

Received Date : 18-Aug-2015

Revised Date: 07-Dec-2015

Accepted Date : 13-Dec-2015

Article type : Original Article-Asthma and Rhinitis

Title: Role of Local Eosinophilopoietic Processes in the Development of Airway Eosinophilia in Prednisone-dependent Severe Asthma

Authors: Roma Sehmi PhD¹, Steven G. Smith PhD¹, Melanie Kjarsgaard RRT¹, Katherine Radford MSc¹, Louis-Philippe Boulet, MD², Catherine Lemiere, MD³, Charlene M. Prazma, PhD⁴, Hector Ortega, MD, ScD⁴, James G. Martin, MD, DSc⁵, and Parameswaran Nair, MD, PhD

Address: ¹Division of Respiriology, McMaster University, Hamilton, ON, Canada. ² Institut universitaire de cardiologie et de pneumologie de Québec, Québec, Canada. ³Division of Pneumologie, University of Montreal, PQ, Canada, ⁴GlaxoSmithKline, Research Triangle Park, North Carolina, USA, ⁵Meakins-Christie Laboratories, Research Institute of the McGill University Health Center, PQ, Canada

Declaration of Funding Sources: Clinical Trials.gov: NCT01691508

MEA115575 Canadian sub-study was funded by GlaxoSmithKline. Dr. Steven Smith is supported by a Father Sean O'Sullivan Postdoctoral Fellowship award. Dr Nair is supported by a Canada Research Chair in Airway Inflammometry.

Running Title: In-situ airway eosinophilopoiesis in prednisone-dependent severe eosinophilic asthma

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cea.12695

This article is protected by copyright. All rights reserved.

Address correspondence and reprint requests to Dr Parameswaran Nair, (E-mail: parames@mcmaster.ca) or Dr Roma Sehmi (E-mail: sehmir@mcmaster.ca) Firestone Institute for Respiratory Health, St Joseph's Healthcare, 50 Charlton Avenue East, Hamilton, ON, L8N 4A6, Canada.

Key Words: anti-IL5 therapy, *in situ* eosinophilopoiesis, severe eosinophilic asthma,

ABSTRACT

Rationale: In severe asthmatics with persistent airway eosinophilia, blockade of the eosinophilopoietin, interleukin-5 has significant steroid sparing effects and attenuates blood and sputum eosinophilia. The contribution of local maturational processes of progenitors within the airways relative to the recruitment of mature cells from the peripheral circulation to airway eosinophilia in these patients is not known. We hypothesize that local eosinophilopoietic processes may be the predominant process that drives persistent airway eosinophilia and corticosteroid requirement in severe asthmatics.

Methods: In a cross-sectional study, the number and growth potential of eosinophil-lineage committed progenitors (EoP) were assayed in 21 severe eosinophilic asthmatics, 19 mild asthmatics, 8 COPD patients and 8 normal subjects. The effect of anti-IL-5 treatment on mature eosinophils and EoP numbers was made in severe eosinophilic asthmatics who participated in a randomized clinical trial of mepolizumab (sub-study of a larger GSK sponsored global phase III trial, MEA115575) where subjects received mepolizumab (100 mg, n=9) or placebo (n=8), as six monthly subcutaneous injections.

Results: Mature eosinophil and EoP numbers were significantly greater in the sputum of severe asthmatics compared with all other subject groups. In colony forming assays, EoP from blood of severe asthmatics demonstrated a greater response to IL-5 than mild

asthmatics. Treatment of severe asthmatics with mepolizumab significantly attenuated blood eosinophils and increased EoP. There was however no significant treatment effect on mature eosinophils, sputum EoP numbers or the prednisone maintenance dose.

Conclusions: Patients with severe eosinophilic asthma have an exaggerated eosinophilopoietic process in their airways. Treatment with 100 mg sub-cutaneous mepolizumab significantly attenuated systemic differentiation of eosinophils, but did not suppress local airway eosinophil differentiation to mature cells. Targeting IL-5 driven eosinophil differentiation locally within the lung maybe of relevance for optimal control of airway eosinophilia and asthma.

INTRODUCTION

Asthma is a chronic inflammatory disease of the airways that is well controlled by inhaled therapy in most patients (1). However, a subset of patients continues to have symptoms despite being on high doses of oral corticosteroids and although fewer than 10% of total asthma sufferers, this group accounts for over half the costs related to asthma care (2). In prednisone-dependent asthmatics with airway eosinophilia, sputum eosinophils are increased when asthma is uncontrolled (3) or severe (4) and decreased when asthma is controlled (5). In these patients, treatment strategies that aim to control airway eosinophilia are steroid-sparing, effective at decreasing asthma exacerbations and cost-effective in improving asthma control (6-8). It is therefore important to investigate the pathobiological processes that promote airway eosinophilia in order to develop effective treatment strategies for severe eosinophilic asthma.

Eosinophil differentiation and maturation from CD34⁺ hematopoietic progenitors (HPC) occurs largely in the bone marrow stimulated by the eosinophilopoietin, interleukin-5 (IL-5) (9) (10). The IL-5 receptor is comprised of a unique binding α -chain and a common

signaling β -chain that is shared with IL-3 and GM-CSF (11). Up-regulation of IL-5R α on CD34⁺ cells, as determined by flow cytometry occurs at an early stage in eosinophil development and denotes the phenotype of eosinophil-lineage committed progenitor cells (EoP) (12, 13). Precursor cells can also be enumerated in semi-solid cultures as eosinophil/basophil colony forming units (Eo/B-CFU) where each colony represents the outgrowth of daughter cells from a single precursor (14) or by sequential immunocytochemistry and *in situ* hybridization as CD34 immunopositive/ IL-5R α mRNA⁺ cells in tissue (15).

