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Mepolizumab attenuates airway eosinophil numbers, but not their functional phenotype in asthma

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At a Glance Commentary: (168 words, 200 max)

Scientific Knowledge on the Subject: Interleukin (IL-5)-blocking antibodies, such as mepolizumab, nearly eliminate circulating eosinophils and reduce the frequency and severity of exacerbations in patients with severe eosinophilic asthma. However, the effect of *in vivo* IL-5 neutralizing antibodies on the functional phenotype of eosinophils in the circulation and airway remains largely unknown.

What This Study Adds to the Field: This study of subjects with mild allergic asthma shows that mepolizumab prevents the slight rise in circulating eosinophils and decreases the dramatic influx of eosinophils into the airway 48 h after segmental bronchoprovocation with allergen. Eosinophils circulating after mepolizumab are shown to have less mRNA encoding eosinophil-derived neurotoxin and reduced surface expression of the IL-3 receptor. In contrast, airway eosinophil expression of the IL-5-responsive activation markers CD23, CD44, and CD69, and receptors for the IL-5-family cytokines IL-5, IL-3, and GM-CSF is not altered by mepolizumab indicating that airway eosinophils can be readily activated after administration of anti-IL-5 antibody. These observations may explain why some patients continue to have asthma exacerbations after anti-IL-5 treatment despite diminished numbers and activity of circulating eosinophils.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

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Abstract (243 words/250 max)

Rationale: Mepolizumab, an interleukin-5 blocking antibody, reduces exacerbations in severe eosinophilic asthma patients. Mepolizumab arrests eosinophil maturation; however, the functional phenotype of eosinophils that persist in the blood and airway after administration of interleukin-5 neutralizing antibodies has not been reported.

Objective: To determine the effect of anti-IL-5 antibody on the numbers and phenotypes of allergen-induced circulating and airway eosinophils.

Methods: Airway inflammation was elicited in mild allergic asthma participants by segmental allergen challenge before and one month after a single intravenous 750 milligram dose of mepolizumab. Eosinophils were examined in blood, bronchoalveolar lavage, and endobronchial biopsies forty-eight hours after challenge.

Measurements and Main Results: Segmental challenge without mepolizumab induced a rise in circulating eosinophils, bronchoalveolar lavage eosinophilia, and eosinophil peroxidase deposition in bronchial mucosa. Interleukin-5 neutralization before allergen challenge abolished the allergen-induced rise in circulating eosinophils and expression of interleukin-3 receptor; whereas, airway eosinophilia and eosinophil peroxidase deposition were blunted but not eliminated. Before mepolizumab treatment, bronchoalveolar lavage eosinophils had more surface interleukin-3 and granulocyte-monocyte colony-stimulating factor receptors, CD69, CD44, and CD23 and decreased

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interleukin-5 and eotaxin receptors than blood eosinophils. This activation phenotype indicated by bronchoalveolar lavage eosinophil surface markers, as well as the release of eosinophil peroxidase by eosinophils in the bronchial mucosa was maintained after mepolizumab.

Conclusions: Mepolizumab reduced airway eosinophil numbers, but had limited effect on airway eosinophil activation markers suggesting these cells retain functionality. This observation may explain why interleukin-5 neutralization reduces but does not completely eradicate asthma exacerbations.

Study is registered with www.clinicaltrials.gov (NCT00802438)

Key words: Allergen challenge, IL-5, IL-3 receptor

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INTRODUCTION (462 words/450 max)

Airway eosinophils are a prominent component of allergic asthma. Asthma patients with persistent eosinophilic airway inflammation tend to have more severe disease and frequent exacerbations (1). In some patients, titrating inhaled corticosteroid dose to reduce sputum eosinophils is an effective approach to lessen exacerbations (2). Nonetheless, in a subset of patients, elevated eosinophils persist despite high-dose corticosteroids treatment. Mepolizumab, a humanized monoclonal antibody with specificity for the pro-eosinophilic cytokine IL-5, was developed to target eosinophils (3), and received FDA approval under the trade name Nucala (GlaxoSmithKline, 2015)(4). In patients with severe eosinophilic asthma, mepolizumab treatment reduced corticosteroid requirement (5, 6) and exacerbation frequency/severity (7-10). Collectively, these studies point to airway eosinophilia as a key component of some asthma exacerbations.

How eosinophils contribute to exacerbations is not completely understood. Eosinophil degranulation/cytolysis releases the cationic granule proteins major basic protein (MBP)-1, eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), and eosinophil peroxidase (EPX) that may contribute to epithelial damage and excessive repair (11, 12). *Ex vivo* studies demonstrating eosinophil generation of MMP-9 (13), TGF-ß1 (14), activin A (15), and semaphorin 7A (CD108) (16) support their role in tissue injury, repair, and remodeling. Furthermore, human eosinophils can influence

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airway inflammation by releasing cytokines (17), chemoattractants (18), and IL-1ß (19), which is essential for Th17 development (20).

In addition to being required for eosinophil maturation and egress from the bone marrow (21), IL-5 promotes eosinophil survival and migration; release of granule proteins, cytokines, and growth factors (21); and surface expression of activation markers including CD69, CD44, and CD23; and the integrins αM (CD11b) and β2 (CD18) (22). The IL-5 receptor is composed of an IL-5-specific α-chain and a signal-transducing ß-chain that is common to IL-3 and GM-CSF receptors (21). Thus, the common ß-chain/IL-5-family cytokines have similar and overlapping effects on mature eosinophils (21). Compared to their circulating counterparts, bronchoalveolar lavage (BAL) eosinophils have heightened levels of IL-3Rα and GM-CSFRα; whereas, IL-5Rα appears to be down-regulated (23). *Ex vivo* exposure of blood eosinophils to IL-5 upregulates GM-CSFRα and IL-3Rα while reducing IL-5Rα (24, 25). Consequently, expression of IL-5-family cytokine receptors on airway eosinophils may be controlled, in part, by *in vivo* IL-5 exposure. We hypothesized that in addition to reducing eosinophil numbers, mepolizumab would affect the activation phenotype of circulating and airway eosinophils and impact responsiveness to IL-3 and/or GM-CSF by altering receptor expression.

To test this hypothesis, we used segmental bronchoprovocation with allergen (SBP-Ag) before and after anti-IL-5 (mepolizumab) administration. Compared to the more commonly used whole lung inhalation challenge, SBP-Ag induces an intense, but

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localized airway eosinophilia in allergic subjects (26). This approach permitted evaluation of allergen-induced activation and recruitment of eosinophils in subjects who have received anti-IL-5.

Some of the results of these studies have been previously reported in the form of an abstract (27).

METHODS (845 words)

Participants

The University of Wisconsin-Madison Health Sciences Institutional Review Board approved the study and each participant provided written informed consent. Subjects had mild allergic asthma (aeroallergen skin prick-test positive, FEV_1 albuterol reversibility ≥12% or methacholine PC_{20} ≤8 mg/mL, pre-albuterol FEV_1 ≥70% and post-albuterol FEV_1 ≥80%) and were not receiving asthma controller medications. Additional inclusion/exclusion criteria and participant disposition (Figure E1) are provided in Online Supplement.

Study design

The design of this experimental, non-clinical study is shown in Figure 1. Participants with mild allergic asthma underwent whole lung inhalation challenge (WLAC) to determine $AgPD_{20}$, the allergen provocation dose resulting in a 20% reduction in FEV_1. An early allergic reaction, defined as a drop in FEV_1 ≥20% within 1 hour of challenge

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(EAR>20%), was required for continuation. One month later, a baseline bronchoscopy with bronchoalveolar lavage (BAL) was performed in two subsegments followed by segmental bronchoprovocation with allergen (SBP-Ag) at a dose of 1 (low dose) or 20% (high dose) of each subject's Ag-PD_{20}. Forty-eight hours later, bronchoscopy with BAL was performed in each subsegment and biopsies (Bx) were taken from an Ag-naïve site and from the high-dose segment. A BAL eosinophilia >50% in the high dose segment was required for subject continuation in the study. One month after SBP-Ag, spirometry was performed to confirm continued asthma stability and then a single 750 mg iv dose of mepolizumab was administered. One month after dosing, circulating eosinophils were required to be ≤80% of the baseline value, or <100 eosinophil/µl. If the threshold was not met, mepolizumab dosing was repeated. If the threshold was met, participants underwent bronchoscopy with BAL performed in two subsegments followed by SBP-Ag at the same 1 and 20% Ag-PD_{20} doses administered for the baseline challenge before mepolizumab. 48 hours after SBP-Ag, bronchoscopy with BAL was performed in each subsegment and Bx were taken from an Ag-naïve site and from the high-dose segment.

WLAC

WLAC was performed to determine the AgPD_{20} for SBP-Ag and to screen for subjects with an airway response to Fel d1 (Bayer Allergy Products), GS Ragweed mix (Greer Labs), or *Dermatophagoides farinae* (Miles Allergy Products). To determine the AgPD_{20}, consecutively greater concentrations of nebulized allergen were inhaled at 15-minute intervals until FEV_1 fell ≥ 20% compared to baseline diluent challenge.

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SBP, BAL, and biopsy, and anti-IL-5 (mepolizumab) dosing

A baseline (D0) bronchoscopy with BAL (4X40 mL of 0.9% NaCl) was performed in two different bronchopulmonary subsegments that were in close proximity. Due to concern that mepolizumab's anti-eosinophil effect might be overcome by an intense SBP-Ag-induced airway eosinophilia, two doses of allergen were administered in two separate segments. Allergen was instilled through a wedged bronchoscope into each of the two subsegments at either 1% or 20% of the participant's $AgPD_{20.}$ Forty-eight hours later (D2), bronchoscopy with BAL (4X40 mL of 0.9% NaCl) was performed in each subsegment and endobronchial biopsies were obtained at the site of the 20% SBP-Ag challenge and from an allergen-naïve (control) site. One month after SBP-Ag, a single 750 mg dose (or in one participant, 2 doses) of mepolizumab (generously donated by GlaxoSmithKline, study No. MEA110170) was administered intravenously. Circulating eosinophils were monitored weekly for 6 months following mepolizumab administration. One month after administration of mepolizumab, bronchoscopy, BAL, SBP-Ag, and Bx were performed by the same methods as before mepolizumab, except in the contralateral lung. SBP-Ag was administered at identical concentrations in segments/subsegments in the contralateral lung.

Immunocytochemistry

EPX was detected as described by Protheroe, et al. (28) and in the Online Supplement using monoclonal anti-EPX (clone MM25-82.2.1) antibody (28) provided by J.J. and N.A. Lee, Mayo Clinic, AZ).

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IHC scoring

A numerical value (0-10) was assigned based on modification of the semiquantitative scoring system established by Protheroe, *et al*. (28) and described in Online Supplement. The staining score was calculated as the sum of each of these criteria: 1) the gross EPX score (0, 0.5, or 1) based on the overall staining profile; 2) the percent area (0-4) of mucosal EPX staining; and 3) the degree (0-5) of EPX staining.

Flow cytometric analysis

See Online Supplement for methods and gating strategy (Figure E2).

Eosinophil purification

Blood and BAL eosinophils were purified by negative selection and density-gradient centrifugation, respectively (See Online Supplement). Only cells with >96% purity were used for qPCR analysis (Table EI).

Reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed as previously reported (15) using specific primers described in the Online Supplement. Data were normalized to ß-glucuronidase and presented as fold change of $2^{-\Delta\Delta Ct}$ compared to each individual's pre-mepolizumab circulating eosinophils, which was fixed at 100%.

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ELISAs

See Online Supplement for the highly sensitive “in-house” sandwich ELISAs.

