eosinophil clone. In these disorders, eosinophils represent the predominant cell type involved or one of several proliferating cell lines. Primary causes of HE include myeloid and stem-cell neoplasms, grouped collectively into the category of myeloproliferative HE/HES (M-HE or M-HES) (10). This group of disorders includes both definitive and presumed eosinophilic myeloproliferative neoplasms, including hematopoietic neoplasms with eosinophilia resulting from fusion genes or mutations leading to the constitutive activation of oncogenic tyrosine kinase receptors such as PDGFRA, PDGFRB, or FGFR1, eosinophilic leukemia, myeloid leukemia, mast cell leukemia, myelodysplastic syndromes and systemic mastocytosis. Primary causes of HE represent a minority of pediatric HE cases (12, 13).

Secondary (Reactive) HE

Secondary (or reactive) HE results from disease states that drive the polyclonal expansion of eosinophils, typically through the increased production of cytokines such as IL-3, IL-5, and GM-CSF that promote increased eosinophil production and survival (14). Most cases of pediatric HE have an underlying secondary etiology.

Infection

Normal immunological responses to certain infectious pathogens remain among the most common etiologies of secondary HE in children worldwide. In particular, invasive helminth infections (Strongyloidiasis, Schistosomiasis, Hookworm, Filariasis, Ascariasis, Toxocariasis, and Trichinosis) can cause pronounced eosinophilia early in infection as the larvae migrate through tissues (15). Some parasites are endemic worldwide and should be considered in all children with eosinophilia, regardless of their travel or exposure history. For example, *Strongyloides stercoralis* is endemic in areas with hot, humid climates (including the southeastern United States) and can directly penetrate the skin upon contact with soil or water contaminated with human feces (15). This parasite can have a long latency period of years between the initial exposure and symptom development, so an infection can easily be missed if the clinician does not routinely test for this infection (16). *Toxocara canis* and *cati* (the etiologic causes of visceral larva migrans) are also endemic worldwide and can be ingested in soil or food contaminated by dog or cat feces (15). Toxocariasis disproportionately affects the pediatric population, particularly toddler-aged children given their unsanitary ingestion habits (15, 17, 18). This parasite

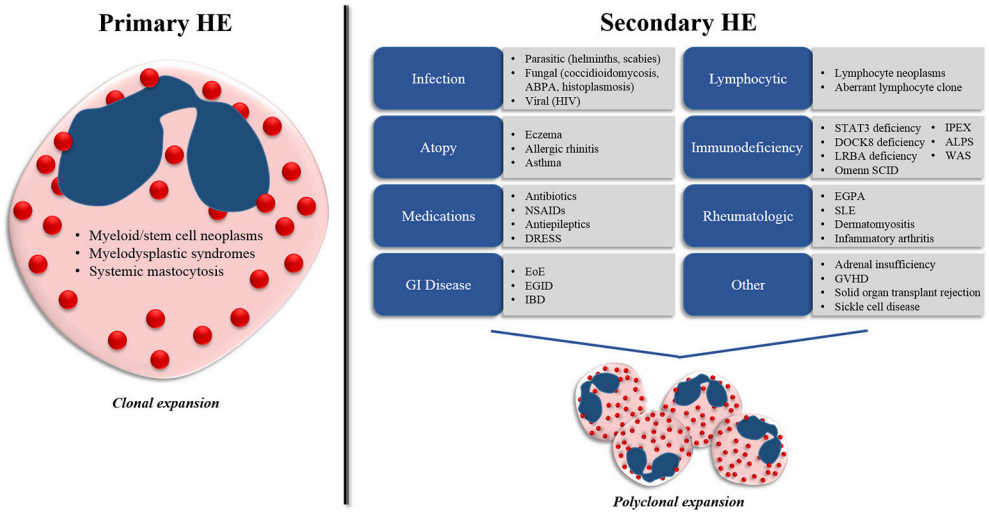

FIGURE 1 | Differential diagnosis for hypereosinophilia (HE) in children. Pediatric HE can be separated into two main categories (primary and secondary HE) on the basis of the underlying mechanisms driving the eosinophil expansion. Primary HE results from myeloid and stem cell abnormalities that propagate the expansion of an eosinophil clone. Secondary HE results from a diverse group of disease states that drive the expansion of the eosinophil population through increased eosinophilopoietic cytokine (IL-3, IL-5, and GM-CSF) production. ABPA, allergic bronchopulmonary aspergillosis; HIV, human immunodeficiency virus; NSAIDs, nonsteroidal anti-inflammatory drugs; DRESS, drug reaction with eosinophilia and systemic symptoms; EoE, eosinophilic esophagitis; EGID, eosinophilic gastrointestinal disease; IBD, inflammatory bowel disease; STAT3**,** signal transducer and activator of transcription 3, DOCK8, dedicator of cytokinesis 8; LRBA, lipopolysaccharide-responsive-beige-like-anchor; WAS, Wiskott-Aldrich syndrome; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome; ALPS, autoimmune lymphoproliferative syndrome; EGPA, eosinophilic granulomatosis with polyangiitis; SLE, systemic lupus erythematosus, GVHD, graft-vs.-host disease.

should be considered in any young child with HE, particularly if the child has a history of pica as these children are at increased risk for ingesting contaminated soil (17, 18). Nonhelminth infections can also trigger HE in the pediatric population. Scabies mite infection should be considered in children who present with a concomitant pruritic skin rash (19). Fungal etiologies, including allergic bronchopulmonary aspergillosis (ABPA), which can be seen in children with chronic lung disease (asthma, cystic fibrosis), and disseminated Coccidioidomycosis and Histoplasmosis infections can trigger HE (20–23). Finally, HIV is a rare cause of HE in the pediatric population and should be considered in patients with unexplained HE and risk factors (24–26). Notably, protozoal parasites that often affect the pediatric population (*Giardia, Cryptosporidium, Entamoeba*) generally do not produce peripheral HE (3, 15).

Immune Disorders

Atopic disease (eczema, allergic rhinitis, asthma) is a common cause of mild-to-moderate eosinophilia in the pediatric population and a minority of these patients can meet criteria for HE. However, the presence of severe, persistent eosinophilia (i.e., eosinophils >5,000/microL) is unlikely secondary to atopy and should prompt additional evaluation for another etiology. Importantly, several immunodeficiency syndromes (signal transducer and activator of transcription 3 [STAT3] deficiency, dedicator of cytokinesis 8 [DOCK8] deficiency, lipopolysaccharide-responsive-beige-like-anchor [LRBA] deficiency, Wiskott-Aldrich syndrome [WAS], and immune dysregulation, polyendocrinopathy, enteropathy, X-linked [IPEX] syndrome) present in childhood with atopy, elevated IgE and peripheral blood eosinophilia (27). Consequently, a thorough infection history (with focus on the frequency and etiology of infections) should be obtained in any pediatric patient with HE and atopy. Other immunodeficiencies that can present with HE in the pediatric population include autoimmune lymphoproliferative syndrome (ALPS) and Omenn syndrome associated with severe combined immunodeficiency (27).