In assessing EoPs, it has become evident that the development of eosinophilic airway inflammation in asthma is associated with the ability of the bone marrow and affected tissues to support the mobilization, proliferation and local differentiation of EoP (16). In mild allergic asthmatics, bone marrow-derived IL-5 responsive EoP are elevated in blood (17), mucosa of the upper and lower airways and airway secretions of allergic asthmatics (15, 18-20) and have been shown to correlate with asthma severity (15). Following allergen or IL-5 inhalational challenge of atopic asthmatics, the number of airway EoP is further enhanced and precedes the development of airway eosinophilia (21-23). In allergic rhinitis patients, local IL-5-dependent differentiation of EoP was observed when nasal biopsies were cultured *ex vivo* with IL-5 or ragweed allergen resulting in a reduction in CD34 immunopositive/IL-5R α mRNA⁺ cells and a concurrent increase in the number of MBP immunoreactive cells (24). This showed that local differentiation, termed *in situ* eosinophilopoiesis, could rapidly increase mucosal levels of mature eosinophils during an inflammatory response (24). In all, these data support the view that local maturational processes promote development of eosinophilia in the lung and that targeting local IL-5 driven expansion of EoP may be an important strategy to control persistent eosinophilia in severe asthmatics.

In mild asthmatics, clinical studies with intravenous administration of high doses of mepolizumab (750 mg), a humanized monoclonal antibody that binds and inactivates IL-5 caused a complete maturational arrest of eosinophils in the bone marrow and blood but only partially depleted mature eosinophils in the bronchial mucosa (25, 26). This was associated with a significant, albeit incomplete, attenuation of EoP numbers within the bronchial mucosa suggesting that local IL-5-driven differentiation may play an important contributory role in the development of airway eosinophilia. Furthermore, this may explain findings from a study where anti-CCR3 treatment failed to clear luminal airway eosinophils in moderately severe asthmatics, likely because the treatment did not attenuate local differentiation reflected in the lack of effect of the drug on sputum levels of either EoP or mature eosinophils (27). Although a number of studies have reported that anti-IL-5 therapy attenuates blood eosinophilia in severe asthmatics with eosinophilic bronchitis, the effect of treatment on systemic versus local eosinophilopoietic processes within this endotype of patients remains unknown (8, 28-31).

The current study has investigated the contribution of *in-situ* eosinophilopoiesis in asthmatics with persistent airway eosinophilia despite treatment with potent anti-inflammatory therapy. We hypothesize that local maturation rather than systemic recruitment of mature cells may contribute to ongoing airway eosinophilia and corticosteroid treatment requirement in severe asthma. The aims of the current study were to 1) to compare EoP numbers in severe prednisone-dependent asthmatics with mild asthmatics, COPD subjects and normal healthy volunteers, and 2) to investigate in a randomized, double-blind, placebo-controlled parallel group clinical trial of severe prednisone-dependent asthmatics, the effect of mepolizumab treatment delivered subcutaneously, on local and systemic eosinophilopoietic processes.

METHODS

Subjects for cross-sectional study

For the baseline cross-sectional study of progenitor cell populations, sputum and blood samples were collected from severe prednisone-dependent eosinophilic asthmatics (n=21), mild atopic asthmatics (n=19), subjects with chronic obstructive pulmonary disease (COPD; n=8) and non-atopic healthy controls (n=8). All except COPD subjects were current non-smokers (<10/pack year) and all were healthy for 4 weeks prior to the study and provided written informed consent. Subject characteristics are summarized in Table 1. All experimental procedures were reviewed and approved by the Institutional Research Ethics Board.

All severe asthmatics included in this baseline cross-sectional study fitted the inclusion criteria for the GSK sponsored MEA115575 sub-study and samples were collected at visit 1 prior to steroid reduction (study design - supplemental Figure S1). A sub-set of these subjects was randomized into the clinical sub-study described below.

Randomized clinical trial and Study Design

The results for mepolizumab treatment described herein are a sub-study of a GSK sponsored global phase III trial, MEA115575 (30). Seventeen subjects who consented to participate and were eligible for inclusion in MEA115575 were invited to participate in the sub-study that investigated the effect IL5 blockade on local and systemic eosinophil differentiation in severe prednisone-dependent asthma. The subject characteristics are summarized in Table 2.

Seventeen volunteers with prednisone-dependent severe asthma with persistent blood eosinophilia as defined per protocol (30) were recruited to a four-center, randomized, double-blind, placebo-controlled parallel group sub-study (described in supplemental Figure S1). Sputum eosinophilia was not an inclusion criterion. The sub-study included venous blood and

sputum sampling for EoP, and mature eosinophil enumerated at Visit 1 (pre-treatment, steroid dose not optimized), Visit 4 (steroid dose optimized and 4 weeks after first injection) and Visit 9 (4 weeks after the last injection). Following a corticosteroid optimization period of 3 - 8 weeks in duration, subjects were randomized to receive either mepolizumab (100 mg) or placebo by subcutaneous injection. Subsequent injections were administered every 4 weeks.

In this study, 9 subjects received mepolizumab (administered in six monthly subcutaneous injections of 100 mg each) and 8 patients received placebo. Two subjects in the placebo group withdrew early due to unrelated illness and these data were not included in the final analyses of the sub-study.

Methods for enumerating progenitor cells by flow cytometry (supplemental Figure S2) and methylcellulose culture and clinical outcomes are described in the online supplement.

Statistical analyses

Baseline clinical and laboratory data are expressed as mean ($\pm$ SEM) unless otherwise stated. Within-group comparisons were made using a paired t-test for normally distributed data or the Wilcoxon rank test for non-normally distributed data. Between-group comparisons were made using the Mann Whitney U test for non-normally distributed data or unpaired t-test for normally distributed data. A MANOVA test was performed to assess eosinophil colony counts in blood methylcellulose cultures and Tukey's post-hoc analyses. Post-hoc tests were carried out for planned comparisons that were determined *a priori*. A two-sided P value of 0.05 was considered to indicate statistical significance. All analyses were performed using the PRISM software, version 6.