Statistical analysis

Baseline subject characteristics were compared by study completion status using the two-sample t-test for continuous measures and Fisher's exact test for categorical measures. Outcome data were compared using longitudinal mixed-effects models with fixed-effect terms for pre/post mepolizumab, pre/post antigen challenge, and antigen dose subsegment and a random-effect subject term (29). Further details are in Online Supplement. A two-sided p-value<0.05 was considered significant. Analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC.).

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RESULTS

Subject Characteristics

As shown in Figure E1, 38 participants were enrolled for screening. Twenty-six participants met the inclusion criteria and underwent WLAC, 17 qualified to undergo bronchoscopy, and 10 had a sufficient eosinophil response following SBP-Ag to continue the study and receive mepolizumab. Baseline characteristics of participants who completed the study were not significantly different from those who were discontinued (Table 1). The subjects who completed the study had mild asthma, which was stable at the time of mepolizumab infusion (Table E1). Only one participant required a second dose of mepolizumab to achieve an 80% reduction in circulating eosinophils one month after dosing. 48 hours after SBP-Ag without mepolizumab, there was a small, but statistically significant decrease in FEV_1 % predicted and an increase in fractional exhaled nitric oxide (FeNO) (Table II). After mepolizumab, allergen challenge still induced a significant rise in FeNO, and levels did not correlate with BAL eosinophil numbers or percentage after segmental allergen challenge (not shown). In these subjects with mild allergic asthma, mepolizumab had no significant effect on pulmonary function before or after challenge (Table II).

Effect of anti-IL-5 on circulating and airway eosinophils

One week after mepolizumab administration, the median value for circulating eosinophils decreased by ~75% (Figure E3). The median % decrease in eosinophil levels remained >75% for 3 months (n=10) and returned toward the pre-dosing level by 6 months (n=8). Table EII shows there were no significant changes in absolute numbers

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of neutrophils, monocytes, or lymphocytes; however, basophils, which are known to express IL-5Rα (3) were reduced by ~33% at 1 week and this reduction persisted for 2 months after dosing.

While there was no change in total circulating WBCs (Figure 2 and Table EII), localized airway allergen challenge induced an increase in circulating eosinophils ($p=0.006$) that did not occur when allergen challenge was performed after mepolizumab (Figure 2).

Forty-eight hours after SBP-Ag without mepolizumab, there was an allergen dose-dependent influx of inflammatory cells with eosinophils accounting for a median (range) of 24 (5 to 49) % and 73 (52 to 81) % of the total BAL cells in the low- and high-dose segments respectively (Figure 3A). Despite the prolonged abrogation of blood eosinophils by mepolizumab (Figure S3), SBP-Ag still induced a dose-dependent increase in BAL eosinophils, which rose from a baseline median (range) of 0.1 (0-0.1) % to 5 (2 to 31) % and 31 (4 to 49) % of the total BAL cells following low- and high-dose challenge respectively. Nonetheless, allergen-induced differences (pre/post SBP-Ag deltas) in eosinophil total numbers and percentage in both the low- and high-dose segments were significantly less ($p\leq0.001$) after mepolizumab. Compared to the response before mepolizumab, there was a median decrease in eosinophils per ml of BAL fluid of 62% and 84% in the low and high dose segments respectively (Figure 3B). Mepolizumab had no effect on other BAL cell populations in the naïve and low-dose segment, but slightly increased the percentages of lymphocytes in the high-dose segment (Table EIII).

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Effect of mepolizumab on eosinophil expression of IL-5-inducible cell surface receptors and release of granule proteins

Flow cytometric comparison of BAL versus circulating eosinophils obtained 48 h (D2) after SBP-Ag showed elevated levels of IL-3Rα and GM-CSFRα, whereas IL-5Rα and ßc were diminished (Figure 4A), which is similar to our prior work (23). Mepolizumab did not significantly alter the patterns of cytokine receptor expression on the cell surface of airway eosinophils (Figure 4A). Circulating eosinophils obtained after administration of mepolizumab had significantly lower levels of surface IL-3Rα ($p=0.0001$, Figure 4A) and mRNA ($p=0.002$, Figure 4B), along with decreased surface ßc ($p=0.0004$, Figure 4A), and a trend toward increased surface IL-5Rα ($p=0.16$). The decreased surface density of IL-3Rα was limited to eosinophils (Figure E2).

Although mepolizumab attenuated IL-3R expression on blood eosinophils, further analysis of the phenotype of blood and BAL eosinophils demonstrated no significant changes in the level of the IL-5-inducible (22, 25, 30, 31) activation markers CD69, CD44, CD23 (Figure 5), CD66e, and CD108 (not shown). Flow cytometric comparison of BAL versus circulating eosinophils showed that levels of CD69, CD44, and CD23 were significantly elevated, while the level of CD193 (CCR3), which is down-regulated by IL-5 (25), was decreased on BAL compared to blood eosinophils. This BAL versus blood eosinophil pattern did not change after mepolizumab (Figure 5).

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The flow cytometric studies suggested that mepolizumab does not affect the surface activation phenotype of airway eosinophils. Therefore, endobronchial biopsies obtained 48h after the high-dose challenge were interrogated for the presence of EPX, which is the only granule protein with absolute specificity for eosinophils (11). Release of EPX and cell-free EPX-containing granules by tissue eosinophils was evident when SBP-Ag was performed after mepolizumab. Similar to reports in eosinophil esophagitis (28) EPX-stained sections of endobronchial biopsies displayed four staining patterns (Figure 6 A) including intact EPX-positive eosinophils, cell-free granules localized near eosinophils, extensive deposition of cell-free EPX-containing granules, and/or extensive EPX deposition within the mucosa. In these allergic asthmatics, samples obtained from the allergen-naïve segment displayed clusters of eosinophils and, in some cases, widespread distribution of cell-free granules (Figure E4). In segments that had received allergen challenge, there was pronounced EPX staining demonstrating granule release and extensive EPX-deposition within the mucosa. One month after mepolizumab, the EPX staining-score was significantly less in the Ag-naïve site ($p=0.01$, $n=8$), and there was a decrease in the pre/post SBP-Ag delta after mepolizumab ($p=0.02$, $n=8$) (Figure 6B). Although the staining score was decreased, granule release was evident in most subjects and there was impressive EPX staining of mucosal tissue from 25% of the subjects (Figure E4).

Detailed functional assays were limited by the low number of circulating eosinophils available for purification after mepolizumab (Table EI). However, sufficient eosinophils of very high purity were obtained from BAL (n=3 participants) and blood (n=6

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participants) for RT-qPCR analysis. Although eosinophils synthesize and store granule proteins (MBP-1 and EPX) during eosinophilopoiesis, we previous established that only *RNASE2* (EDN) mRNA is detectable in unstimulated BAL (32) and blood eosinophils (33). After mepolizumab, EDN mRNA (n=6) was significantly decreased in blood eosinophils (Figure 6C). Due to the small sample size for highly pure BAL eosinophils, meaningful changes could not be assessed.

Effect of mepolizumab on BAL fluid concentrations of IL-5 family cytokines

Prior to administration of mepolizumab, there was an allergen dose-dependent increase in BAL fluid concentrations of IL-5, IL-3, and GM-CSF (Table EIV). After mepolizumab, IL-5 levels in the low-dose subsegment were modestly, but significantly increased. To assess how IL-5/mepolizumab complexes might affect the IL-5 values obtained by ELISA, BAL fluids were acid treated prior to performing the ELISA. Dissociation of IL-5/mepolizumab complexes by pH shock resulted in a small, but statistically significant increase in IL-5 detection from a median (1^{st} and 3^{rd} quartiles) of 409 (49, 741) pg/ml to 447 (66, 1107) pg/ml (p=0.01) in BAL fluid from the segment challenged with a high dose of allergen after administration of mepolizumab. Acid treatment had no effect on IL-5 detection in BAL fluid from the low dose or control segments after mepolizumab or BAL fluid obtained when SBP-Ag was performed before mepolizumab, indicating that IL-5/mepolizumab complexes only slightly reduce the values of the IL-5 ELISA.

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DISCUSSION

Capitalizing on the unique approach of segmental allergen challenge to induce a robust localized airway eosinophilia, we observed that, in addition to preventing the allergen-induced rise in circulating eosinophils, IL-5 blockade with mepolizumab resulted in reduced allergen-induced BAL eosinophilia. Remarkably, the eosinophil-reducing effect of a single 750 mg dose of mepolizumab was maintained for 4 weeks (at the time of allergen challenge) and was not completely overpowered by the potent signal provided by SBP-Ag. Our estimates of the magnitude of the mepolizumab-mediated decrease in airway eosinophil numbers was in line with clinical efficacy studies showing >75% reduction in BAL eosinophils in patients with asthma who received three monthly infusions of mepolizumab (7, 34).

While the eosinophil reducing effect of mepolizumab was remarkable, sufficient numbers remained in the circulation and airway allowing investigation of mepolizumab-induced changes in the activation phenotype of eosinophils before and after airway allergen challenge. The most striking effect of mepolizumab was attenuation of IL-3Rα levels on circulating eosinophils, which suggests that IL-5 neutralization affects IL-3Rα expression during hematopoiesis rather than simply preventing the allergen-induced rise in levels of surface IL-3Rα that we previously reported (23). Based on e*x vivo* studies of bone marrow- or cord blood-derived progenitors, it is thought that IL-3 and GM-CSF induce proliferation of eosinophil lineage-committed IL-5Rα-positive progenitors, whereas IL-5 induces terminal differentiation to mature eosinophils (35, 36). It is tempting to speculate that in addition to preventing IL-5-mediated terminal

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differentiation, mepolizumab also reduces IL-3 receptor expression thereby restricting expansion of eosinophil-committed progenitors. Menzies-Gow and colleagues have shown that mepolizumab arrests eosinophil maturation (37). Studies characterizing the cell surface phenotype of eosinophil-committed progenitors would be warranted to more fully understand the role of IL-5 (and IL-3) in eosinophil hematopoiesis and the effect of mepolizumab on this process.

In contrast to circulating eosinophils, and contrary to our hypothesis, administration of mepolizumab did not reduce expression of the GM-CSF or IL-3 receptor, nor the increase in IL-5-inducible membrane proteins on airway eosinophils. Based on previous studies showing attenuated IL-5Rα expression (protein and mRNA) on BAL eosinophils (23) and persistent down-regulation of IL-5Rα and enhanced IL-3Rα and GM-CSFRα levels following e*x vivo* exposure of eosinophils to IL-5 (24, 25), we were surprised that neutralization of IL-5 did not affect levels of any of the IL-5-cytokine family receptors on BAL eosinophils. These observations are consistent with studies showing no mepolizumab-mediated change in circulating eosinophils' expression of IL-5Rα in asthma (38) or eosinophilic esophagitis (39), but are in opposition to a report that IL-5Rα is increased (40) after mepolizumab treatment of patients with various hypereosinophil-related diseases. In addition to a lack of an effect on IL-5-family cytokine receptors, mepolizumab had little effect on BAL eosinophil surface expression of IL-5-inducible activation markers such as CD69, CD44, and CD23. These observations are in accord with our finding that mepolizumab does not blunt increased expression and activation of β2-integrins on BAL eosinophils (41).

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The observation that mepolizumab did not affect surface expression of IL-5-inducible activation markers on BAL eosinophils raises the possibility that, while their numbers are substantially reduced, the remaining airway eosinophils retain their functionality. This notion is supported by evidence of eosinophil degranulation in the bronchial mucosa when allergen challenge occurred after mepolizumab, along with the clinical efficacy studies showing persistence of mucosal eosinophil granules following mepolizumab treatment of patients with mild (34) or severe eosinophilic asthma (7). Whether granule release occurred via exocytosis, piecemeal degranulation, or cytolysis (12, 42-44) is not known. Regardless, cell-free granules express surface receptors and can release toxic granule proteins as well as cytokines in response to a variety of agonists including IFN-γ, eotaxin, and cysteinyl leukotrienes (45, 46). Thus, the residual cell-free granules present after mepolizumab could contribute to persistent airway inflammation and damage to mucosal tissue. The preservation of the active eosinophil phenotype in the airway following mepolizumab may explain why some patients continue to have asthma exacerbations following treatment with mepolizumab, despite the significant reduction in eosinophil number.