Drug Hypersensitivity

Drug hypersensitivity reactions are also a frequent cause of HE in children. Antibiotics (particularly penicillins, cephalosporins, and vancomycin), NSAIDs, and antiepileptic medications are commonly implicated as causes of eosinophilia, but almost any prescription or nonprescription drug, herbal remedy, or dietary supplement can be a trigger (16, 28, 29). A temporal relationship between drug initiation and development of eosinophilia is helpful in identifying a drug reaction, although latency between exposure and eosinophilia can vary from days to months. Notably, drug reaction with eosinophilia and systemic symptoms (DRESS) is a potentially life-threatening systemic hypersensitivity reaction associated with peripheral HE that typically presents after a latency period of 2–8 weeks between drug exposure and clinical manifestations (fever, malaise, lymphadenopathy, elevated liver enzymes and morbilliform skin eruption that can progress to an exfoliative dermatitis) (30).

Gastrointestinal Disorders

Eosinophilic esophagitis (EoE) is also a common cause of HE in the pediatric age group. This diagnosis can often be missed if an appropriate history is not obtained. The primary symptoms of

EoE vary with age, with younger patients presenting with feeding difficulties, frequent vomiting, food refusal/selective eating, and failure to thrive (31). As these children increase in age, complaints of abdominal pain and dysphagia increase and adolescents can develop food impactions. Other primary gastrointestinal eosinophilic disorders (eosinophilic gastrointestinal disease) can also cause HE in children, although these disorders are less common than EoE. Additionally, inflammatory bowel disease can be associated with a peripheral eosinophilia.

Neoplasms

Lymphocytic variant HE/HES (L-HE or L-HES) is a category of disorders characterized by a clonal or aberrant lymphocyte population that produces cytokines that propagate eosinophil production and survival (10). Included within this category are lymphoid neoplasms, some of which are more common in children (ex. pre-B cell acute lymphoblastic leukemia [ALL]). ALL can present with HE in children, in some cases months before the underlying malignancy is detected (32, 33). In these situations, eosinophils are not part of the neoplastic clone and represent a secondary response to the malignancy.

Autoimmune Disorders

Less common etiologies of secondary HE in children include rheumatologic disease. Notably, eosinophilic granulomatosis with polyangiitis (EGPA, previously called Churg-Strauss syndrome) is a potentially life-threatening vasculitis rarely seen in the pediatric population and is commonly associated with moderate-to-severe peripheral blood eosinophilia, allergic rhinitis, and asthma. The most commonly involved organs include the lung and skin, although this disease can affect virtually any organ system, including the cardiovascular, gastrointestinal, renal, and central nervous systems (34). Other autoimmune diseases associated with HE in children include systemic lupus erythematosus, dermatomyositis, and inflammatory arthritis.

Other

Adrenal insufficiency has been associated with eosinophilia, possibly due to the loss of endogenous glucocorticoids. Other secondary causes of HE to consider in certain clinical situations include graft-vs.-host disease following hematopoietic stem cell transplantation, solid-organ transplant rejection, and sickle cell disease.

EPIDEMIOLOGY

The overall prevalence of HE within the pediatric population remains unknown as no population-based studies have been completed. Several retrospective cohort studies have focused on characterizing HE within the adult population (12, 35). However, a paucity of data on this subject exists within the pediatric literature, which is largely composed of single case reports and a small number of case series (36–38). Consequently, very little data exist with regards to the clinical presentation, underlying etiology, and prognosis of HE in the pediatric population and how it may differ from the adult population. Recently, Willams *et al.* published the largest retrospective cohort analysis comparing children and adults with HE. The study evaluated 291 patients (37 children, 254 adults) who presented to the National Institutes of Health for evaluation of unexplained HE between 1994 and 2012 (12). The most common diagnosis in both patient cohorts was idiopathic HES (46% of children and 47% of adults). A secondary cause for the HE was identified in only 14% of the children vs. 10% of the adults, with the most common etiology in both populations being helminth infection. Notably, all of the helminth infections in the pediatric cohort were *Toxocara* species compared to none in the adult cohort, providing evidence that an increased suspicion for *Toxocara* infection in children with HE is warranted. Primary immunodeficiency was noted more frequently in the pediatric vs. adult cohorts, although the number of cases was still limited (2/37 cases in pediatric cohort vs. 1/254 cases in adult cohort). In the patients that met criteria for HES, notable differences in the baseline characteristics in the pediatric population included male predominance, higher median peak AEC levels and higher median serum vitamin B12 levels. Clonal T-cell receptor rearrangement abnormalities were overrepresented in the adult population. The clinical manifestations were relatively similar between the two cohorts, with the exception of increased gastrointestinal involvement in children and increased pulmonary involvement in the adult cohort. Although the median peak AEC was almost twice as high in the pediatric cohort, mortality was low and similar to that in the adult cohort. Given that this study was compiled from a single-center, tertiary referral center (NIH), a referral bias is likely to exist within the study population, which thus may not completely reflect the true epidemiology of pediatric HE in the general population. Xiaohong *et al.* completed a retrospective analysis of the etiology of HE in 88 children admitted to the Children's Hospital of Zhejiang University School of Medicine in China between 2009 and 2015 (13). The most common etiology identified was infectious (parasite infections were the most common), followed by allergy and EGID. Immunodeficiency, hematologic neoplasms and EGPA were also noted in the study group. The limited amount of published data in children regarding the epidemiology and prognosis of HE makes definitive conclusions difficult; consequently, there remains a clear need for additional studies in this area to be completed.

CLINICAL EVALUATION

Defining the underlying mechanism propagating a child's eosinophilia is an important first step in the management of pediatric HE, as effective therapy depends upon knowing whether to target the eosinophils themselves or a secondary condition that is driving eosinophil production. Given the broad differential for pediatric HE, a systematic diagnostic approach is necessary.

In general, the degree of eosinophilia is rarely useful for identifying the underlying cause of the eosinophilia, with the exceptions occurring at the extremes of the AEC spectrum (ex. persistent mild eosinophilia [500–1,500 Eos/microL] is more likely to be seen in atopic disease; severe eosinophilia [≥100,000 Eos/microL] is more likely to be caused by a myeloid neoplasm)

(16, 39). Though much attention has focused on classifying patients on the basis of their blood AEC levels, organ dysfunction is ultimately caused by activated eosinophils infiltrating into the tissue, which is not always reflected by a concomitant increase in the AEC (16, 21). Conversely, patients with markedly elevated peripheral blood eosinophil counts may have little to no clinical symptoms (40). Consequently, the most important step in the initial evaluation of a child who presents with HE is to assess the presence and degree of illness symptoms, including signs of tissue/organ involvement. The urgency of the evaluation depends upon the acuity of the illness symptoms, the type of tissue/organ affected and the degree of organ dysfunction.