Role of the sponsors

For the MEA115575 sub-study the academic investigators designed the protocol in collaboration with employees of GlaxoSmithKline. GSK sponsored the larger randomized clinical trial (MEA115575) for which GlaxoSmithKline supplied the study drug and collected and analyzed the data. The GSK funded sub-study sputum samples were collected from 4 centers in Canada and samples were sent to the Firestone Institute for Respiratory Health for processing and analyses. All authors contributed to interpretation of the data. The study was approved by the hospital's research ethics board. All patients provided written informed consent. ClinicalTrials.gov number, NCT01691508.

RESULTS

Increased Luminal Eosinophil Progenitor Cells in Severe Asthmatics

In the baseline cross-sectional study, COPD subjects were included as a non-eosinophilic airways disease comparator and mild asthmatics were included as a disease-severity control group to severe asthmatics. There were significantly great numbers of mature eosinophils in blood and sputum from severe eosinophilic asthmatics compared to mild asthmatics, COPD and normal healthy volunteers (Table 3). While hemopoietic progenitor cell numbers (HPC; CD34+45+) in blood did not differ between the subject groups, in sputum, HPC numbers from severe asthmatics were significantly greater than COPD and normal subjects. The number of EoP (CD34+45+IL5R α + cells) in blood from severe asthmatics was significantly greater than normal and COPD subjects but not mild asthmatics. In sputum, EoP from severe asthmatics were significantly greater than all other groups including patients with mild asthma where the proportional frequency of EoP was 10-fold higher (Table 3).

In 14 day methylcellulose colony assays, circulating progenitors from severe eosinophilic asthmatics showed a significantly greater clonogenic response than mild asthmatic subjects when cultured with IL-5 (1 and 10 ng/ml) (Figure 1a). IL-5-, but not diluent-, stimulated growth of Eo/B-CFU from severe asthmatics was significantly attenuated in the presence of an optimal concentration of an IL-5 receptor blocking antibody (20ng/ml). These data demonstrate the specificity of IL-5 driven outgrowth of EoP in methylcellulose cultures (Figure 2).

The marked increase in sputum EoP in severe asthmatics suggests that extramedullary eosinophilopoietic processes may be an important pathological process contributing to persistent airway eosinophilia (Table 1). To test this hypothesis, we investigated the effect of blockade of interleukin-5 activity on local and systemic levels of EoP and mature eosinophils in severe prednisone dependent asthmatics. (Study design – supplemental Figure S1)

Effect of mepolizumab treatment on mature eosinophils in the blood and sputum

Blood eosinophils levels at the beginning of the study (visit 1: V1) were comparable between the mepolizumab and placebo treated groups (Figure 3). Following treatment with mepolizumab but not placebo (V1 versus V9) there was a significant decline in the number of mature eosinophils circulating in the blood (Figure 3).

At V1, sputum eosinophil levels were comparable in the mepolizumab- and placebo-treatment group (V1 drug: $7.4 \pm 3.3\%$ vs V1 placebo: $22.4 \pm 8.6\%$, $p=0.08$) (Figure 3). Following treatment with mepolizumab there was a trend for a reduction in sputum levels of eosinophils, although this effect was not statistically significant when either percent values were compared (V9 drug: $3.9 \pm 3.0\%$ vs placebo: $17.3 \pm 10.5\%$, $p=0.19$) (Figure 3), or ratios to baseline were compared as V9/V1 (drug vs placebo, median and range: 21.95 (3.2-49.08%) vs 11.67 (0 -75%, respectively), $P=0.73$).

Effect of mepolizumab treatment on eosinophils progenitors in the blood and sputum

By flow cytometry, we found a significant increase in blood levels of EoP expressed as a proportion of the total progenitor cell numbers when baseline (V1) levels were compared to the end treatment (V9) in the mepolizumab but not the placebo treated group (Figure 4). Similar findings were observed when absolute numbers of blood EoP were compared, discounting the effect of changes in total leukocyte cell counts contributing to proportional increases in blood EoP. In contrast, treatment with mepolizumab had no effect on sputum levels of EoP when pre-treatment levels were compared to post-treatment levels (Figure 4). In addition, no statistically significant changes were found when mepolizumab induced changes were compared to the placebo control group (Figure 4).

In methycellulose culture assays, compared to pre-treatment levels there was a significant increase in Eo/B-CFU grown with IL-5 (0.1 ng/ml) in the mepolizumab-treated but not the placebo treated group (Figure5). Similar trends were observed in the Eo/B-CFU grown in diluent and in higher doses of IL-5 (1 and 10 ng/ml) in the mepolizumab-treated group (supplemental Figure S3). Similar culture assays were not performed on sputum extracted cells due to the lack of sterility of the sample and the low cell recovery.

Clinical Outcomes: Steroid sparing effect, asthma control and airway function

The steroid sparing effect and ACQ data have previously been reported in the main global clinical trial report by Bel et al.(30). For the cohort of subjects included in this study, the steroid dose was reduced compared to pre-treatment levels in the mepolizumab treated group(V1 vs V9 drug: 15(7.5-25) vs 5(0-20) mg; placebo: 15(7.5-30) vs 15 (7.5-25). However, when the prednisone dose was expressed as % reduction of the *optimized* steroid dose (V4), this effect was not significant (drug: 33±22% vs. placebo: -3±8.3%, P=0.23). The ACQ data showed an improvement in asthma control score when pre-treatment baseline

levels (V1) were compared to end of treatment (V9) in the mepolizumab treated group (drug: 1.9 ± 0.4 vs 0.8 ± 0.4 ACQ Score) but not in the placebo-treated group (placebo: 1.6 ± 0.2 vs 1.9 ± 0.4 ACQ score) . FEV1 (% predicted) showed modest changes that were not statistically significant when V1 levels were compared to V9 levels in the mepolizumab treated group (drug: 46.4 ± 5.6 vs $54.8 \pm 7.9\%$; placebo: 52.1 ± 6.9 vs $54.1 \pm 9\%$ predicted). In addition, no significant difference was observed when changes in the drug-treated group were compared to the placebo-treated group. There were 6 eosinophilic asthma exacerbations in the 6 patients who received placebo and completed the study. In comparison 4 of the 9 patients who received mepolizumab and completed the study had an eosinophilic asthma exacerbations and one patient had a neutrophilic asthma exacerbation.