The post-mepolizumab observations that SBP-Ag-induced airway eosinophilia occurs despite the near ablation of circulating eosinophils, and the striking difference between surface levels of IL-3Rα on circulating versus BAL eosinophils, raise questions concerning the origin and activation mechanism(s) of eosinophils that are in the airway lumen after mepolizumab. It is possible that a limited number of circulating eosinophils

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do express a high level of IL-3Rα and are selectively recruited/retained in the airway. However, we believe it is more likely that at least a portion of BAL eosinophils originate from allergen-induced release of circulating eosinophil progenitors (47) and/or expansion of resident eosinophil progenitors in the bronchial mucosa (48). In non-allergic individuals, local airway eosinophil differentiation can occur in response to inhaled IL-5 (49) and in allergic asthmatics, recruitment/differentiation of eosinophil progenitors in the airway is reduced following mepolizumab treatment (37) suggesting that *in situ* maturation of eosinophils is, to some extent, governed by IL-5. Nonetheless, patients with severe eosinophilic asthma have exaggerated eosinophilopoiesis in the bronchial mucosa that is not affected by treatment with mepolizumab (50). This may be due to inadequate neutralization of IL-5 that is produced locally by stromal cells (51), innate lymphoid cells (52), or eosinophil progenitors (53) themselves. Additionally, there may be an IL-5-independent component of bronchial tissue eosinophilopoiesis. Candidate factors include the combination of GM-CSF and leukotriene D4 (54) or IL-3 plus TSLP or IL-33 (53).

A major strength of this study was our ability to examine both circulating and airway eosinophils after administration of mepolizumab, and, unlike previously published studies of patients with severe asthma, the effects of mepolizumab on eosinophils were not confounded by corticosteroid effects. Another strength is the use of a well-established experimental model of allergen-induced eosinophilic airway inflammation in patients with allergic asthma, which allowed interrogation of mepolizumab on airway cell numbers and functional phenotype. However, we recognize that our study design has

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limitations. First, the induction of profound type 2 inflammation may have limited the IL-5-neutralizing capacity of mepolizumab. Levels of IL-5 in BAL fluid were increased to the same extent by SBP-Ag before and after mepolizumab. Others have demonstrated that IL-5-mepolizumab complexes are present in serum (40) and we noted a small but significant increase in detection of IL-5 following acid treatment of BAL fluid. Although we believe that the majority of IL-5 in the BAL fluid is neutralized by mepolizumab, we cannot be completely certain that there is not a small amount of free and functional IL-5 in the airway. Second, SBP-Ag induces intense local airway eosinophilia that is much greater than what is typically seen in asthma. When planning the study design, we felt the unique opportunity to study airway eosinophils outweighed the physiological limitations. This is the first report evaluating the effect of mepolizumab on the functional phenotype of airway eosinophils. Induction of airway eosinophilia by SBP-Ag does not model the induction of non-allergic eosinophilic asthma (55). While the sources of IL-5 may differ in non-allergic vs allergic asthma (innate ILC2 vs ILC2 plus adaptive Th2 cells) (56), there are no reported differences in eosinophils, and mepolizumab significantly reduces asthma exacerbations in eosinophil-associated asthma regardless of allergen sensitization (55). Notwithstanding its shortcomings, using the combined approach of SBP-Ag before and after mepolizumab permitted purification of circulating and BAL eosinophils from a limited number of subjects. While current technologies did not permit us to study the *ex vivo* potential of eosinophils to respond to IL-5-family cytokines, *in vivo* activation could be inferred from RT-qPCR analysis.

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We realize that our small sample size limits any meaningful consideration about the mechanisms of mepolizumab's clinical action. The prolonged time commitment and inclusion of four bronchoscopies affected subject retention and limited the sample size. Nonetheless, statistically significant differences in numbers of BAL eosinophils pre versus post mepolizumab were of sufficient power to halt recruitment when 10 participants had completed the study. Finally, given the experimental nature of our intervention, we cannot directly extrapolate our findings to the bedside. However, it is tempting to speculate that our study may explain why some patients continue to have asthma exacerbations despite the near elimination of circulating eosinophil following anti-IL5 therapy. We also recognize that exacerbations could be due to neutrophilic airway inflammation that occurs in response to infection (57).

In summary, using an experimental model to induce intense eosinophilic airway inflammation in asthmatics, we demonstrated that mepolizumab has potent anti-eosinophil effects leading to dramatic reduction in circulating and airway eosinophilia. Although it does not completely eliminate circulating eosinophils, mepolizumab affects their activation status as assessed by EDN mRNA expression, and may attenuate their response to IL-3 by preventing their expression of IL-3 receptors. Despite the low level of circulating eosinophils after mepolizumab, SBP-Ag still induced recruitment, and/or maturation of eosinophils in the airway. The observation that BAL eosinophil expression of cytokine receptors and IL-5-responsive activation markers is not altered by mepolizumab may indicate that airway eosinophils retain their specific functions (release of granule proteins, cytokines, chemokines, and growth factors) and may

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explain why some patients continue to have asthma exacerbations after anti-IL-5 treatment despite diminished numbers and activity of circulating eosinophils.

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FIGURE LEGENDS

Figure 1. Study design. This was an experimental, non-clinical study rather than a traditional pharmaceutical industry-sponsored "clinical trial". Participants with mild allergic asthma underwent whole lung inhalation challenge (WLAC) to determine allergen provocation dose resulting in a 20% reduction in FEV_1 ($AgPD_{20}$). An early allergic reaction defined as a drop in FEV_1 ≥20% within 1 hour of challenge (EAR>20%) was required for continuation. One month later, bronchoscopy with bronchoalveolar lavage (BAL) was performed in two subsegment followed by segmental bronchoprovocation with allergen (SBP-Ag) at a dose of 1 (low dose) or 20% (high dose) of the subject's Ag-PD_{20}. 48 hours later, bronchoscopy with BAL was performed in each subsegment and biopsies (Bx) were taken from an Ag-naïve site and from the high-dose segment. A BAL eosinophilia >50% in the high dose segment was required for continuation. One month after SBP-Ag, spirometry was performed to confirm continued asthma stability and then a single 750 mg iv dose of mepolizumab was administered. One month after dosing, circulating eosinophils were required to be ≤80% of the baseline value, or <100 eosinophils/µl. If the threshold was not met, mepolizumab dosing was repeated. If the threshold was met, participants underwent bronchoscopy with BAL in two subsegments followed by SBP-Ag at the same 1 and 20% Ag-PD_{20} doses administered for the baseline challenge before mepolizumab. The post-mepolizumab challenge was performed in the contralateral lung. 48 hours after SBP-Ag,

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bronchoscopy with BAL was performed in each subsegment and Bx were taken from an Ag-naïve site and from the high-dose segment.

Figure 2. Mepolizumab prevents an allergen-induced rise in circulating eosinophils. A. Prior to administration of mepolizumab, there was a significant rise in circulating eosinophil counts 48 h (D2) after airway allergen challenge. The allergen-induced rise did not occur when challenge was given 1-month post mepolizumab. B. WBC counts were not changed by allergen challenge or mepolizumab. Data are medians with 1st and 3rd quartiles; whisker lines represent the 10th and 90th percentile, n=10. Pre vs post allergen p values are indicated by solid lines; pre vs post mepolizumab p values are indicated by dashed lines.

Figure 3. Mepolizumab decreases, but does not eliminate allergen-induced BAL eosinophils. Both before and after mepolizumab, there was an antigen dose-dependent increase in the percentage of BAL eosinophils **(A)** and total numbers of BAL cells per ml of BAL fluid **(B)** 48 hours (D2) after SBP-Ag. The magnitude of the Ag-induced eosinophilia was significantly lower after mepolizumab. Data are medians with 1st and 3rd quartiles; whisker lines represent the 10th and 90th percentile, (n=10). Pre vs post 1% and post 1% vs 20% SBP-Ag p values are indicated by solid lines; pre vs post mepolizumab p values are indicated by dotted lines.

Figure 4. Mepolizumab decreases expression of the IL-3 receptor on circulating eosinophils, but has no effect on IL-5-family cytokine receptor expression on BAL eosinophils. 48 h after SBP-Ag, the cytokine receptor expression pattern was

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assessed on blood eosinophils and on airway eosinophils obtained from the segment challenged with 20% of the participants $AgPD_{20}$ (p values indicated above the solid lines). After mepolizumab, IL-3 receptor alpha (IL3RA/CD123) and beta (ßc/CD131/CSF2RB) chains were significantly diminished on blood eosinophils. Overall, however, mepolizumab did not alter the pattern of receptor expression on blood versus BAL eosinophils (p value indicated above hashed line). Data are medians with 1st and 3rd quartiles for **(A)** cell surface protein (n=9) and **(B)** mRNA fold-change normalized to each individual's pre-mepolizumab circulating eosinophils (n=6 for blood and n=3 for BAL eosinophils). LAD, ≤ the level of accurate detection.

Figure 5. Mepolizumab has no significant effect on IL-5-responsive cell surface activation markers on blood or BAL eosinophils. 48 h after SBP-Ag, the expression pattern of activation markers was assessed on blood eosinophils and on airway eosinophils obtained from the segment challenged with 20% of the participants $AgPD_{20}$ (p values indicated above the solid lines). The differences in activation marker expression did not change after mepolizumab (p value indicated above hashed line). Data are medians with 1st and 3rd quartiles; whisker lines represent the 10th and 90th percentile. CD44 and CD23, n=8; CCR3, n=7; CD69, n=6.

Figure 6. Eosinophil release of EPX and EPX-containing granules is reduced, but still present in bronchial mucosa after mepolizumab. (A) A representative photomicrograph, taken using 10X (rear photograph) and 40X (forward photograph) objectives, demonstrating EPX staining patterns. **(B)** After mepolizumab, the EPX

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staining score (medians with 1st and 3rd quartiles) was significantly less for the Ag-naïve site and tended to decrease for the site that had been challenged with 20% of the participants $AgPD_{20}$ (n=8). **(C)** EDN mRNA fold-change normalized to each individual's pre-mepolizumab circulating eosinophils (n=6 for blood and n=3 for BAL eosinophils). Pre vs post SBP-Ag p values are indicated above solid lines, pre vs post mepolizumab p values are indicated above dashed lines.