History

All children with HE should undergo a thorough history to address symptoms indicating possible organ involvement, prior medical history, exposures (dietary, travel, medications), and prior eosinophil counts. Symptoms that may identify specific organ system involvement include fever, weight loss, fatigue, skin rash, nasal congestion, wheezing, cough, dyspnea, chest pain, dysphagia, vomiting, loss of appetite, abdominal pain, diarrhea and arthralgias/myalgias. Medical history, such as recurrent infections, atopy, inflammatory bowel disease, prior malignancy, or failure to thrive, can also be important when considering the differential diagnosis. Dietary history should include risk for ingestion of raw or undercooked meat, particularly wild game meat that can increase risk for Trichinosis. Ingestion of fruits, vegetables, or soil (i.e., a child with pica) possibly contaminated by dog or cat feces can be a risk factor for Toxocariasis. A history of travel to parasite-endemic areas may also suggest risk for other parasitic etiologies. As noted above, however, lack of travel or specific risk factors does not necessarily eliminate parasitic infection as some helminths are endemic worldwide and can have long latency periods (i.e., *Strongyloides*). Medication exposure is also important to evaluate, particularly in those children who are taking medications regularly and therefore have ongoing exposure. Finally, it is often useful to review prior AEC data if available. Chronic HE in the absence of symptoms is reassuring and suggests that the evaluation can be done less urgently if the child is otherwise healthy. When reviewing AEC trends, it is important to remember that several factors can transiently decrease eosinophil counts (i.e., steroids, bacterial or viral infections) causing the appearance of an eosinophilia that is waxing and waning.

Diagnostic Testing

All children who meet diagnostic criteria for HE (i.e., blood AEC ≥1,500 cells/microL on at least 2 separate occasions [interval ≥1 month] or marked tissue eosinophilia) or moderate-to-severe eosinophilia with illness symptoms should undergo an initial diagnostic evaluation to try to determine the underlying etiology (**Figure 2**). A careful physical exam should be completed at every visit, noting any fever, nasal obstruction, abnormal or decreased lung sounds, skin rashes, abdominal tenderness, hepatosplenomegaly, lymphadenopathy or joint redness/swelling. Laboratory evaluation should include complete blood count with differential to evaluate for abnormalities in the other blood cell lines. A peripheral blood smear should be reviewed to evaluate for white blood cell blasts or other blood dyscrasias that could suggest a primary hematologic disorder. If blasts are noted, LDH, uric acid and hematology/oncology consultation is indicated. Bone marrow examination (aspiration and biopsy) should be considered for any child whose initial evaluation demonstrates no clear secondary etiology and a primary hematologic cause of the eosinophilia remains possible. In addition, bone marrow

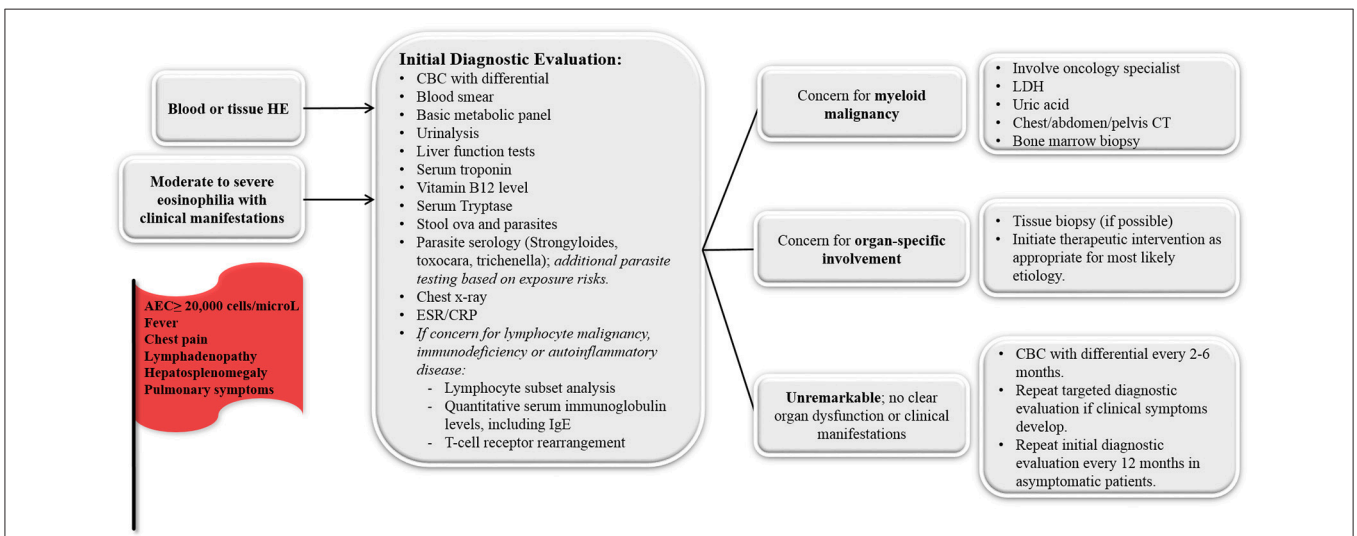

FIGURE 2 | Diagnostic approach for the child who presents with unexplained hypereosinophilia (HE) and/or moderate-to-severe eosinophilia with clinical manifestations. Concerning symptoms/laboratory findings that should prompt medical providers to pursue more intensive evaluation are noted in the red flag. ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; LDH, lactate dehydrogenase.

examination is appropriate for any acutely ill child with specific organ involvement and no clear underlying diagnosis, children with an eosinophil count >100,000 eosinophils/microL, or children with abnormal features on their peripheral blood smear (immature or dysplastic white blood cells, thrombocytopenia, or unexplained anemia). Serum chemistries, creatinine, and urinalysis should be completed to evaluate for evidence of renal or bladder involvement. Abnormal serum chemistries could also suggest underlying adrenal insufficiency. Liver function tests (to determine hepatic involvement) and cardiac troponin levels (for evidence of subclinical myocardial disease) should also be obtained. Patients with an elevated troponin levels should be further evaluated with electrocardiography and echocardiography. Serum B12 level should be obtained as a screening marker for myeloproliferative neoplasms and autoimmune lymphoproliferative syndrome (ALPS). Serum tryptase can be obtained to screen for systemic mastocytosis. Stool testing for ova and parasites and serologic testing for endemic parasites should also be routinely completed (*Strongyloides*, *Toxocara*, *Trichenella*). The indication for additional parasite testing is typically determined by exposure (diet, travel). Chest radiography should be completed to evaluate pulmonary involvement. Finally, in patients with a history of recurrent infections, lymphadenopathy, and/or hepatosplenomegaly, flow cytometry to evaluate lymphocyte subsets and immunoglobulin levels can be sent to screen for lymphocyte clonality and selective lymphocyte and immunoglobulin deficiencies. Additionally, T-cell receptor rearrangement studies can be useful to provide evidence of oligoclonality in the lymphocyte compartment. Finally, depending on risk factors, HIV testing may be indicated.

For those in whom the above evaluation is unremarkable and have no signs of organ involvement, repeat screening with a CBC with differential every 2–6 months to monitor AEC levels is a reasonable approach. If the AEC remains stable and the child remains healthy, repeating the above testing at 12-month intervals is appropriate. The development of new symptoms or an increasing AEC should prompt more immediate reevaluation.