DISCUSSION

Eosinophil progenitor cells have been demonstrated in the airways, blood and bone marrow of patients with mild atopic asthma (21, 32). However, there is no information on the numbers, clonogenic potential, or clinical relevance of these lineage-committed progenitors in patients with severe prednisone-dependent eosinophilic asthma. We show for the first time, in a cross-sectional study, a marked luminal increase in EoP in severe asthmatics compared to subjects with mild atopic asthma, COPD and normal healthy controls. In clonogenic assays, circulating progenitors from severe asthmatics demonstrated a greater IL-5-driven proliferative potential (that could be attenuated by an anti-IL5 antibody, *in vitro*) compared to cells from mild atopic asthmatics. Persistence of EoP in sputum despite anti-IL5 therapy was associated with persistence of sputum mature eosinophils and lower prednisone-sparing effect. These observations support the view that IL-5-driven *in situ* eosinophilopoiesis is clinically relevant in modulating airway eosinophilia and clinical response to anti-eosinophil therapies.

In a randomized placebo-controlled clinical trial of mepolizumab administered in 6 monthly subcutaneous injections of 100 mg each, we found that there was a rapid and sustained reduction in blood levels of mature eosinophils and an associated increase in eosinophil progenitors in prednisone dependent severe eosinophilic asthmatics. In contrast, there was no significant treatment effect on sputum levels of mature eosinophils or their precursors in these subjects. The observed dissociation between luminal and systemic effects of this treatment suggest that *in situ* differentiation may be the predominant process contributing to persistent airway eosinophilia. For the optimal control of symptoms and exacerbations, we propose that local eosinophilopoietic processes this should be considered as an important therapeutic target in severe eosinophilic asthmatics.

Clinical trials evaluating the therapeutic relevance of anti-IL5 biologics over the past decade have been variously successful with recent studies indicating that this therapy is most effective in severe asthmatics with eosinophil-driven airway inflammatory disease (33, 34). Two independent small group studies showed that treatment with monthly intravenous infusions of mepolizumab (750 mg iv) in this endotype of patients was steroid sparing, associated with 84% reduction in exacerbations and improved asthma control (28, 35). Importantly, Nair et al, showed that these clinical outcomes were associated with a significant attenuation of both blood and sputum eosinophil numbers (8). Subsequent studies have endeavored to optimize the dose and delivery platforms for mepolizumab (29-31). In the recent study, Bel et al., showed that monthly subcutaneous injections of a lower dose of mepolizumab (100 mg sc) attenuated the exacerbation rates by 32% and were steroid-sparing in severe eosinophilic asthmatics (30) albeit to a lower level than those reported by Nair et al. (750 mg, i.v.) (8). A sub-group of 17 patients from this trial consented to participate in a sub-study investigating the effect of mepolizumab treatment on local and systemic eosinophilopoietic processes.

The systemic reduction of mature blood eosinophils from the current study is consistent with a previous study by Menzies-Gow et al., that demonstrated a significant reduction of circulating mature eosinophil numbers as a result of maturational arrest of eosinophils at the myelocyte and metamyelocyte stage within the bone marrow following intravenous treatment with mepolizumab (750 mg) in asymptomatic mild asthmatics (26). In addition, our findings of increased blood EoP numbers, determined by flow cytometry and colony-forming assays, align with the view of systemic arrest of eosinophil maturation by anti-IL-5 treatment. That IL-5 can upregulate its own receptor expression on CD34⁺ cells predicts that anti-IL-5 therapy would attenuate EoP numbers (36). However eosinophil-lineage commitment involving the earliest expression of IL-5R α on CD34⁺ cells is controlled by a delicate combinatorial balance between the threshold expression levels of fate-determining transcription factors including CCAAT/enhancer binding protein (C/EBP), GATA-1 (a zinc finger family member), PU.1 (an Ets family member) and friend of GATA (FOG) which serve as master regulators for eosinophil-lineage commitment from common myeloid precursors (37). Little is known about upstream activators of these transcription factors, although these events precede up-regulation of IL-5 receptor α chain (IL-5R α) surface expression on CD34⁺ cells which in turn acts as a permissive rather than instructive signal for IL-5 mediated terminal differentiation of EoP. This may explain the observed effect of anti-IL-5 therapy which arrested terminal differentiation of eosinophils thus explaining the observed reduction in mature eosinophil and increased EoP numbers in the blood.

The observed dissociation between the systemic and local airway effects of mepolizumab therapy suggest that local maturation rather than systemic recruitment of mature cells may be the predominant process promoting persistent airway eosinophilia in severe asthmatics. This is in line with studies that have shown significant modulation of local airway eosinophilia in asthmatics using biologics that targeted IL-5, (26) (38) while those that

targeted the recruitment of mature eosinophils by chemoattractants such as eotaxin were ineffective (27). There may, however, be several alternate explanations for the lack of a significant attenuation of airway levels of mature eosinophils and their precursors following mepolizumab therapy. Firstly, this may be due to the small sample size of the current study. However a report by Dasgupta et al has shown that examining 6 subjects in each group is adequate to detect a 50% reduction in sputum eosinophils numbers (39) and this analysis was based on fluctuations in airway eosinophils in the mepolizumab treatment study performed by Nair et al (8). Secondly, reduced bioavailability of mepolizumab within the tissue mucosa consequent to the lower dose of drug (100 mg sc) used in this study may have contributed to this effect although this requires further investigation in severe asthmatics. The argument against this is that even with high doses of mepolizumab (750 mg iv) while systemic levels of eosinophils fell precipitously, levels of mature eosinophils and CD34+IL-5R α mRNA+ cells in the bronchial mucosa were only modestly attenuated (26). The compensatory effect of eosinophilopoietic cytokines produced locally within the tissue may have counteracted the effect of anti-IL-5 (26). It has been shown that CD34⁺ cells appearing in sputum of allergic asthmatic subjects post-allergen challenge stain positively for intracellular IL-5 and IL-13 suggesting that precursor cells may themselves act as pro-inflammatory effectors in the microenvironment of the inflamed tissue, being able to autologously replenish IL-5 depleted by antibody treatment(40).