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Table I. Participants' baseline characteristics*

	Total enrolled	**Completed**	**Screen failed**	***p* value**
Number	38	10	28	
Age (years)	26 ± 7	25 ± 5	26 ± 8	0.90
Female/male	18/20	3/7	15/13	0.28
FEV_1 (% predicted)	90 ±16	96 ± 19	88 ± 15	0.21
FEV_1/FVC	0.77 ± 0.07	0.78 ± 0.08	0.77 ± 0.07	0.75
Methacholine PC_{20} (mg/mL)†	1.7 ± 2.8	1.5 ± 2.7	1.8 ± 2.8	0.90

*Data are expressed as mean ± SD unless indicated otherwise, †geometric mean ± median absolute deviation, n=13 participants for screen failed

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Table II. Effect of mepolizumab on SBP-Ag-induced changes in pulmonary function*

	Pre mepolizumab			Post mepolizumab			*p* value Pre mepo vs post mepo	
	Pre SBP-Ag	**48 hours Post SPB-Ag**	***p* value**	**Pre SBP-Ag**	**48 hours Post SPB-Ag**	***p* value**	**Pre SBP-Ag**	**Post SBP-Ag**
FEV_1;L	4.1 (3.4, 4.7)	4.0 (3.3, 4.6)	*0.08*	4.1 (3.4, 4.7)	4.0 (3.4, 4.6)	0.10	0.80	0.90
FEV_1; %predicted	96.3 (85.0, 107.6)	92.7 (82.1, 103.3)	**0.04**	95.1 (84.2, 106.0)	92.7 (84.0,101.4)	*0.08*	0.38	1.00
FVC; L	5.5 (4.4, 6.5)	5.4 (4.3, 6.5)	0.34	5.4 (4.4, 6.4)	5.4 (4.4, 6.4)	0.38	0.77	0.85
FEV_1/FVC	0.76 (0.70, 0.82)	0.76 (0.68, 0.84)	0.81	0.76 (0.70, 0.83)	0.75 (0.69, 0.80)	0.14	0.74	0.48
PEFR; L/min	3.4 (2.7, 4.0)	3.3 (2.6, 4.0)	0.12	3.4 (2.7, 4.1)	3.2 (2.6, 3.7)	*0.07*	0.74	0.50
Broncho-dilator reversibility† %	6.8 ± 4.8	9.3 ± 5.0	*0.05*	6.7 ± 4.7	8.2 ± 5.1	0.23	0.94	0.41
FeNO; ppb‡	40 (29, 54)	62 (49, 77)	**0.003**	32 (22, 47)	58 (39, 85)	**0.0002**	0.11	0.56

*least squares means with 95% confidence intervals
†180 µg albuterol
‡geometric means with 95% confidence intervals
FeNO, fraction of exhaled nitric oxide; *FVC*, forced vital capacity, mepo, mepolizumab; *PEFR*, peak expiratory flow rate; *SBP-Ag*, segmental bronchoprovocation with allergen

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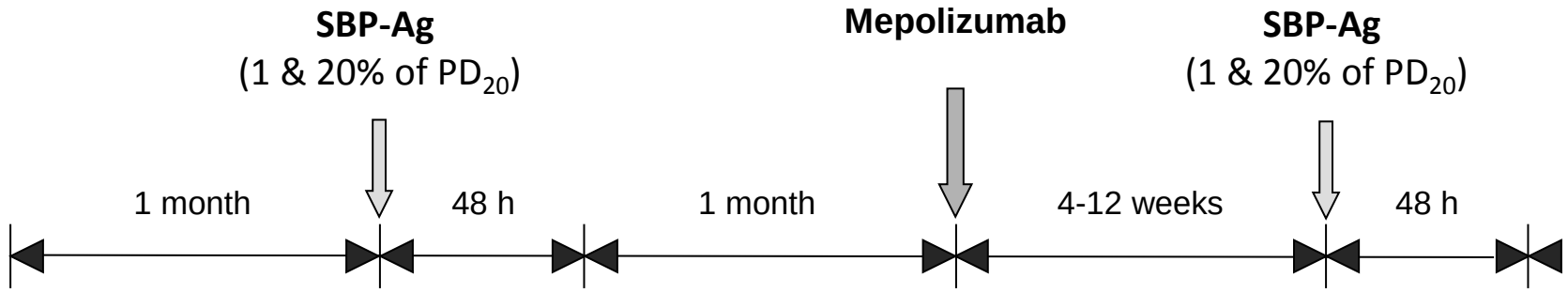
Figure 1

Recruitment:
History of allergic asthma?

Screening Visit:
History + Physical
Spirometry ± albuterol
Skin testing
Exhaled nitric oxide

Mild allergic asthma criteria:
+skin test
FEV_1 albuterol reversibility ≥ 12% or methacholine PC_{20} ≤ 8 mg/mL
Pre-albuterol FEV_1 ≥ 70%
Post-albuterol FEV_1 ≥ 80%
No controller medication



Continuation criteria:

EAR
>20%

BAL EOS
>50% in 20% seg

blood EOS
≤80% of baseline

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Figure 2

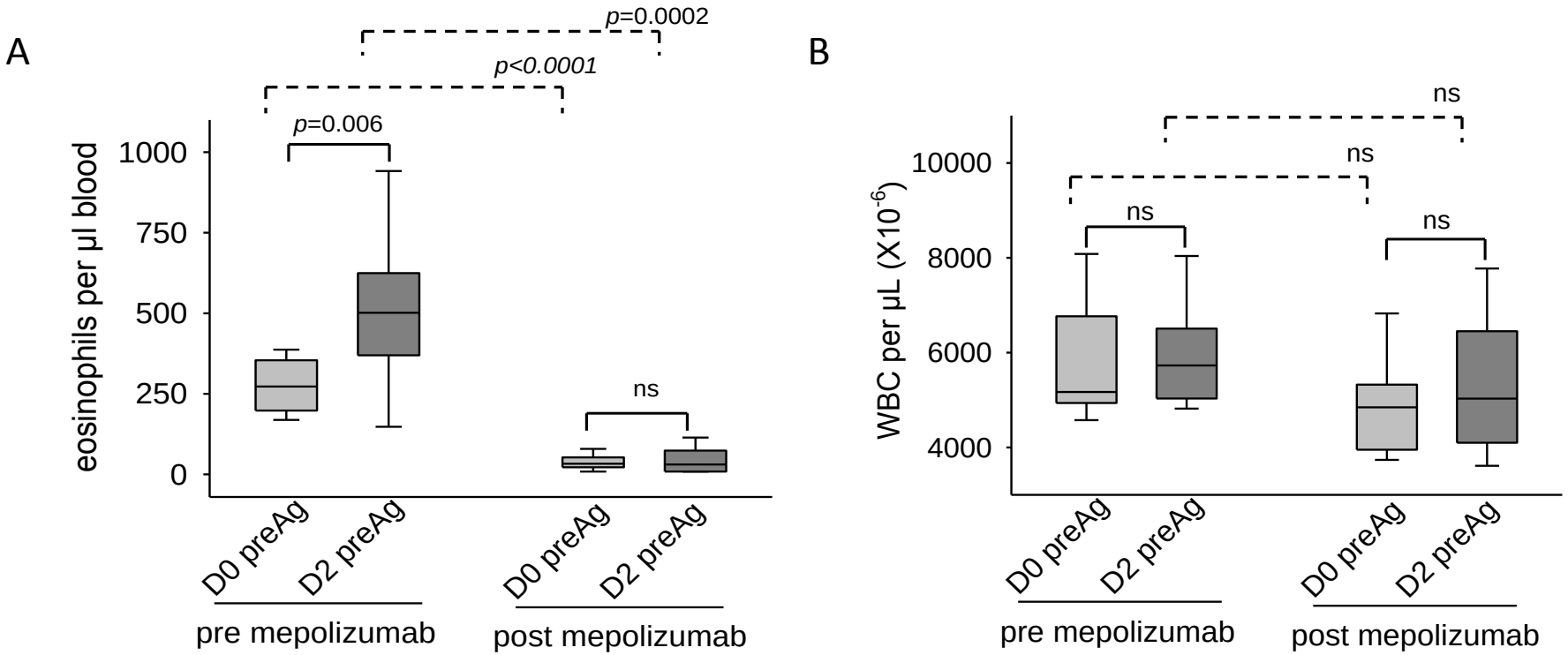

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Figure 3

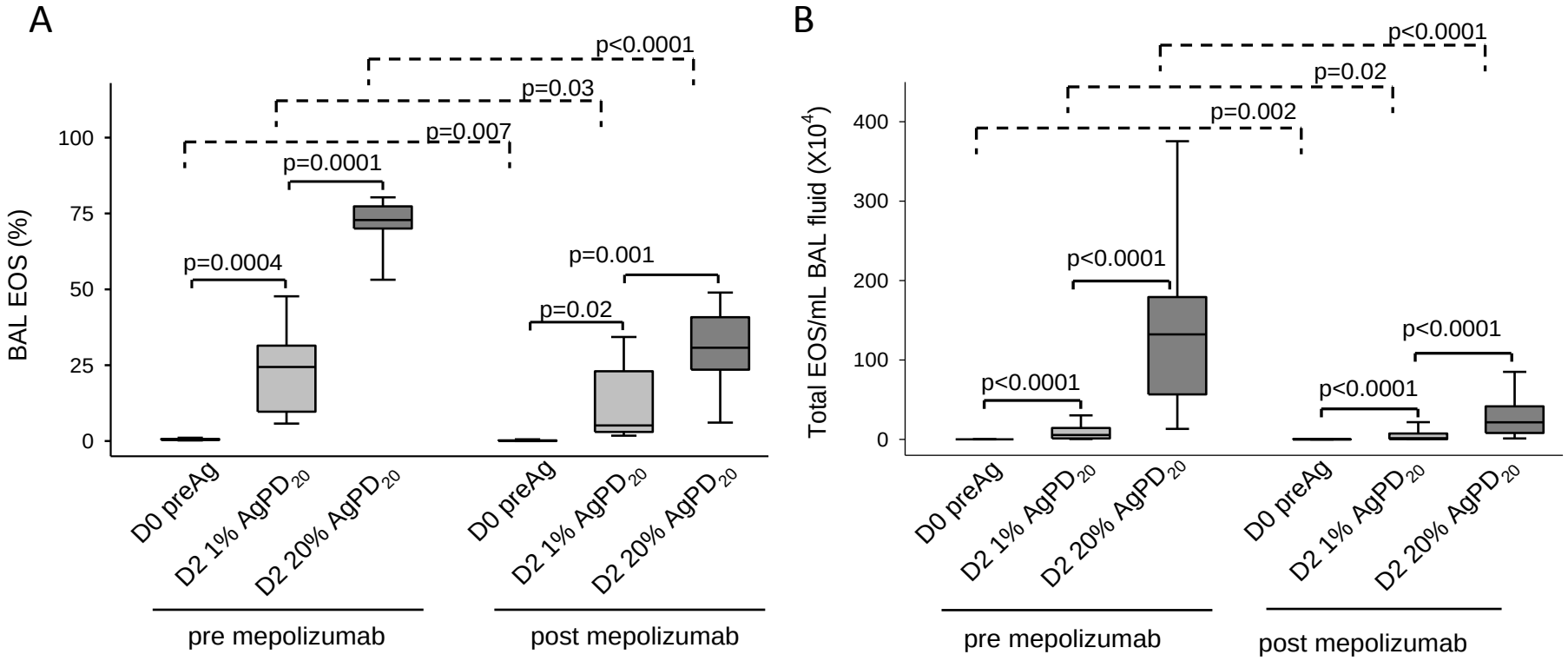

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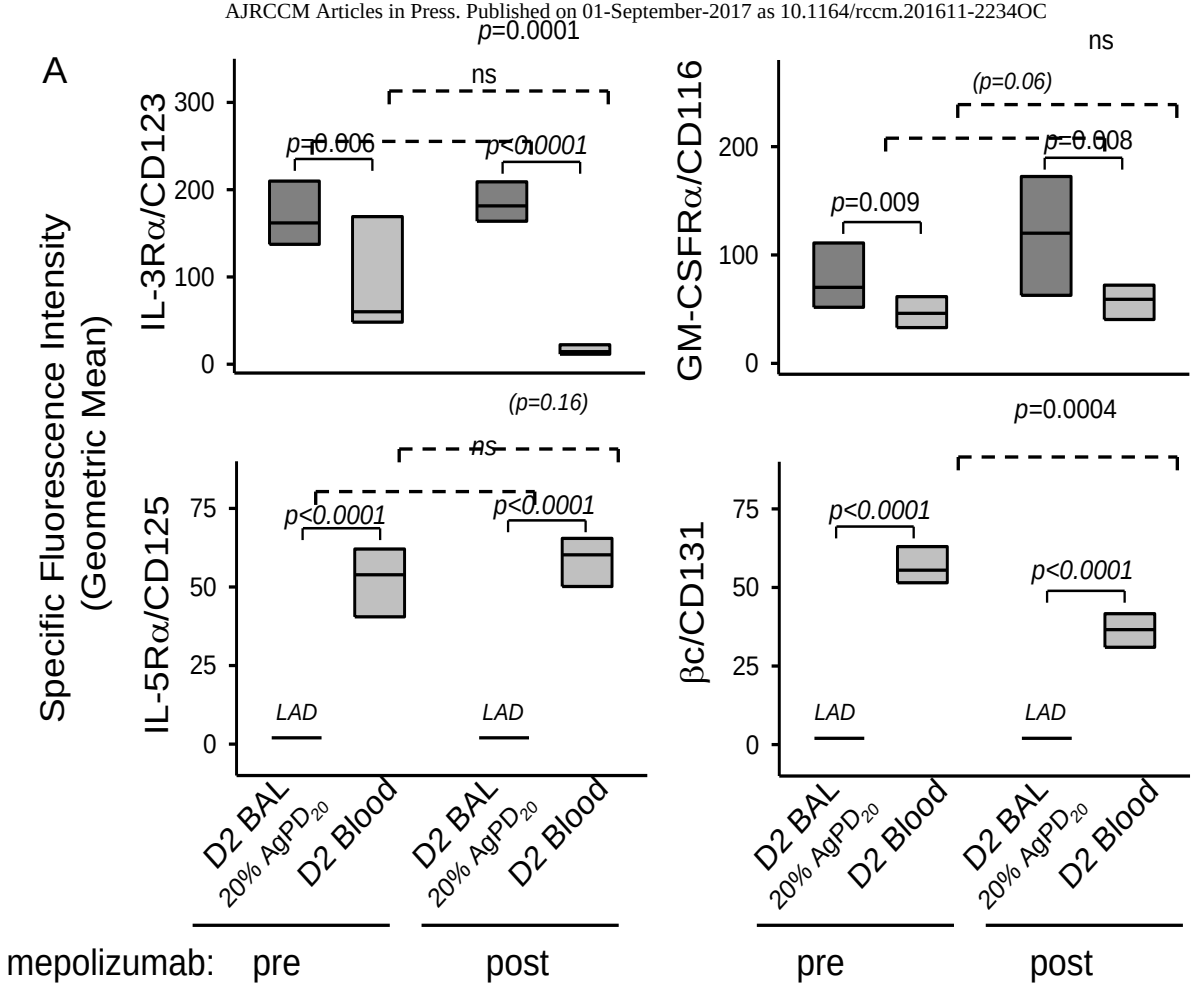
Figure 4



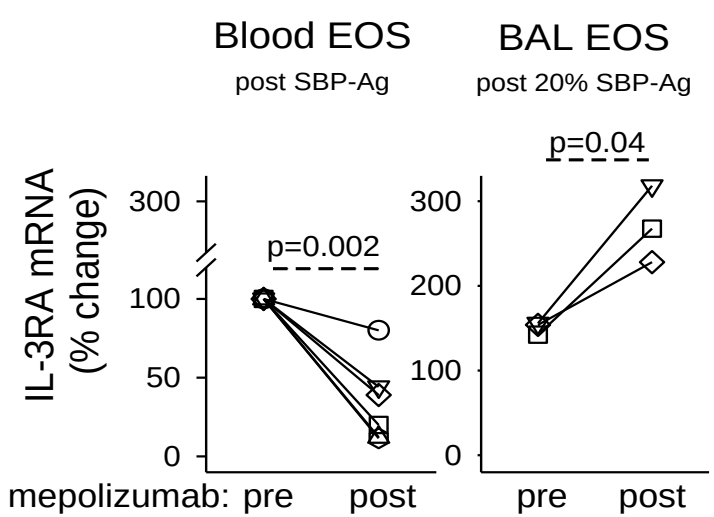


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Figure 5

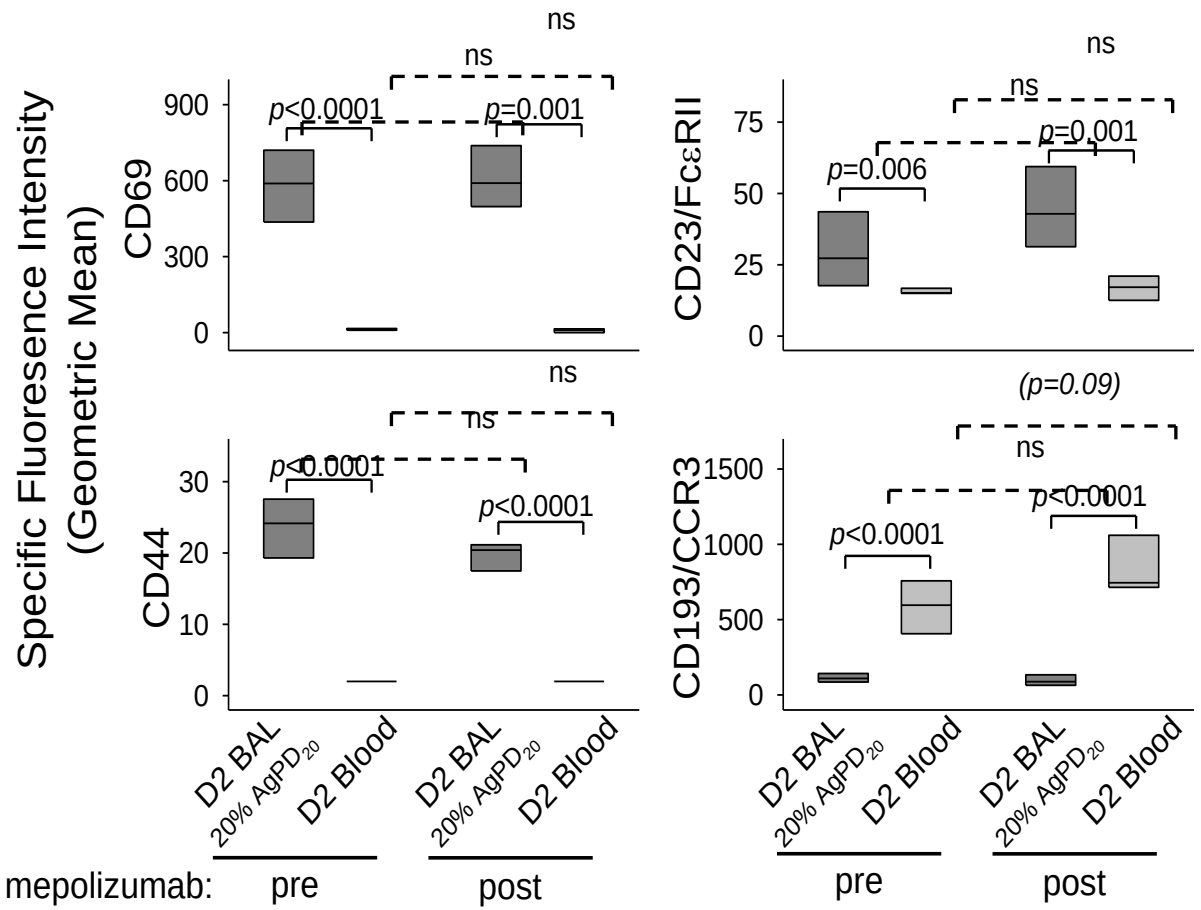

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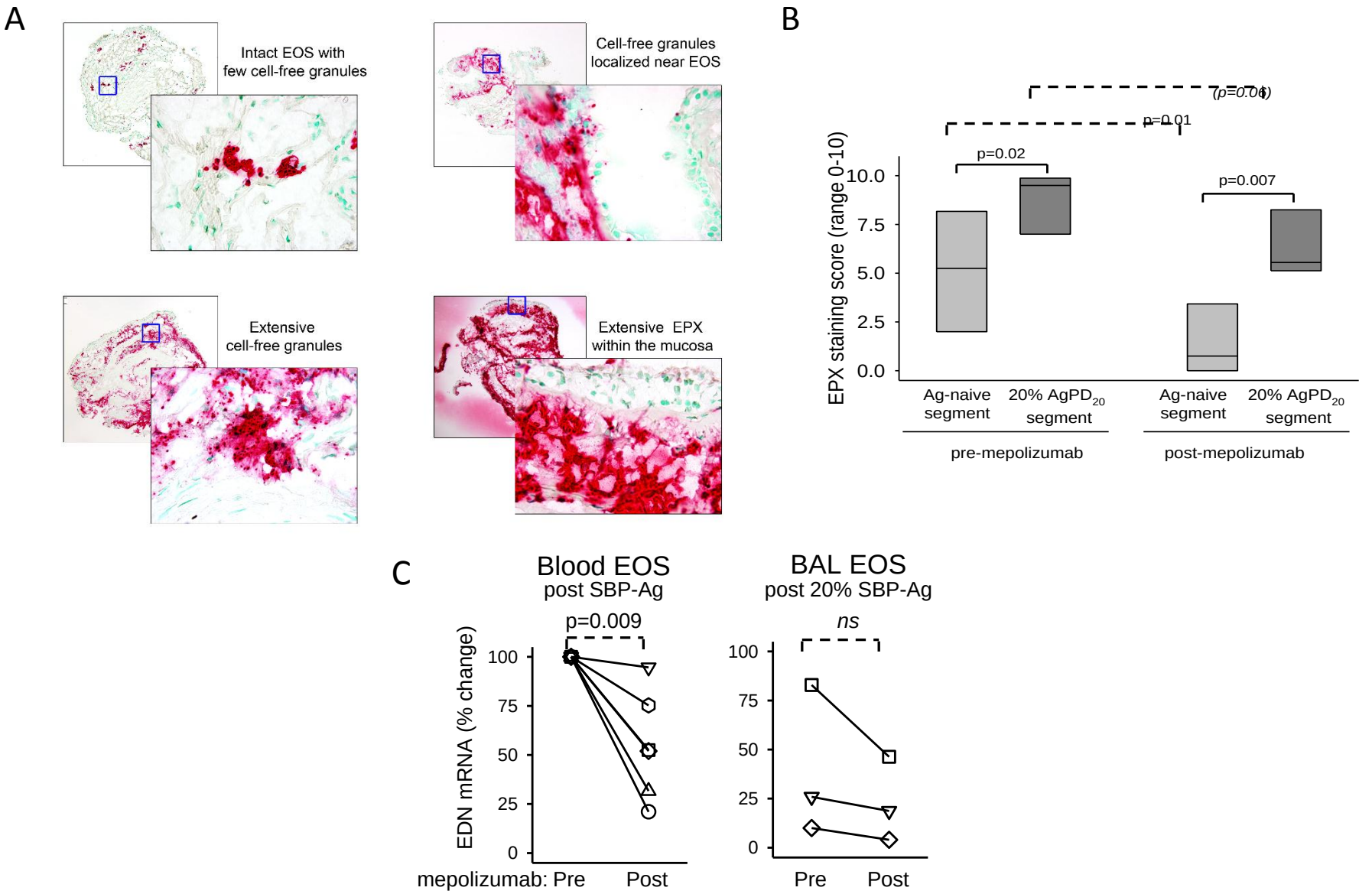
Figure 6



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Online Data Supplement

Mepolizumab attenuates airway eosinophil numbers, but not their functional phenotype in asthma

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Participants

Participants had mild allergic asthma with a positive skin-prick test (wheal compared to negative >3mm) to house dust mite (*Dermatophagoides farinae*), ragweed (short *Ambrosia artemisiifolia* containing antigen E), or a standardized extract of cat (*Felis domesticus*) pelt and cat dander containing Fel d I), a pre-180 µg albuterol FEV_1 ≥70%, a post-albuterol FEV_1 ≥ 80%; airway reversibility to albuterol (180 µg) ≥12% and/or airway hyper-reactivity to methacholine (PC_{20} ≤8 mg/ml).