Exceptions for the Acutely Ill Child With Eosinophilia

Any child with acute illness symptoms (fever, evidence of end-organ dysfunction) and unexplained eosinophilia or a child with an extremely high blood eosinophil count ($\geq$20,000 Eos/microL) requires hospitalization for immediate evaluation to determine the underlying cause. Notably, bacterial and viral infections typically cause a decrease in the blood eosinophil count, so the combination of fever and eosinophilia is an important red flag that should prompt consideration of other etiologies (15). Irrespective of the underlying etiology, the potential complications related to eosinophil tissue infiltration are similar (16). The most serious complications associated with HE are myocardial damage (i.e., myocarditis), pulmonary involvement and neurological involvement. Splinter hemorrhages and elevated serum troponin levels are indicative of cardiac involvement. Respiratory failure with pulmonary infiltrates are suggestive of pulmonary involvement. Neurological manifestations of HE include encephalopathy, sensory polyneuropathy and cerebral infarction**.** If evidence exists to suspect that the acute illness or organ dysfunction is secondary to tissue eosinophil infiltration, urgent therapy directed at reducing the eosinophilia (i.e., high dose glucocorticoids) is indicated (10). Patients with potential exposure to *Strongyloides* should receive concomitant empiric therapy with ivermectin to prevent corticosteroid-associated hyperinfection syndrome. In most situations, diagnostic laboratory testing to determine the underlying etiology of the eosinophilia should be obtained before initiating urgent empiric therapy, but treatment should not be delayed while awaiting the results of these studies.

CONCLUSIONS

There is a paucity of data focused on HE in children, and we continue to have much to learn about the differences between HE in adults and children. The evaluation of HE in children can be challenging given the broad differential diagnosis and the wide range of clinical consequences, which include self-resolving elevations in the AEC and life-threatening disorders. Given the broad differential diagnosis of HE, it remains essential to systematically approach the diagnostic evaluation of unexplained HE in children.

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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CASE REPORT
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Severe Eosinophilia in Myelodysplastic Syndrome With a Defined and Rare Cytogenetic Abnormality

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Myelodysplastic syndromes (MDS) are a heterogeneous group clonal disorders of hematopoietic stem cells (HSC) characterized by ineffective hematopoiesis that lead to variable grades of impaired blood cell production. Chromosomal aberrations are often detected in MDS patients and thus cytogenetic analysis is useful for the diagnosis of these disorders. Common recurring chromosomal defects, such as the −5/5q- and −7/7q- are relatively well characterized cytogenetic abnormalities in MDS, however, the biological significance of uncommon cytogenetic alterations is unknown. We report here, two cases of peripheral blood and bone marrow hypereosinophilia in patients with MDS harboring the unbalanced translocation der(1;7)(q10;p10), a poorly characterized cytogenetic abnormality that is found in certain myeloid malignancies, including MDS. The patients reported here presented hypereosinophilia that was refractory to steroids and cytotoxic therapy, leading to severe target tissue damage that ultimately resulted in fatal end-organ failure. Potential roles of the der(1;7)(q10;p10) aberrations in the pathogenesis of aggressive eosinophilia and disease prognosis are discussed here.

Keywords: eosinophilia, cytogenetic (CG) analyses, eosinophilic pneumonia, myelofibrosis, membranoproliferative glomerulonephritis (MPGN), myelodisdplastic/myeloproliferative disorders

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INTRODUCTION

Eosinophilia, defined as a peripheral blood eosinophil count exceeding 500 μl, can be associated with a large number of disorders and is causally classified into two general categories, (1) primary eosinophilia that results from disorders that are intrinsic to the eosinophil lineage and (2) secondary eosinophilia, which is caused by factors outside the eosinophil lineage (1). In most cases secondary eosinophilia represents a reaction to the overproduction of the eosinophilopoietic cytokines such as interleukin 3 (IL-3), interleukin 15 (IL-5), and granulocyte colony stimulating factor (GM-CSF), such as the eosinophilia observed in response to allergens, drugs or parasitic infections. Secondary eosinophilia may also develop in the context of autoimmune disorders or certain malignancies such as Hodgkin's lymphomas (2, 3).

Myeloid neoplasms, including myeloproliferative neoplasms (MPN), acute myeloid leukemia (AML) and MDS are well-known causes of primary eosinophilia, in which defined clonal disorders of HSC lead to the overproduction of eosinophils (4). Hence, myeloid malignancies carrying dysregulated fusion tyrosine kinase genes (TK), including platelet-derived growth factor (PDGF-AB), fibroblast growth factor receptor 1 (FGFR1), and the PCM1-JAK2 fusion product has been recently recognized by the World Health Organization (WHO) category of myeloid neoplasms with eosinophilia and rearrangement of PDGFRA, PDGFRB, or FGFR1, or with PCM1-JAK2. These distinctive group of myeloid

neoplasms present with eosinophilia, ranging from a mild increase in eosinophil count to a marked eosinophilia that can be directly responsible for target organ damage (1). Hypereosinophilia (HE) is defined as a persistent eosinophil count in the peripheral blood that exceeds 1,500 μl and may be associated with life-threatening organ damage as a result of tissue infiltration by eosinophils and the release of their granular contents (5, 6).

MDS are clonal disorders of HSCs characterized by the ineffective blood cells production leading to variable grades of cytopenias in the circulating blood (7). Overall, the incidence of MDS ranges from 3 to 5 per 100,000 individuals, however, in individuals over 70 years old, the incidence increases to about 50 per 100,000 inhabitants (8).

Typically, the bone marrow (BM) of patients with MDS shows increased cellularity with variable grade of dysplasia of bone marrow cells, ranging from erythroid hyperplasia to defective maturation in the myeloid series an increase in the number of blasts, thus patients with MDS have an exceptional increased risk for progression to AML and although most MDS cases arise *de novo*, some cases are caused by mutagenic insults, such as the prolonged exposure of the BM to cytotoxic chemotherapy (9–11).

Chromosomal abnormalities are detected in approximately 30–70% of MDS patients and the presence of cytogenetic abnormalities correlates with disease prognosis, consequently, disease karyotype constitutes an important component of the International Prognostic Scoring System (IPSS) and the revised IPSS-R (IPSS-R), which are the most broadly utilized scoring tools for assessing prognosis in patients with MDS (11, 12). Notably, the prognostic relevance of cytogenetic abnormalities in MDS is largely limited to the most frequent ones, including del(5q),−7/del(7q),−8,-18/del (18q), del (20q),-5,-Y,-17/del (17p) (12, 13) and hence the biological relevance of less common chromosomal alterations in MDS is poorly understood. This is particularly important considering that over 600 different cytogenetic categories had been identified in MDS patients (14).

The unbalanced translocation, der(1;7)(q10;p10), is a rare cytogenetic abnormality that is detectable in some myeloid malignancies, including MDS (1–3%), AML (1–2%), and MPN (1%) (15, 16). MDS patients harboring this translocation appear to have specific molecular and clinical features, however, given its scarceness compared with better characterized cytogenetic groups found in AML/MDS, several aspects of this entity have not been described (15, 17).