Irrespective of the mechanism of persistent airway eosinophilia, our observations suggest that control of airway eosinophilia may be necessary to obtain optimum clinical benefits. While the larger clinical trial demonstrated a significant prednisone sparing effect, this was not apparent in this sub-study in which sputum eosinophils and eosinophil progenitors were not adequately controlled.

In conclusion, the findings of this study suggest that local maturation from eosinophil precursors may play an important role in promoting airway eosinophilia in severe prednisone dependent asthmatics. Further studies that investigate targeting local molecular mechanisms that ameliorate *in-situ* eosinophil differentiation within the lung may help to address whether controlling airway tissue and luminal eosinophilia is relevant to achieving good asthma control and to prevent exacerbations, in addition to controlling blood eosinophilia, in patients with severe prednisone-dependent asthma.

Acknowledgments

We thank all the subjects that volunteered in this study and the staff members at all the study centres.

Reference List

1. Holgate ST. A look at the pathogenesis of asthma: the need for a change in direction. *Discovery Medicine*. 2010;9:439-47.
2. Bateman ED, Boushey HA, Bousquet J, Busse WW, Clark TJH, Pauwels RA, et al. Can Guideline-defined Asthma Control Be Achieved? *American Journal of Respiratory and Critical Care Medicine*. 2004; 170:836-44.
3. Leuppi JD, Salmone CM, Jenkins CR, Anderson SD, Xuan W, Marks GB, et al. Predictive markers of asthma exacerbation during stepwise dose reduction of inhaled corticosteroids. *Am J Respir Crit Care Med*. 2001;163:406-12.
4. van Veen IH, Ten Brinke A, Gauw SA, Sterk PJ, Rabe KF, Bel EH. Consistency of sputum eosinophilia in difficult to treat asthma: a 5-year follow-up study. *J Allergy Clin Immunol*. 2009;124:615-7.
5. Lemièrè C, Ernst P, Olivenstein R, Yamauchi Y, Govindaraju K, Ludwig MS, et al. Airway inflammation assessed by invasive and noninvasive means in severe asthma: eosinophilic and non-eosinophilic phenotypes. *J Allergy Clin Immunol*. 2006;118:1033-9.
6. Green RH, Brightling CE, McKenns S, Hargadon B, Parker D, Bradding P, et al. Asthma exacerbations and sputum eosinophil counts: a randomised control trial. *Lancet*. 2002;360:1715-21.
7. Jayaram L, Pizzichini MM, Cook RJ, Boulet LP, Lemièrè C, Pizzichini E, et al. Determining asthma treatment by monitoring sputum cell counts: effect on exacerbations. *Eur Respir J*. 2006;27:483-94.
8. Nair P, Pizzichini M, Kjarsgaard M, Inman M, Efthimiadis A, Pizzichini E, et al. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *New Engl J Med*. 2009;360:985-93.
9. Clutterbuck EJ, Herst EMA, Sanderson CJ. Human interleukin-5 (IL-5) regulates the production of eosinophils in human bone marrow cultures: comparisons and interaction with IL-1, IL-3, IL-6 and GM-CSF. *Blood*. 1989;73:1504-12.