All participants had stable asthma with no respiratory infection within 4 weeks of study and did not have unstable asthma as indicated by increased symptoms or increased ß-agonist use over the previous two weeks. Participants had not received corticosteroids or leukotriene inhibitors within one month of screening, omalizumab within nine months of screening, an investigational drug within 1 month of screening, did not have any prior treatment with an anti-IL-5 monoclonal antibody, and were not on medications that would affect the outcome of the study. Participants did not a have major health problem or previous malignancy, were not pregnant, had safety lab assessments within normal ranges, had not smoked within the last year or had a smoking history of >5 pack years.

Whole lung allergen inhalation challenge (WLAC)

WLAC was performed to determine allergen dose for segmental bronchoprovocation (SBP-Ag) and to screen for subjects with an airway response to Fel d1 (Bayer Allergy Products), GS Ragweed mix (RW, Greer Labs), or *Dermatophagoides farinae* (Der f*,* Miles Allergy Products). Following baseline spirometry, participants received 5 breaths

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of saline diluent (0.9% sodium chloride, 0.03% human serum albumin, 0.4% phenol) via a breath-actuated nebulizer. Increasing concentrations Der f (3-fold increases), RW (4-fold increases) or Fel d1(3-fold increases) allergenic extract were inhaled at 10 minute intervals until a sustained (20 minute) 20% fall in FEV_1 was achieved. The dose of allergen required to produce a 20% decrease in FEV_1 was calculated and defined as the 20% antigen provocative dose ($AgPD_{20}$).

Bronchoscopy with SBP-Ag, BAL and Bx

The bronchoscopy procedures were performed under conscience sedation that included intramuscular glycopyrolate (0.2 mg) and midazolam (0.5-2.0 mg). To prevent bronchospasm, albuterol was administered by metered-dose inhaler, 2 puffs (180 mcg). Nasopharyngeal anesthesia was achieved by topical lidocaine (1% solution, lidocaine gel, lidocaine spray). Afrin nasal spray was used to reducing nasal congestion and facilitate bronchoscope insertion. The fiberoptic bronchoscope was passed into a pulmonary segment or subsegment. To control cough, lidocaine in 1% solution was instilled through the bronchoscope. The dose of lidocaine was limited to 600 mg or 9 mg/kg, a dose that leads to safe levels of lidocaine in the circulation.

A bronchopulmonary segment was identified, lavaged with 4 X 40 ml of 0.9% NaCl immediately followed by administration of 20% of the subject-specific, predetermined $AgPD_{20}$. A second bronchopulmonary segment, in close proximity, was identified, and the bronchoscope was wedged into that segment and lavage was performed with 4 X 40 ml of saline. A dose equal to 1% of the subject's $AgPD_{20}$ was instilled.

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48 hours after SBP-Ag, the segments that were challenged at the first bronchoscopy were identified and each was lavaged with 4 X 40 ml of 0.9% NaCl. After the lavage, endobronchial biopsies were taken from the segment challenged with 20% $AgPD_{20}$. Control biopsies were taken from a different non-challenged segment of the lung.

One month after administration of mepolizumab, bronchoscopy, BAL, SBP-Ag, and Bx were performed the same as before mepolizumab, except in the contralateral lung. SBP-Ag was administered at identical concentrations in segments/subsegments in the contralateral lung. Forty-eight hours later (D2), bronchoscopy with BAL (4X40 mL of 0.9% NaCl) was performed in each subsegment and endobronchial biopsies were obtained at the site of the 20% challenge and from an allergen-naïve (control) site.

Anti-IL-5 (Mepolizumab) Dosing

Spirometry was obtained prior to and 30 minutes after completion of the mepolizumab dosing. Mepolizumab was generously donated by GlaxoSmithKline (study No. MEA110170). Three 250 mg vials of active drug were diluted according to manufacturer specifications, added to a 100 ml PVC infusion bag, and administered intravenously by gravimetric drip over a period of 30 minutes.

Cell count and differentials

On the day of bronchoscopies, eosinophils were enumerated by hemacytometer using phyloxin staining. BAL cell differentials were determined by counting a total of 1000

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cells on two cytospin preparations stained with the Wright-Giemsa-based Hema-3 (ThermoFisher, Pittsburgh, PA).

Immunocytochemistry

EPX was detected as described by Protheroe, et al. (1) using monoclonal anti-EPX (clone MM25-82.2.1) antibody (1) provided by J.J. and N.A. Lee, Mayo Clinic, AZ).

IHC scoring

A numerical value (0-10) was assigned based on modification of the semiquantitative scoring system established by Protheroe, *et al*. (1) for eosinophilic esophagitis. The staining score was calculated as the sum of each of these criteria: 1) the gross EPX score based on the overall staining profile (0=minimal, 0.5=moderate, 1=excessive) of each slide; 2) the percent area of mucosal EPX staining, which was a numerical value ranging from 0-4 (0=<1%, 1=1-24%, 2=25-49%, 3=50-74%, and 4=75-100%); and 3) the degree of EPX staining scored as 0=no eosinophils/no EPX release, 1=eosinophils present/no detectable EPX release, 2=EPX release limited to areas surrounding intact eosinophils, 3=free EPX granules present/nominal deposition onto the mucosa, 4=free EPX granules present/considerable mucosal deposition, 5=dense EPX mucosal deposition.

Flow cytometry

Unfractionated blood (100 µL) or BAL cells (250,000) were stained with a cocktail of FITC or PE-conjugated anti-CD16 (clone CLB-gran11.5) and -CD14 (clone MφP9) to

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discriminate eosinophils from neutrophils and monocytes, respectively. PE-conjugated anti-IL-5Rα (CD125, clone A14), -IL-3Rα (CD123, clone 6H6), -GM-CSFRα (CD116, clone M5D12), -CCR3 (CD193, clone SE8), and -CD45 (clone HI30) were from BD Biosciences (San Jose, CA); PE conjugated anti-CD69 (cloneFN50) and -common beta chain (CD131, clone 1C1) were from eBioscience, Inc., San Diego, CA. RBCs were lysed using BD Biosciences-lysing solution. Data acquisition was performed on a FACSCalibur (BD Biosciences) after adjusting the instrument voltages with Rainbow fluorescent calibration particles (Spherotech, Lake Forest, IL) and data collection continued until 5-10,000 events within the eosinophil gate were counted. Data were analyzed with FlowJo (FlowJo, LLC Ashland, OR). The gating strategy for a representative subject is shown in Figure E1. Data were expressed as geometric mean of specific stain minus that of the isotype control antibody.

Eosinophil purification

Heparinized blood (280 ml) was centrifuged over Percoll (1.090 g/ml), RBCs in the granulocyte fraction were lysed with water, and eosinophils were negatively selected utilizing anti-CD16, anti-CD3, and anti-CD14 immunomagnetic beads (AutoMac system, Miltenyi Biotec.) to deplete neutrophils, T cells, and monocytes respectively. Airway eosinophils were purified from BAL cells using a two-step Percoll gradient as previously described.(2) Eosinophils were collected from the 1.085/1.100 g/ml interface. Only cells with >96% purity were used for qPCR analysis. Eosinophil yields and purity are provided in Table EII.

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Reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR)

Total RNA preparation, real-time quantitative PCR (RT-qPCR) using SYBR Green Master Mix, and calculation of mRNA fold change using the comparative cycle threshold (ΔΔCT) method, have been described previously.(3) Total RNA was extracted (RNeasy Mini kit, Qiagen) and the reverse transcription reaction was performed (Superscript III system, Invitrogen). Specific primers were designed with Primer Express 3.0 (Applied Biosystems) and blasted against the human genome. The 5' forward primers and 3' reverse primers were CGCCGACTATTCTATGCCGGCAG and TGGCCACTCGGACGGTGTAGT for IL3RA, GCTGCCCCTGAACCCCAGAACA and ACCATGTTTCCCAGTCTCCGCG for EDN, and CAGGACCTGCGCACAAGAG and TCGCACAGCTGGGGTAAG for reference gene, β-glucuronidase (GUSB). Real-time quantitative PCR (qPCR) was performed using SYBR Green Master Mix (SABiosciences). Efficiency for target and reference genes was typically above 94%. Data are expressed as fold change of $2^{-\Delta\Delta Ct}$ compared with expression in each individual's blood eosinophils purified prior to mepolizumab. Data from pre mepolizumab blood eosinophils were fixed at 100%.

ELISA

"In-house" sandwich ELISAs were utilized as previously described (4) with the following modifications. Thawed BAL fluids were vortexed gently and then centrifuged (5,000 x g; 5 min) to remove any cellular debris or aggregated protein. 15 ml of BAL fluid were

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concentrated to 20X using Amicon® Ultra-15 Centrifugal Filter Units with 3 kDa cellulose membranes (Millipore, Billerica, MA).

For ELISA analysis, BAL fluid was prepared in LowCross-Buffer® (Boca Scientific Inc., Boca Raton, FL) to minimize potential matrix effects. A1/2 dilution of 20X concentrated BAL fluid was used for IL-3 and a 1/2 dilution of "1X" BAL fluid was used for IL-5. For GM-CSF, "1X" BAL fluid was diluted 1/2, 1/4, 1/8 and the dilutions resulting in equivalent calculated values were averaged. Data are expressed as pg/ml and are extrapolated to 1X. Unlabeled capture antibodies were rat anti-IL-3 monoclonal antibody clone BVD8-3G11 (BD Biosciences), rat anti-IL-5 monoclonal antibody clone TRFK5 (BD Biosciences), and mouse anti-GM-CSF monoclonal antibody clone 6804.11 (R&D Systems). Biotinylated detection antibodies were rat anti-IL-3 monoclonal antibody clone BVD3-1F9 (BD Biosciences), rat anti-IL-5 monoclonal antibody clone JES1-5A10 (BD Biosciences), and mouse anti-GM-CSF monoclonal antibody clone 3209.1 (R&D Systems). The recombinant IL-5 and GM-CSF were from R&D Systems and IL-3 was from BD Biosciences. The antibody-antigen complex was detected using streptavidin poly-HRP-40 (Fitzgerald, Acton, MA) and TMB substrate (Thermo Fisher Scientific, Grand Island, NY). The assay sensitivities were below 3 pg/ml for GM-CSF and IL-5, and ≤12 pg/ml for IL-3. pH shock (5) was used to dissociate IL-5 from binding proteins such as mepolizumab. Immediately before performing the ELISA, 50 µl of BAL fluid was treated with 10 µl of 0.1M glycine, pH2.5 for 15 min with shaking. The reaction was neutralized by adding 10 µl 0.25M Tris-HCL, pH 8.8.

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Statistical analysis

Baseline subject characteristics were compared by study completion status using the two-sample t-test for continuous measures and Fisher's exact test for categorical measures. Methacholine PC_{20}, exhaled nitric oxide, BAL cell counts, and BAL cytokines measurements were log-transformed for analysis (and were normally distributed after log transformation). Summaries of log-transformed data were back-transformed to the original scale and reported as geometric mean with 95% confidence interval or median within 1st and 3rd quartiles. Outcome data were compared using longitudinal mixed-effects models with fixed-effect terms for pre/post mepolizumab, pre/post antigen challenge, and antigen dose subsegment and a random-effect subject term to account for repeated measures (6). Outcomes are summarized using least squares means with 95% confidence intervals or median with 1st and 3rd quartiles. For RT-qPCR, a paired t-test was used to compare pre/post mepolizumab blood or BAL eosinophils. A two-sided p-value<0.05 was considered significant. Analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC.).

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Legends Online Repository figures

Figure E1. Participant disposition. Thirty-eight participants with a history of allergic asthma were enrolled. Twenty-six participants met screening criteria and underwent a WLAC to determine $AgPD_{20}$. 17 subjects met the criteria of >20% drop in FEV_1 within 1 hour of challenge and agreed to undergo bronchoscopy with BAL and SBP-Ag. Of these, 10 participants met the criteria of a BAL eosinophilia >50% in the subsegment that received a dose of 20% of their $AgPD_{20}$. These 10 subjects received mepolizumab and underwent a second SBP-Ag with BAL to complete the study. In addition to the 18 subjects who did not meet study criteria, 7 subjects withdrew due to scheduling conflicts and 3 did not complete the initial bronchoscopy with BAL.