Here, we report two MDS cases with (1;7)(q10;p10) translocation in previously healthy men who presented with severe hypereosinophilia and target organ damage leading to fatal complications. In both cases, a large number of eosinophils were detectable in the peripheral blood and BM and eosinophilia was refractory to the treatment with corticosteroids.

TABLE 1 | Laboratory test at the diagnosis in case 1.

(Peripheral blood)		(Biochemistry)			
WBC	7,700/μL	CRP	2.66 mg/dL	ANA	Negative
Stab	5.0%	Ca	8.8 mg/dL	Jo-1-Ab	Negative
Seg	33.5%	UA	4.8 mg/dL	RF	<7.0 U/mL
Eo	53.5%	BUN	12.0 mg/dL	PR-ANCA	<1.0 U/mL
Baso	4.0%	Crea	0.90 mg/dL	MPO-ANCA	<1.0 EU
Mono	2.5%	TP	6.6 g/dL	TARC	119 pg/mL
Lymph	1.5%	Alb	3.5 g/dL		
RBC	366 × 10^4/μL	AST	15 U/L		
HGB	8.6 g/dL	ALT	11 U/L	(Arterial blood gas analysis)	
PLT	25.5 × 10^4/μL	LDH	243 U/L	pH	7.549
(Infectious marker)		ALP	176 U/L	pCO2	27.1 mmHg
HBs-Ag	(–)	T-Cho	156 mg/dL	pO2	68.2 mmHg
HBs-Ab	(–)	TG	86 mg/dL	HCO3	23.1 mmol/L
HCV-Ab	(–)	sIL-2R	495 U/mL		
HIV-Ab	(–)	IgE	254 IU/mL		

ANA, anti-nuclear antibody; RF, rheumatoid factor; PR3-ANCA, proteinase3-antineutrophil cytoplasmic antibody; MPO-ANCA, myeroperoxidase-antineutrophil cytoplasmic antibody; TARC, Thymus and activation-regulated chemokine.

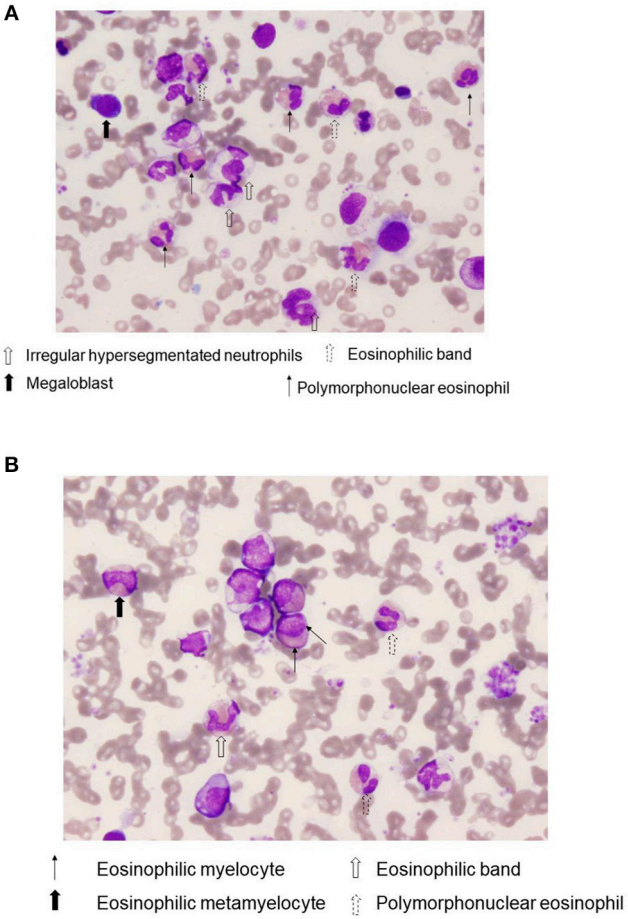

FIGURE 1 | Bone marrow eosinophilia. A representative Wright-Giemsa stain, (×400) of bone marrow aspirate smears from case 1. Notice the presence of various immature eosinophils, including eosinophilic band and Polymorphonuclear eosinophils **(A)**, as well as eosinophilic myelocyte and eosinophilic metamyelocyte **(B)**.

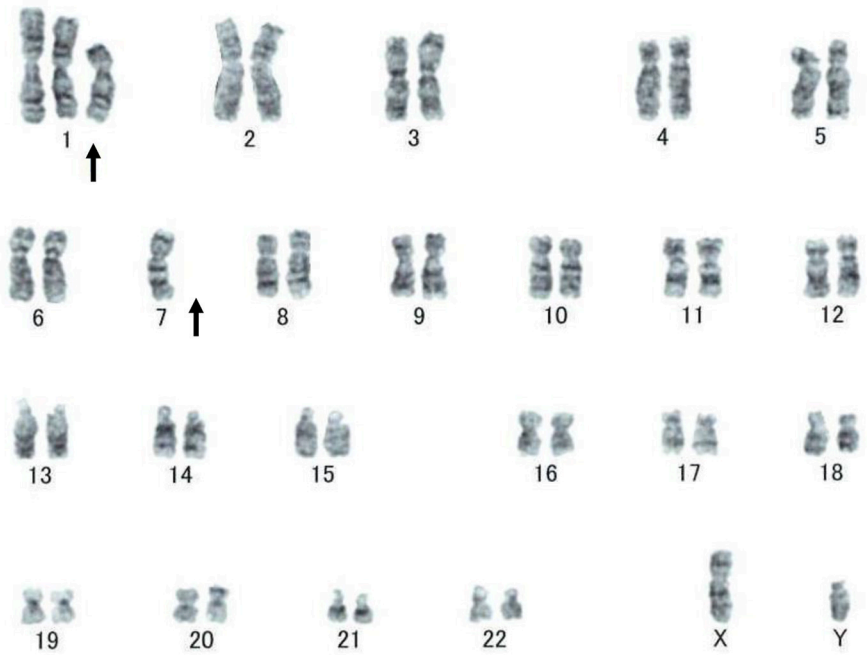

FIGURE 2 | Chromosomal analysis of case 1. A representative metaphase chromosomal analysis of the bone marrow cells derived from case 1. Arrows indicate the allelic imbalance of trisomy 1q and monosomy 7q.