10. Sanderson CJ. Interleukin-5, eosinophils, and disease. *Blood*. 1992;79:3101-9.
11. Tavernier J, Devos R, Cornelis S, Tuypens T, Van der Heyden J, Fiers W, et al. A human high affinity interleukin-5 receptor (IL-5R) is composed of an IL-5 specific α -chain and a β -chain shared with a receptor for GM-CSF. *Cell*. 1991;66:1175-84.
12. Mori Y, Iwasaki H, Kohno K, Yoshimoto G, Kikushige Y, Okeda A, et al. Identification of the human eosinophil lineage-committed progenitor: revision of phenotypic definition of the human common myeloid progenitor. *J Exp Med* 2009;206:183-93.
13. Sehmi R, Wood LJ, Watson RM, Foley R, Hamid Q, O'Byrne PM, et al. Allergen-induced increases in IL-5 receptor α -subunit expression on bone marrow-derived CD34⁺ cells from asthmatic subjects: a novel marker of progenitor cell commitment towards eosinophilic differentiation. *J Clin Invest*. 1997;100:2466-75.
14. Sehmi R, Denburg J. Differentiation of human eosinophils: role in allergic inflammation. In: G.Marone, editor. *Human Eosinophils: Biological and Clinical Aspects*. Chemical Immunology. 2 volume 76 ed. Basel: Karger; 2000. p. 29-44.
15. Robinson DS, Damia R, Zeibecoglou K, Molet S, North J, Yamada T, et al. CD34⁺/Interleukin-5R α messenger RNA⁺ cells in the bronchial mucosa in asthma: Potential airway eosinophil progenitors. *Am J Respir Cell Mol Biol*. 1999;20:9-13.
16. Gauvreau GM, Denburg JA. Hemopoietic progenitors: the role of eosinophil/ basophil progenitors in allergic airway inflammation. *Expert Reviews Clin Immunol* 2005;1:87-101.
17. Sehmi R, Howie K, Sutherland DR, Schragge W, O'Byrne PM, Denburg JA. Increased levels of CD34⁺ hemopoietic progenitor cells in atopic subjects. *Am J Respir Cell Mol Biol*. 1996;15:645-54.
18. Kim YK, Uno M, Hamilos DL, Beck L, Bochner B, Schleimer R, et al. Immunolocalization of CD34 in nasal polyposis. Effect of topical corticosteroids. *Am J Respir Cell Mol Biol*. 1999;20:388-97.
19. Sergejeva S, Johansson AK, Lotvall J. Allergen exposure-induced differences in CD34⁺ cell phenotype: relationship to eosinophilopoietic responses in different compartments. *Blood*. 2004;103:1270-7.
20. Sergejeva S, Malmh  ll C, Lotvall J, Pullerits T. Increased numbers of CD34⁺ cells in nasal mucosa of allergic rhinitis patients: inhibition by a local steroid. *Clin Exp Allergy*. 2005;35:34-8.
21. Dorman SC, Sehmi R, Gauveau GM, Watson R, Foley R, Jones GL, et al. The kinetics of bone marrow eosinophilopoiesis and associated cytokines after allergen inhalation. *Am J Respir Crit Care Med*. 2004;169:565-72.
22. Smith SG, Gugilla A, Mukherjee M, Merim K, Irshad A, Tang W, et al. Thymic stromal lymphopoietin and IL-33 modulate migration of hematopoietic progenitor cells in patients with allergic asthma. *J Allergy Clin Immunol*. 2015;135(6):1594-602
23. Menzies-Gow AN, Flood-Page PT, Robinson DS, Kay AB. Effect of inhaled interleukin-5 on eosinophil progenitors in the bronchi and bone marrow of asthmatic and non-asthmatic volunteers. *Clin Exp Allergy*. 2007;37:1023-32.
24. Cameron L, Christodouloupoulos P, Lavigne F, Nakamura Y, Eidelman D, McEuen A, et al. Evidence of local eosinophil differentiation within allergic nasal mucosa: inhibition with soluble IL-5 receptor. *J Immunol*. 2000;164:1538- 45.
25. Flood-Page P, Menzies-Gow A, Kay A, Robinson D. Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. *Am J Respir Crit Care Med*. 2003;167:199-204.
26. Menzies-Gow AN, Flood-Page PT, Sehmi R, Burman J, Hamid Q, Robinson DS, et al. Anti-IL-5 (mepolizumab) therapy induces bone marrow eosinophil maturational arrest and decreases eosinophil progenitors in the bronchial mucosa of atopic asthmatics. *J Allergy Clin Immunol* 2003;111:714-9.

27. Neighbour H, Boulet LP, Lemiere C, Sehmi R, Leigh R, Sousa AR, et al. Safety and efficacy of an oral CCR3 antagonist in patients with asthma and eosinophilic bronchitis: a randomized, placebo-controlled clinical trial. *Clin Exp Allergy*. 2014;44:508-16.
28. Haldar P, Brightling C, Hargadon B, Gupta S, Monteiro W, Sousa A, et al. Mepolizumab and exacerbations of refractory eosinophilic asthma. *New Engl J Med*. 2009;360:973 - 84.
29. Pavord I, Korn S, Howarth P, Bleecker E, Buhl R, Keene O, et al. Mepolizumab for severe eosinophilic asthma (DREAM): a multicentre, double-blind, placebo-controlled trial. *Lancet*. 2012;380:651-9.
30. Bel EH, Wenzel SE, Thompson PJ, Prazma CM, Keene ON, Yancey SW, et al. Oral glucocorticoid-sparing effect of mepolizumab in eosinophilic asthma. *N Engl J Med*. 2014 Sep 25;371:1189-97.
31. Ortega H, Liu M, Pavord I, Brusselle G, FitzGerald J, Chetta A, et al. Mepolizumab treatment in patients with severe eosinophilic asthma. *N Engl J Med*. 2014;371:1198 - 207.
32. Dorman SC, Babirad I, Post J, Watson RM, Foley R, Jones GL, et al. Progenitor egress from the bone marrow after allergen challenge: role of stromal cell-derived factor 1alpha and eotaxin. *J Allergy Clin Immunol*. 2005;115:501-7. .
33. Nair P. Anti-Interleukin-5 Monoclonal Antibody to Treat Severe Eosinophilic Asthma. *New England Journal of Medicine*. 2014;371:1249-51.
34. Mukherjee M, Sehmi R, Nair P. Anti-IL5 therapy for asthma and beyond. *World Allergy Organization Journal*. 2014;7:32-35.
35. Punia N, Smith S, Thomson J, Irshad A, Nair P, Sehmi R. Interleukin-4 and interleukin-13 prime migrational responses of haemopoietic progenitor cells to stromal cell-derived factor-1alpha. *Clin Exp Allergy*. 2012;42:255 - 64.
36. Tavernier J, Van der Heyden J, Verhee A, Brusselle G, Van Ostade X, Vandekerckhove J, et al. Interleukin-5 regulates the isoform expression of its own receptor a-subunit. *Blood*. 2000;95:1600-7.
37. Uhm TG, Kim BS, Chung IY. Eosinophil development, regulation of eosinophil-specific genes, and role of eosinophils in the pathogenesis of asthma. *Allergy Asthma Immunol Res*. 2012;4:68-79.
38. Imaoka H, Campbell H, Babirad I, Watson RM, Mistry M, Sehmi R, et al. TPI ASM8 reduces eosinophil progenitors in the sputum after allergen challenge in mild asthmatics. *Clin Exp Allergy*. 2011;41:1740-6.
39. Dasgupta A, Zhang S, Thabane L, Nair P. Sample sizes for clinical trials using sputum eosinophils as a primary outcome. *Eur Respir J*. 2013;42:1003-11.
40. Allakhverdi Z, Comeau MR, Smith DE, Toy D, Endam LM, Desrosiers M, et al. CD34+ hemopoietic progenitor cells are potent effectors of allergic inflammation. *J Allergy Clin Immunol*. 2009;123:472-8.