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Figure E2. Gating strategy for flow cytometric analysis. Non-cellular debris was gated out based on forward and side scatter and eosinophils were identified as PE and FITC autofluorescent and CD16/CD14 negative. Backgating on autofluorescence confirmed the location of eosinophils in the forward versus side scatter plot with eosinophils having high side scatter and moderate forward scatter that partially overlapped neutrophils. The selected region was examined using CD16 and CD14 to eliminate neutrophils or monocyte/macrophages, respectively. A tight region was drawn around the CD16-/CD14- eosinophils and this region was examined using a histogram. Note that in the overlays of total cells pre (blue) and post (red) mepolizumab (bottom of figure), the change in IL-3Rα is limited to eosinophils. Data from a representative subject is shown.

Figure E3. Kinetics of circulating eosinophils after one (or two, gray circle) dose of mepolizumab. Heavy lines represent mean, *$p<0.05$ versus mean value at dosing. Eosinophils counts were decreased >75% one week after mepolizumab administration and remained at that level for 3 months (n=10), returning toward the pre-dosing level by 6 months (n=8).

Figure E4. Effect of mepolizumab on EPX staining in bronchial mucosa. Immunohistochemistry using anti-eosinophil peroxidase (EPX) antibody (clone MM25-82.2.1 (1) provided by J.J. and N.A. Lee, Mayo Clinic, AZ) was performed simultaneously on slides from all 8 subjects. EPX staining was visualized using a

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biotinylated antibody, streptavidin-alkaline phosphatase conjugate, and a Permanent Red substrate-chromogen with a methyl green (Dako) counterstain. Photomicrographs were taken using a 10X objective. Biopsy sections were obtained before and after mepolizumab from an allergen-naïve site and the site that had been challenged with a high dose of allergen (20% $AgPD_{20}$). Of the 10 participants who completed the study, an acceptable biopsy was not obtained after SBP-Ag in one subject and another subject opted out of the biopsy procedure.

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Table EI. Eosinophil yield and purity*

	Pre Mepolizumab	Post Mepolizumab
BAL eosinophil yield†, $X10^6$	22 (13-77)	4 (2-8)
BAL eosinophil purity, %	99 (99-99)	94 (81-97)
Circulating eosinophil yield‡, $X10^6$	46 (22-54)	4 (2-5)
Circulating eosinophil purity, %	99 (99,99)	97 (95-99)

*BAL and blood eosinophils were obtained 48 h after SBP-Ag. Data are shown as median with 1st and 3rd quartiles, †per 160 ml BAL fluid, ‡per 240 ml heparinized blood

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Table EII. Effect of mepolizumab on cell counts per µl of blood[†]

	Time post mepolizumab administration							
	Day 0	**1 week**	**2 weeks**	**3 weeks**	**4 weeks**	**2 month**	**3 months**	**6 months**
Eosinophils	200 ± 90	50 ± 30*	40 ± 20*	50 ± 20*	40 ± 20*	40 ± 20*	50 ± 30*	210 ± 120
Basophils	30 ± 10	20 ± 10*	20 ± 10*	20 ± 10*	20 ± 10*	20 ± 10*	20 ± 10	30 ± 20
Lymphocytes	1730 ± 240	1840 ± 280	1750 ± 270	1770 ± 260	1530 ± 230	1790 ± 260	1780 ± 360	1890 ± 390
Neutrophils	3020 ± 470	3320 ± 860	2770 ± 490	2760 ± 430	3180 ± 910	3230 ± 980	3490 ± 1850	2930 ± 470
Monocytes	360 ± 100	420 ± 130	440 ± 150	410 ± 100	470 ± 110	430 ± 90	430 ±120	390 ± 90
Platelets	210 ± 30	240 ± 20	200 ± 10	200 ± 20	220 ± 10	220 ± 40	230 ± 30	230 ± 20
WBC count	5300 ± 400	5700 ± 1000	5000 ± 800	5000 ± 500	5100 ± 1100	5500 ± 900	5800 ± 1900	5500 ± 400

[†]Data are mean ± SD of cells per µL of blood from 10 subjects, *$p<0.05$ versus day of dosing (Day 0)

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Table EIII. Effect of mepolizumab on BAL cell differential*

	Pre mepolizumab			Post mepolizumab			*p* value Pre mepo		*p* value Post mepo		*p* value Pre mepo vs post mepo		
	D0 Pre SBP-Ag	**D2 1% Ag**	**D2 20% Ag**	**D0 Pre SBP-Ag**	**D2 1% Ag**	**D2 20% Ag**	**D0 v D2 1% Ag**	**D0 v D2 20% Ag**	**D0 v D2 1% Ag**	**D0 v D2 20% Ag**	**D0 preAg**	**D2 1% Ag**	**D2 20% Ag**
Eosinophils (%)	0.6 ± 0.4	24 ± 13	72 ± 8	0.2 ± 0.2	12 ± 12	31 ± 13	**<0.001**	**<0.001**	**0.02**	**<0.001**	**0.007**	**0.03**	**<0.001**
Lymphocytes (%)	9 ± 10	10 ± 9	7 ± 4	10 ± 9.8	13 ± 10	14 ± 6	0.57	0.41	0.14	0.12	0.63	0.13	**0.002**
Neutrophils (%)	0.9 ± 0.8	5 ± 3	3 ± 2	1.6 ± 1.4	8 ± 7	7 ± 5	**0.004**	**0.007**	**0.02**	**0.006**	0.14	0.28	0.06
Monocytes/ macrophages (%)	89 ± 10	61 ± 19	18 ± 8	88 ± 10	68 ± 16	49 ± 19	**<0.001**	**<0.001**	**0.003**	**<0.001**	0.5	0.19	**<0.001**

*Data are mean ± SD; *SBP,* segmental bronchoprovocation, *% Ag*, SBP with 1 or 20 of individual's allergen PD_{20}, *D0*, immediately prior to SBP-Ag, *D2*, 48 hours after SBP-Ag. N=10 subjects.

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Table EIV. BAL fluid proteins*

	Pre mepolizumab			**Post mepolizumab**			***P* value Pre mepo vs. Post mepo**		
	Pre Ag	**Post 1% $AgPD_{20}$**	**Post 20% $AgPD_{20}$**	**Pre Ag**	**Post 1% $AgPD_{20}$**	**Post 20% $AgPD_{20}$**	**Pre Ag**	**Post 1% $AgPD_{20}$**	**Post 20% $AgPD_{20}$**
IL-5 (pg/mL)	0 ± 0	24 ± 47	305 ± 555	0 ± 0	71 ± 84	241 ± 172	1.0	**0.02**	0.43
IL-3 (pg/mL)	0 ± 0	0 ± 0	15 ± 28	0 ± 0	0 ± 0	12 ± 12	1.0	0.83	0.10
GM-CSF (pg/mL)	3 ± 3	7 ± 9	22 ± 14	0 ± 0	15 ± 27	29 ± 20	0.61	0.76	0.66
Total protein (µg/mL)	100 ± 100	300 ± 200	2,400 ± 310	100 ± 100	400 ± 500	1,700 ± 2,700	0.79	0.35	0.32
Volume return (%)	71 ± 9	69 ± 10	66 ± 11	72 ± 7	75 ± 10	66 ± 10	0.82	**0.04**	0.97

*mean ± SD, n-10 subjects.

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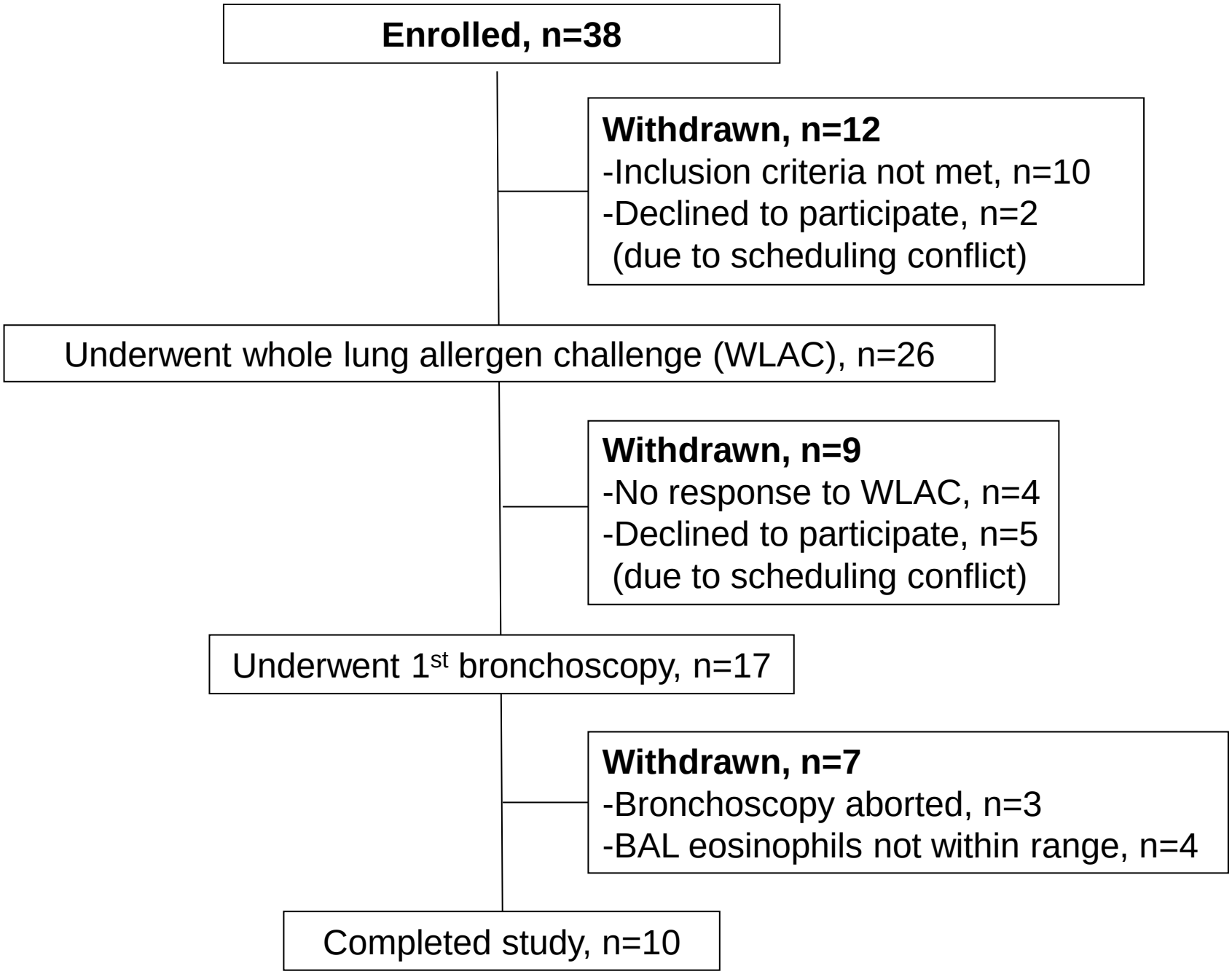
Supplemental Figure 1