CASE 1

A previously healthy 67-year old male presented to another hospital complaining of dry cough, wheezing and mild dyspnea. Physical examination was unremarkable, except for the signs of bronchoconstriction. The laboratory tests revealed a marked increase in the number of eosinophils in the peripheral blood and thus a diagnosis of eosinophilic asthma was made. He was given inhaled bronchodilators and corticosteroids which induced a moderate improvement of symptoms. Four months later his symptoms worsened and was then diagnosed as Chronic Eosinophilic Pneumonia and oral methylprednisolone was added, which induced a minor improvement of symptoms without affecting eosinophilia. In addition, dry cough and respiratory discomfort reoccurred along with tapering the methylprednisolone to 10 mg/day. He was referred to our hospital in July 2016 for further evaluation. He had no smoking history and his medical history was unremarkable. On examination, vital signs were stable except for requiring 1L of nasal cannula oxygen. The SaO2 was 96% on 1L oxygen. He had decreased breath sounds in the lower right lung field with fine crackles. He had no raised JVP, murmurs, gallop or peripheral edema. Chest x-ray revealed right ground glass opacities (GGOs). A high-resolution CT scan revealed GGOs surrounded by consolidation in the right lower lung field.

Main laboratory findings were as follows: WBC 7,770/μl, with eosinophils 52.3%; red blood cells (RBC) 366 × 10^4/μl; hemoglobin (Hb) 8.6 g/dl; Platelets (Plt) 25.5 × 10^4/μl; C-reactive protein 2.66 mg/dl (normal <0.3 mg/dl); lactate dehydrogenase (LDH) 243 IU/L (normal range <225 IU/L); IgE 254 IU/ml (normal <232 IU/ml); Soluble IL-2 receptor (sIL-2R) 495 U/ml (normal 150–505 U/ml); serum thymus and activation-regulated chemokine (TARC) 119 pg/mL (**Table 1**).

Bone marrow (BM) aspirate demonstrated infiltration of eosinophils (26.2% of the total BM cells) without dysplasia. The percentage of blast cells in the BM was 2.4% with trilineage dysplasia seen in megakaryocytes, as well as in myeloid and erythroid lineages (**Figures 1A,B**). Chromosomal analysis of BM cells showed 46, XY, +1, der(1;7) (q10;p10) in 13 of 20 metaphases (**Figure 2**). He was negative for PDGFRA, PDGFRB rearrangement or FGFR1, or with JAK2 mutations. Accordingly, a diagnosis of MDS (refractory cytopenias with multilineage dysplasia type) was made, and consequently was categorized as intermediate risk, according to IPSS-R and intermediate-1 risk according to the IPSS scoring.

The patient was treated with 75 mg/m^2 azacitidine (AZA) once daily for five consecutive days on a 28-day cycle based (**Figure 3**). After the first cycle of AZA, the number of eosinophils further increased (WBC 6,570/μl with eosinophils 60.2%), which coincided with the tapering of 10 mg/day. After completing the

second cycle of AZA treatment and increasing prednisolone to 30 mg/day, we noticed a substantial decrease in the right lung infiltrate. However, before starting the third cycle of AZA, dyspnea, cough and wheezing significantly worsened and a new infiltrate was detected in the left lower lung field (**Figure 4**). The infiltrate was refractory to various antimicrobial regimens combined with methylprednisolone and the patient's condition deteriorated, leading to respiratory failure and death.

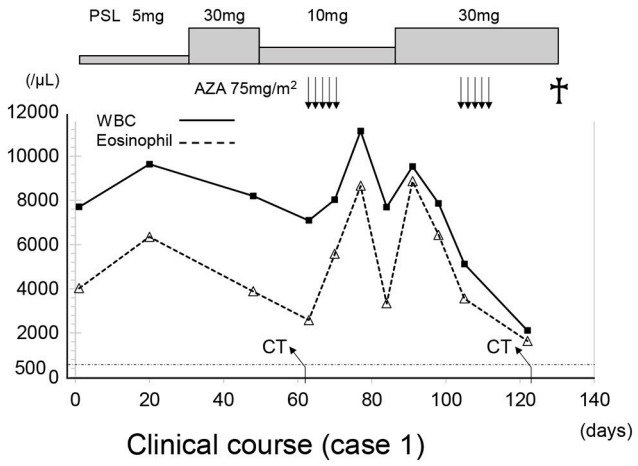

FIGURE 3 | The clinical course of case 1. The X axis indicate the time (days). AZA, azacitidine; WBC, white blood cells; PLS, prednisolone.

CASE 2

A 23-year old male presented to our hospital in April 2005 with maculopapular rash involving >50% of his body and intermittent fever of several weeks of evolution. He had no significant past medical history and denied symptoms of fatigue, body weight loss, or night sweats. Physical examination was unremarkable, except for the presence of a maculopapular rash covering nearly 50% of the skin surface.

Laboratory findings were as follows: WBC 24,300/μl with eosinophils 39.0%; RBC 263 × 10^4/μl; Hb 10.0 g/dl; Plt 12.5 × 10^4/μl; C-reactive protein 1.66 mg/dl (normal <0.3 mg/dl); LDH 363 IU/L (normal range <225 IU/L); creatinine 0.95 mg/dl and estimated glomerular filtration rate (eGFR) of 65.7 ml/min/1.73 m^2 (according to the modification of the CKD Epidemiology Collaboration Equation for Japanese); IgE 1,156 IU/ml (normal <232 IU/mL). A BM aspirate demonstrated significant infiltration of eosinophils (23% of total BM cells) without dysplasia and a 0.3% of blast cells with dysplasia in the erythroid lineages. Chromosomal analysis of BM cells showed 46, XY, +1, der(1;7) (q10;p10) in 4 of 20 metaphases (**Figure 5**). He was negative for PDGFRA, PDGFRB rearrangement or FGFR1, or with JAK2 mutations.

A diagnosis of MDS (Refractory anemia type) with hypereosinophilic syndrome (HES) was made and was subsequently categorized as low risk according to IPSS-R and intermediate-1 according to the IPSS scoring.

He was initially treated with methylprednisolone (1.0 mg/kg/day) in an attempt to control HES. Within approximately

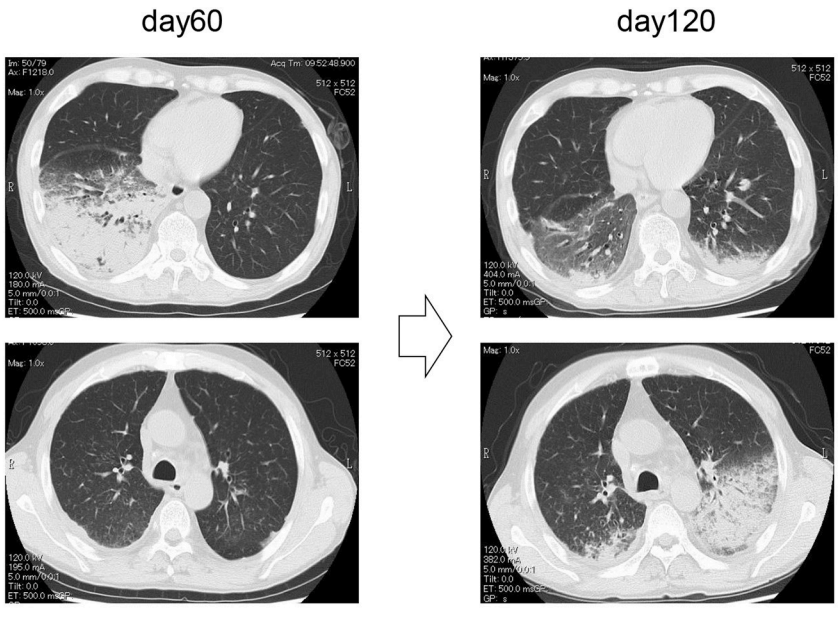

FIGURE 4 | Representative chest computer tomography of case 1, showing consolidation in the right lower lung field at day 60 and consolidation in the left lower lung field at day 120.