FIGURE LEGENDS

Figure 1: *Increased clonogenic potential of progenitor cells from blood of severe versus mild asthmatics.* (a) Blood derived progenitors were cultured in methylcellulose culture with diluent or IL-5. Compared to mild asthmatics (open bars, n=5), progenitor cells from severe asthmatics (closed bars, n=7) showed a greater proliferative potential in IL-5 stimulated conditions (*p<0.05 within group comparison to diluent; #p<0.05 between group comparisons).

Figure 2: Attenuation of IL-5 driven clonogenic expansion of EoP by anti-IL5 In blood from severe asthmatics (n=5), IL-5 stimulated Eo/B-CFU colony growth was inhibited in the presence of an optimal concentration of IL-5R blocking antibody (20 ng/ml). This demonstrates the specificity of IL-5 driven eosinophilopoietic potential of progenitor cells from severe asthmatics. Data are presented as mean $\pm$ SEM.

Figure 3: Blood and sputum eosinophil counts. Following treatment with mepolizumab there was a significant decline in circulating levels of mature eosinophils while the numbers in the placebo treatment group remained unchanged. Treatment with mepolizumab treatment did not significantly attenuate sputum eosinophil levels compared to placebo treatment arm and no significant between group effect was found. Data are presented as mean $\pm$ SEM.

Figure 4: EoP enumeration by FACS. Blood and sputum eosinophil-lineage committed progenitors (EoP) were enumerated by flow cytometry (CD34+45+IL5R α + cells and expressed as a percentage of the total hematopoietic progenitor cell population (CD34+45+ cells). Compared to baseline screening levels, a significant increase in blood levels of EoP was observed post-treatment in the mepolizumab but not placebo treated group. In contrast, drug treatment had no effect on sputum levels of EoP using a similar comparison.

Figure 5: EoP clonogenic assay. Blood eosinophil progenitor cells (EoP) were enumerated by clonogenic expansion in methylcellulose culture. Compared to pre-treatment levels there was a significant increase in Eo/B-CFU grown with sub-optimal dose of IL-5 (0.1 ng/ml) in the drug but not the placebo treated group. These data suggest that inhibition of IL-5-driven terminal differentiation of eosinophils results in the increased circulating levels of EoP.

Table 1: Baseline Subject Characteristics for EoP Enumerations

Characteristic	Severe Asthmatics (n=21)	Mild Atopic Asthmatics (n=19)	COPD (n = 8)	Normals (n = 8)
Age, years	50.1 $\pm$ 2.7	29.9 $\pm$ 3.9	64.2 $\pm$ 5.4*	31.3 $\pm$ 4.5
Gender, % male	53.3	46.7	85.7	42.9
BMI, kg·m ²	29.4 $\pm$ 1.3	24.3 $\pm$ 1.2	26.7 $\pm$ 4.6	27.2 $\pm$ 5.2
Pulmonary function				
Pre-bronchodilator				
FEV ₁ , L	1.7 $\pm$ 0.2*	3.7 $\pm$ 0.2	1.4 $\pm$ 0.8*	3.4 $\pm$ 1.1
FEV ₁ % predicted	52.2 $\pm$ 4.1*	95.0 $\pm$ 3.4	40.4 $\pm$ 20.1*	101.8 $\pm$ 15.2
FVC, L	2.9 $\pm$ 0.2*	4.8 $\pm$ 0.2	2.6 $\pm$ 0.7*	4.4 $\pm$ 1.1
FVC % predicted	67.7 $\pm$ 3.3*	103.8 $\pm$ 3.0	56.4 $\pm$ 13.8*	114.6 $\pm$ 15.2
FEV ₁ /FVC %	61.4 $\pm$ 3.2*	76.5 $\pm$ 1.8	63.2 $\pm$ 23.8	82.4 $\pm$ 19.0
Comorbidities				
Hypertension, n %	20.0	0.0	25.0	0.0
Hyperlipidemia, n %	20.0	0.0	25.0	0.0

Smoking Status				
Current, n %	0.0	0.0	66.7	0.0
Ex-smoker, n %	53.0	0.0	33.3	16.7
Non-smoker, n %	47.0	100.0	0.0	83.3
Cigarette pack-years	2.9 ± 1.0	0.0	60.6 ± 22.3*	1.3 ± 2.2
Statin use, n %	20.0	0.0	16.7	0.0

Severe eosinophilic asthmatics had pre-bronchodilator FEV₁<80% predicted, >12% reversibility of FEV₁ upon challenge with short-acting bronchodilator, PC₂₀ <8mg/ml, at least 6 months history of treatment with oral steroids (5-35 mg daily prednisone or equivalent), inhaled corticosteroids (>880mcg/day) and eosinophilic inflammation determined by blood eosinophils ≥150/μl currently or <300/μl within the past 12 months. Mild atopic asthmatics were skin prick test positive, FEV₁≥70% predicted, >12% reversibility of FEV₁, PC₂₀≤16 mg/ml, and were steroid-naïve with infrequent use of inhaled β₂-agonists. Subjects with COPD were skin prick test negative, FEV₁<70% predicted, <12% reversibility of FEV₁. Normal healthy controls (n=8) were skin prick test negative, FEV₁≥70% predicted, baseline PC₂₀>16 mg/ml. Values are expressed as mean ± SD

* Significant difference (p<0.05) compared to normal subjects

Table 2: Baseline Demographic and Clinical Characteristics of Severe Asthmatics Patients in Mepolizumab Treatment Study