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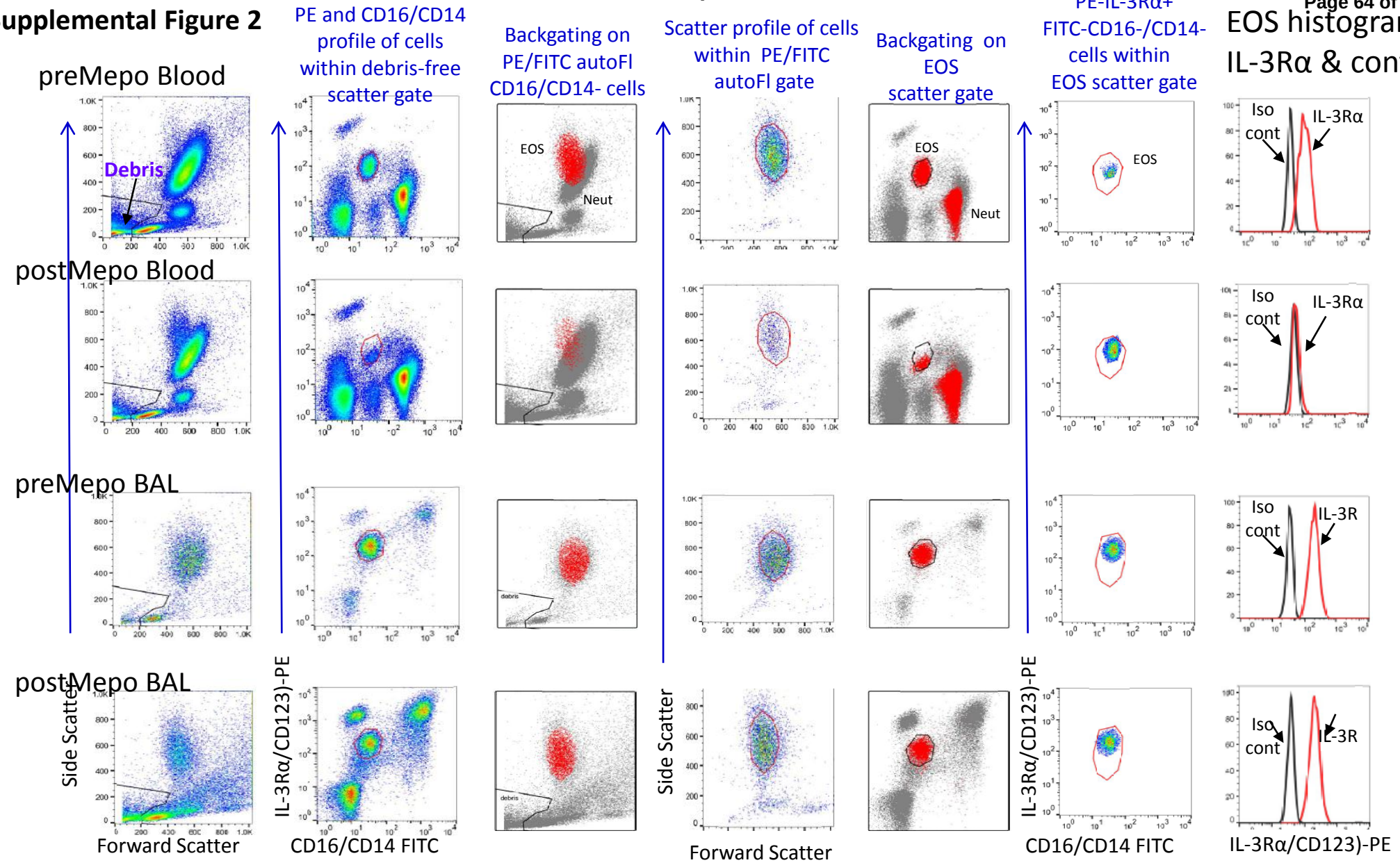
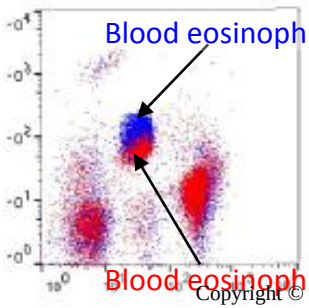
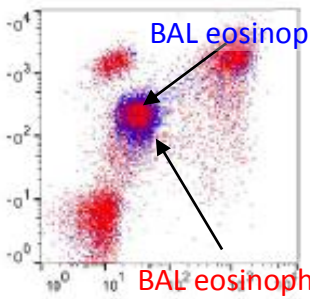
Supplemental Figure 2



IL-3Rα on all blood cells pre/post mepolizumab



IL-3Rα on all BAL cells pre/post mepolizumab



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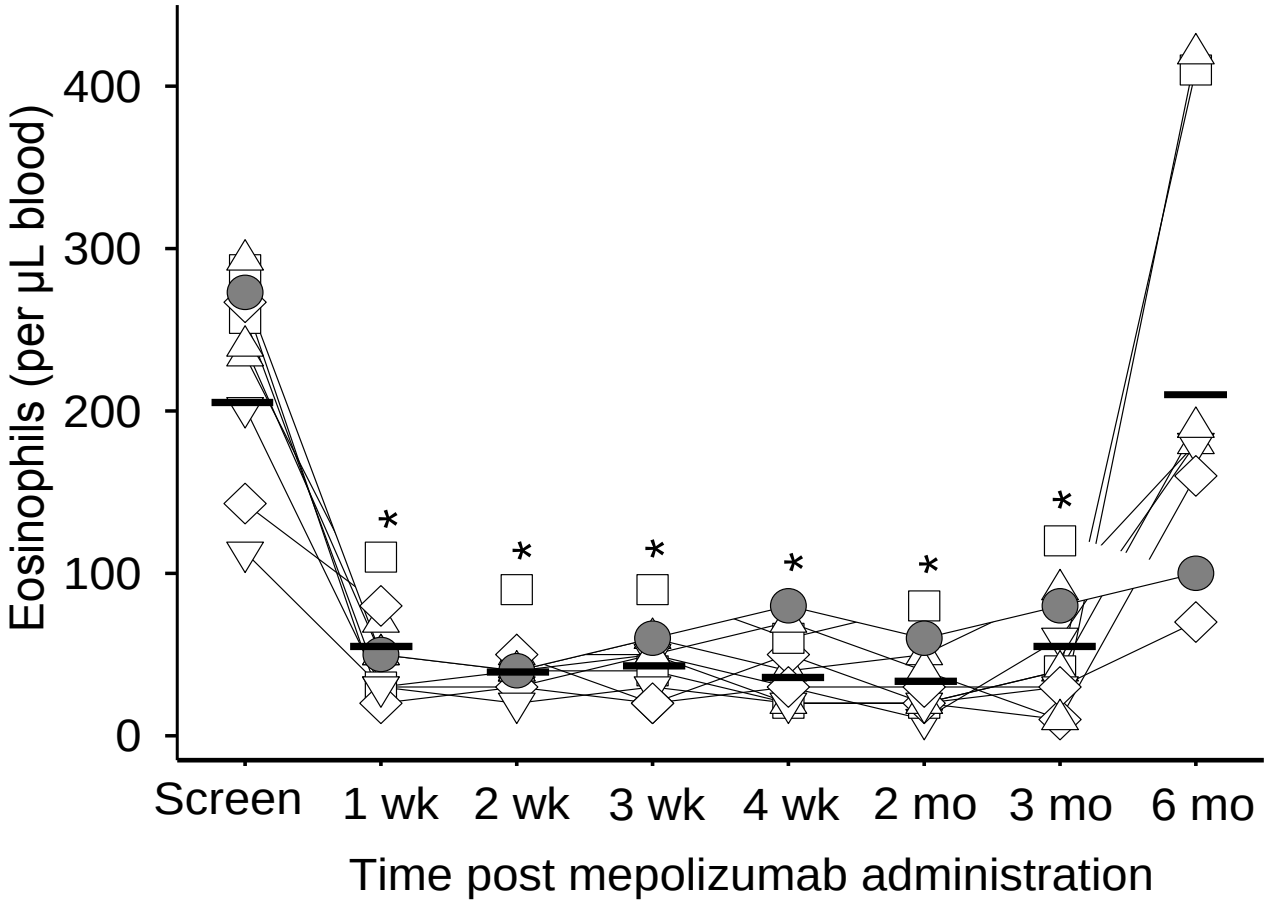

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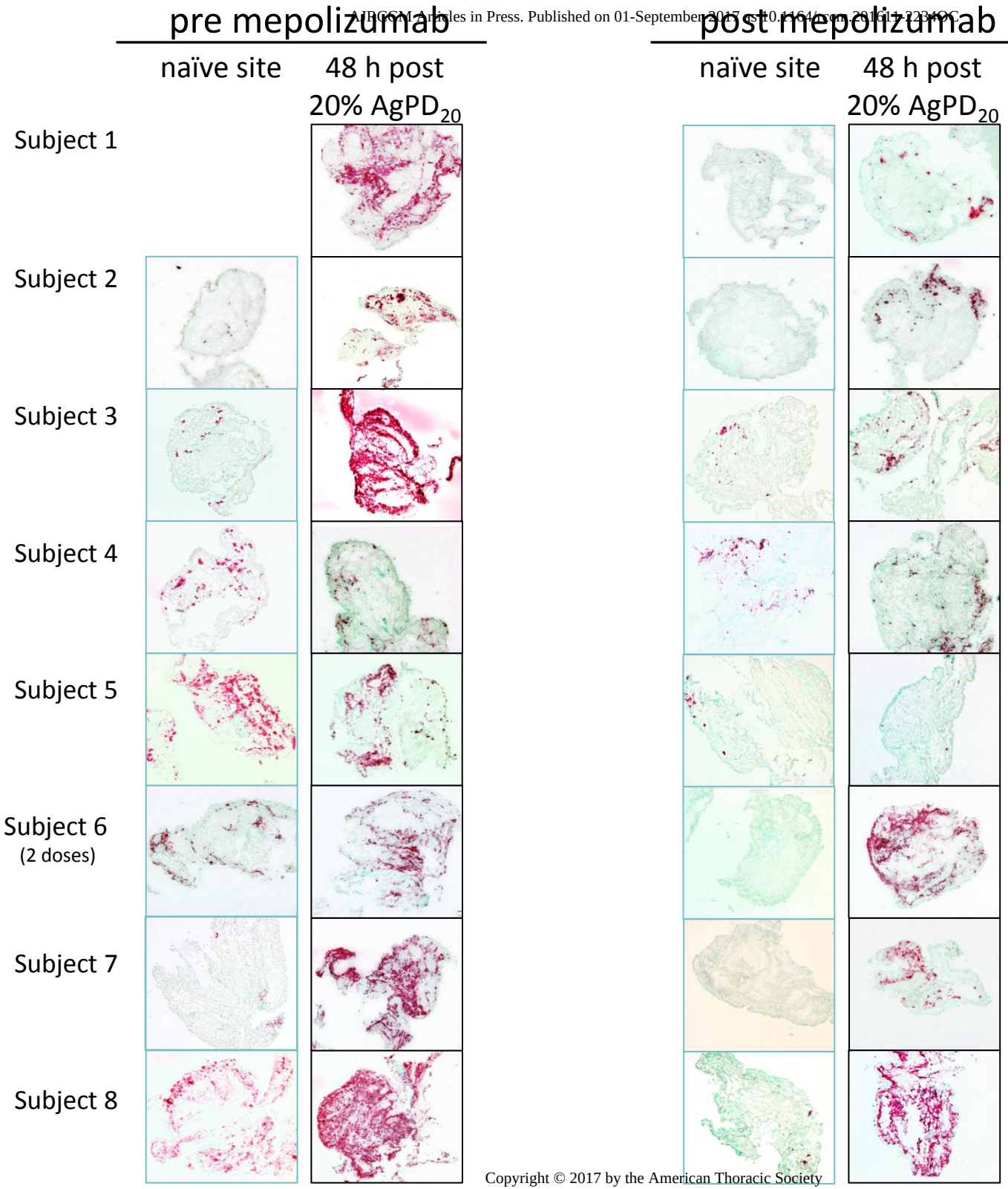

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