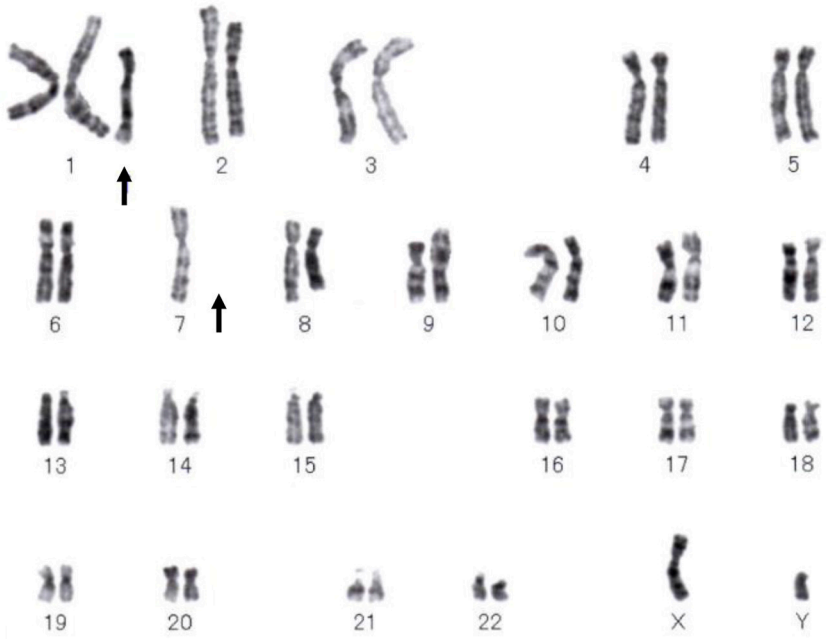

FIGURE 5 | Chromosomal analysis of case 2. A representative metaphase chromosomal analysis of the bone marrow cells derived from case 2. Arrows indicate the allelic imbalance of trisomy 1q and monosomy 7q.

1 week, significant improvement in the clinical condition was observed, and eosinophil count returned to normal values. However, the maculopapular rash and eosinophilia reoccurred along with tapering the methylprednisolone to 25 mg/day along with a rapid and progressive increase in the serum levels of creatinine (7.61 mg/dl) (**Table 2** and **Figure 6**). A renal biopsy was performed for histopathological diagnosis, which showed globally sclerotic glomerulus in four out of 14 glomeruli analyzed by light microscopy. The remaining 10 glomeruli appeared enlarged and the marked diffuse thickening of glomerular basement membranes (GBM) and mesangial cell proliferation were also noted. Mesangial interposition neither endocapillary hypercellularity nor crescent formation in these glomeruli were noted periodic acid methenamine silver staining revealed duplication of the capillary wall (**Figure 7**). A Congo red staining for amyloid protein was negative. A diagnosis of membranoproliferative glomerulonephritis (MPGN) was made, however the patient's condition deteriorated and died from septicemia.

DISCUSSION

Cytogenetic aberrations are frequently detectable in patients with MDS. Some of those molecular changes have been linked to disease pathogenesis and their presence is utilized for assessing disease prognosis (13). Here we report two male patients who presented with *de novo* MDS, harboring the unbalanced translocation der(1;7)(q10;p10) associated with aggressive hypereosinophilic syndromes. In both cases, eosinophilia was not responsive to corticosteroids treatment and ultimately lead to severe target tissue damage and fatal end-organ failure.

In contrast to other hematological malignancies, in which specific chromosomal arrangements are distinctive molecular features of the disease, MDS are frequently associated with a variable number of cytogenetic abnormalities, which appear to determine the heterogeneous clinical phenotype of these disorders (18, 19).

A recurrent molecular characteristic of MDS is the loss of genetic material, via deletions and monosomies, while the gain of genetic material is uncommon. Consequently, such a loss of genetic material is consistent with the assumption that the deletion or inactivation of tumor suppressor genes, rather than the activation of oncogenes, constitutes the main molecular mechanism implicated in the development of MDS (12, 20).

The unbalanced translocation, der(1;7)(q10;p10), is a nonrandom chromosomal abnormality that occurs through a mitotic recombination between chromosome 1 and chromosome 7 that generates two copies of chromosome 1 and a single copy

TABLE 2 | Laboratory test at the diagnosis of MPGN in case 2.

(Peripheral blood)		(Biochemistry)			
WBC	22,300/μL	CRP	15.18 mg/dL	ANA	Negative
Stab	1.3%	Ca	7.6 mg/dL	RF	<7.0 U/mL
Seg	43.3%	UA	4.8 mg/dL	C3	73 mg/dL
Eo	26.0%	BUN	53.0 mg/dL	C4	25 mg/dL
Baso	0.0%	Crea	7.61 mg/dL	CH50	40.1 U/mL
Mono	9.3%	TP	5.3 g/dL	PR-ANCA	<1.0 U/mL
Lymph	4.3%	Alb	2.4 g/dL	MPO-ANCA	<1.0 EU
RBC	290×10^4/μL	AST	22 U/L	Anti-GBM	<2.0 U/mL
HGB	9.4 g/dL	ALP	37 U/L	(Urinalysis)	
PLT	17.8×10^4/μL	LDH	536 U/L	pH	5.5
(Infectious marker)		ALP	700 U/L	Protein	3+
HBs-Ag	(–)	T-Cho	204 mg/dL	Sugar	±
HBs-Ab	(–)	HDL-Cho	29 mg/dL	RBC	30–49/HPF
HCV-Ab	(–)	TG	182 mg/dL	WBC	1–4/HPF
HIV-Ab	(–)	sIL-2R	4,273 U/mL	(Urine chemistry)	
		IgG	502 mg/dL	NAG	10.0 U/L
		IgA	130 mg/dL	β-2MG	54,985 μg/day
		IgM	79 mg/dL	Protein	7.9 g/day
		IgE	6,760 IU/mL		

C3, complement 3; C4, complement 4; CH50, complement hemolytic activity; Anti-GBM, anti-glomerular basement membrane.

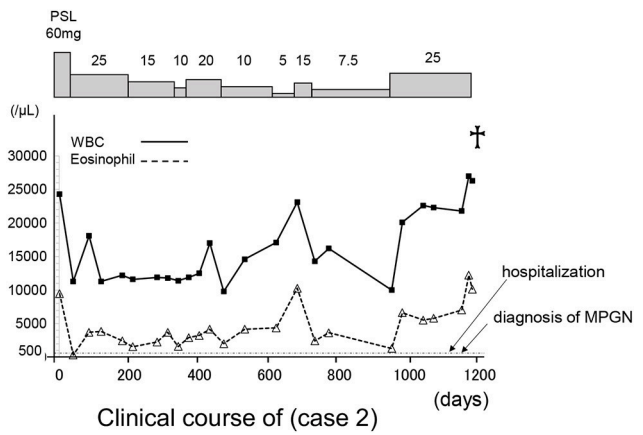

FIGURE 6 | The clinical course of case 2. The X axis indicate the time (days). WBC, white blood cells; PLS, prednisolone.

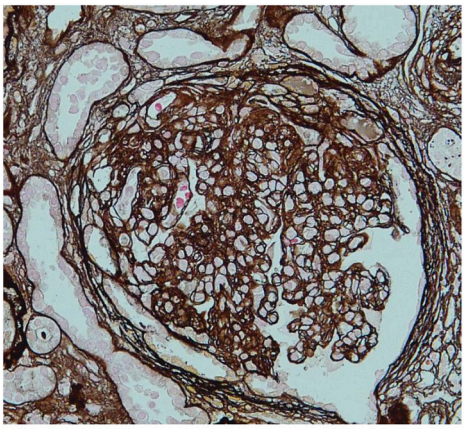

FIGURE 7 | Representative images of periodic acid methenamine silver staining (high magnification) of renal specimen from patient 2 showing duplication of the capillary wall consistent with membranoproliferative glomerulonephritis.

of the intact chromosome 7 leading to an allelic imbalance of trisomy 1q and monosomy 7q. (15–17).

There is some controversy regarding the prognosis of der(1;7)(q10;p10). Early reports involving small numbers of patients with der(1;7)(q10;p10) suggested this entity correlates with unfavorable prognosis and increased risk of progression to AML (21, 22), however, in subsequent studies that included relatively larger number of cases, the presence of this translocation in MDS indeed correlated with a better clinical outcome, with patients showing milder anemia and lower blast counts at diagnosis and a tendency to have less trilineage dysplasia and a slower progression to AML (15, 17). Similarly, in a more recent study, newly diagnosed MDS patients with der(1;7)(q10;p10) were less likely to have excess blasts or multilineage dysplasia and overall showed higher hemoglobin levels compared to patients with monosomy 7 or those with 7q. However, the three groups were otherwise similar in regard to other laboratory and clinical features, including overall survival (23). These findings are consistent with the results of large study involving a cohort of 1,593 MDS patients (944 Germans and 695 Japanese). In this study, clinical outcomes of der(1;7)(q10;p10) patients were significantly better than those having−7/del(7q) or 1q gain alone. Interestingly, der(1;7)(q10;p10) was found to be 10 times more frequent in Japanese than in Germans (4.5 vs. 0.43%) and the strong male predominance (86% of cases) of this entity was also confirmed (Okuda et al. The 80th annual meeting of the Japanese Society of Hematology, 2018, abstract OS3-5C-3).

Most MDS patients develop symptoms related to cytopenias and anemia, although isolated neutropenia and thrombocytopenia can also occur. In addition, some MDS patients may also present with eosinophilia (7). Among 288 patients with *de novo* MDS retrospectively analyzed by Matsushima and colleagues, 36 (12.5%) fulfilled the criterion for BM eosinophilia (eosinophils in BM exceeding 5%) and those with BM eosinophilia showed a higher tendency to evolve to AML and had a worst overall survival. In the same study, specific cytogenetic aberrations, especially, abnormalities in chromosome 7, complex karyotypes and i(17q), were associated with an increase in BM eosinophils (24). Nonetheless, it must be noted that the frequency of eosinophilia in MDS der(1;7)(q10;p10) has not been comprehensively investigated, likely due to the rarity of this entity. In the study by Slovak and colleagues, none of the 12 MDS patients with der(1;7)(q10;p10) showed eosinophilia (17). On the other hand, Sanada and

colleagues documented eosinophilia in the peripheral blood of six out of 77 patients with der(1;7)(q10;p10), however none of those patients had eosinophilia in the BM (15).

The optimal treatment for MDS patients with der(1;7)(q10;p10) is another important aspect that has not been defined and patients are currently managed following the current treatment algorithm for MDS. In isolated case reports, the response with AZA was good (Imi et al. The 75th annual meeting of the Japanese Society of Hematology, 2013, abstract PS2-90), although the limited number of cases and the lack of controlled studies is a handicap to assume that AZA is the optimal treatment for this entity. In the cases reported here, AZA was ineffective in case 1 and by the time of diagnosis of case 2, AZA has not been approved for clinical use in Japan.

Corticosteroids are the first line therapy for all types of hypereosinophilia preventing end-organ damage caused by these disorders, however recent studies suggest that certain subtypes are less responsive to these agents and many either exhibit resistance or relapse during steroid tapering or withdrawal (4, 25, 26), as observed in the case presented here. On the other hand, tyrosine kinase inhibitors are highly effective in those cases harboring fusion gene as a cause of hypereosinophilia. For example, imatinib is the drug of choice for patients with FIP1L1-PDGFRA and ruxolitinib is the first drug of choice in hypereosinophilia driven by gain of function mutations involving JAK/STAT axis (4, 25, 26). None of those mutations were detectable in the cases presented here. Novel monoclonal antibodies capable of depleting circulating eosinophils, such as mepolizumab (targeting IL-5), omalizumab (anti-IgE), and dupilumab (anti-IL4α receptor subunit) have been recently approved by the FDA have therapeutic potential for the management of hypereosinophilia, especially those cases refractory to corticosteroids and not associated with tyrosine kinase mutations (25, 26).

Does der(1;7)(q10;p10) translocation play a role in the pathogenesis of MDS or in the development of eosinophilia? So far, no specific molecular targets have been identified for this translocation and it is unknown if the loss of 7q and/or gain of 1q play a direct role in the pathogenesis of this entity. It is plausible that those chromosomal aberrations may generate genetic rearrangement of genes encoding eosinophilopoietic cytokines. Alternatively, prominent eosinophilia has been reported in myeloid leukemia with translocations or deletions of chromosome 7 (27). Interestingly, the deletion of the long arm of chromosome 7 (7q) has been reported in association with eosinophilia in isolated cases of myeloid malignancies such as myelomonocytic leukemia with BM eosinophilia (28).

MDS with der(1;7)(q10;p10) has distinctive clinical and pathological characteristics, however, this translocation is found in a very small fraction of patients, and its pathogenic relevance to MDS and eosinophilia is unclear. Newer high-resolution whole-genome molecular approaches, such as comparative genomic hybridization and gene expression microarray studies, are expected to provide a more comprehensive analysis to unravel a potential role of this particular cytogenetic abnormality in disease pathogenesis.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Kindai University ethical committee. The protocol was approved by the Kindai University ethical committee. Written informed consent in accordance with the Declaration of Helsinki was obtained from the patient for analysis, publication of this report, and any accompanying images.

AUTHOR CONTRIBUTIONS

SR collected information and data and wrote the manuscript. JE conceived the study and wrote the manuscript. YM collected data. HT collected and analyzed data. IM analyzed data and supervised the study.

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