Characteristics	Mepolizumab	Placebo
Pt. Number	n=9	n=6
Age (yrs)	50.1 ± 14.5	48.8 ± 5.9
Gender	M=3, F=6	M=5, F=1
Duration of Asthma (yrs)	21.1 ± 11.7	10.3 ± 6.3
Prednisone (mg/day)		
Median	15.0	15.0
Range	7.5 – 25.0	7.5 – 30.0
† ICS Dose (Total daily dose)		
Median	2000	1000
Range	1000 – 2500	500 - 1500
Long-acting β agonist (no. of patients)	9.0	6.0
Leukotriene-receptor antagonist (no. of patients)	4.0	3.0
Blood Eos x10 ⁶ /ml		

Median Range	0.4 0.03 – 0.75	0.44 0.33 – 0.51
Sputum Eos (%) Median Range	4.5 0 – 28.5	16.3 3.3 – 50.4
FEV ₁ % predicted (pre-BD)	46.4 ± 15.8	52.1 ± 15.4
FEV ₁ Abs (L)	1.43 ± 0.34	1.98 ± 0.65
FVC (L)	2.29 ± 0.52	3.33 ± 0.51
FEV ₁ /FVC (%)	67.79 ± 15.38	57.84 ± 12.93
FEV ₁ reversibility (%)	22.37 ± 8.46	15.6 ± 1.52

* Plus-minus values are mean ± SD. Values are those recorded at the time of screening unless otherwise stated. No significant differences between the groups for any of the variables. FEV₁ denotes forced expiratory volume in 1 second. † Dose of inhaled corticosteroid is the equivalent of inhaled fluticasone. *P<0.05 for between group comparisons.

Table 3: Increased Luminal Eosinophil Progenitor Cells in Severe Asthma

	SEVERE ASTHMA n=21	MILD ASTHMA n=19	COPD n=8	NORMALS n=8
BLOOD				
Eosinophils (per mm ³)	920 ± 250*†\$	270 ± 60*	220 ± 40	70 ± 10
HPC Cells/10 ⁶ WBC)	1436 ± 201	2093 ± 427	1726 ± 151	1131 ± 227
EoP (Cells/10 ⁶ WBC)	213 ± 54*†\$	293 ± 194*	12.6 ± 3.9	38 ± 26
EoP (% of total HPC)	13.9 ± 1.9%*†\$	10 ± 6%*	1.75 ± 0.48%	3.0 ± 2.6%
SPUTUM				
TCC (x10 ⁶ /ml)	11.8±4.7*†	4.86±2.82*	5.89±3.22*	2.75±2.33
Eos (% of TCC)	14 ± 3.4%*†\$	8.9 ± 1.9%*	4.4 ± 2%	1.1 ± 0.5%
HPC Cells/10 ⁶ WBC)	9997 ± 2530*†\$	8270 ± 1230*	2154 ± 1140	1805 ± 930
EoP (Cells/10 ⁶ WBC)	6621 ± 2000*†\$	631 ± 341*	30 ± 17	9 ± 5
EoP (% of total HPC)	70.1 ± 6.3%*†\$	6 ± 2%*	3.4 ± 2.8%	3.4 ± 2.8%

In a baseline study hematopoietic progenitor cells (HPC; CD34+45+ cells) and Eosinophil-lineage committed progenitor cells (EoP; CD34+45+IL5R α + cells) were enumerated in blood and sputum samples by flow cytometry. Baseline subject characteristics of each group are listed in Table 1. Data are expressed as mean $\pm$ SD; * (p<0.05) compared to normal controls; † (p<0.05) severe asthmatics compared to mild asthmatics; § (p<0.05) severe asthmatics compared to COPD.

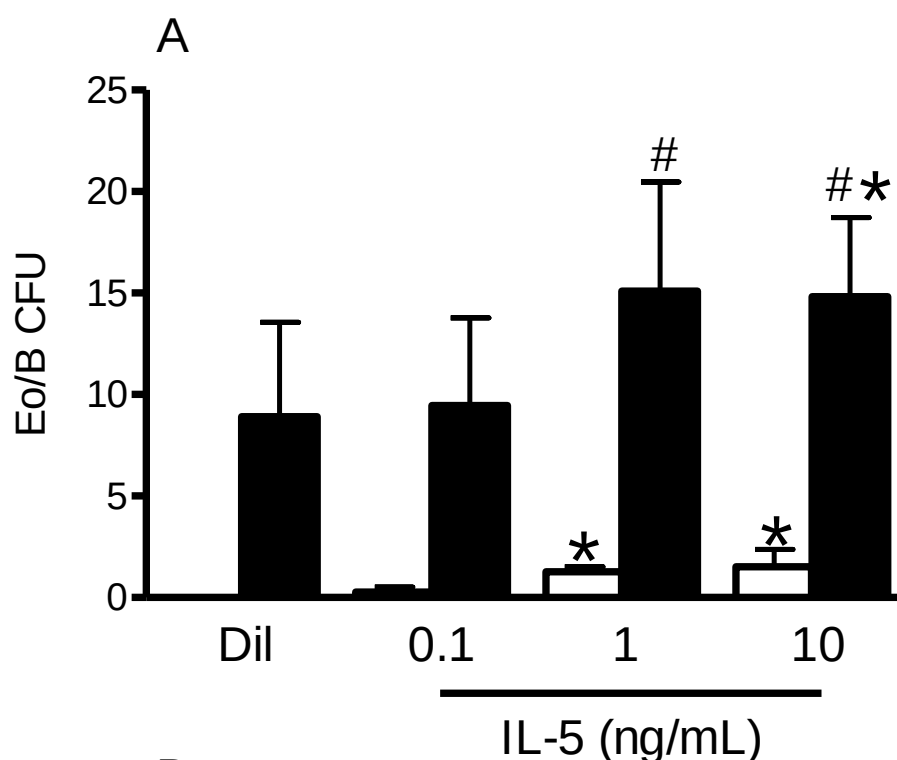


Figure 1

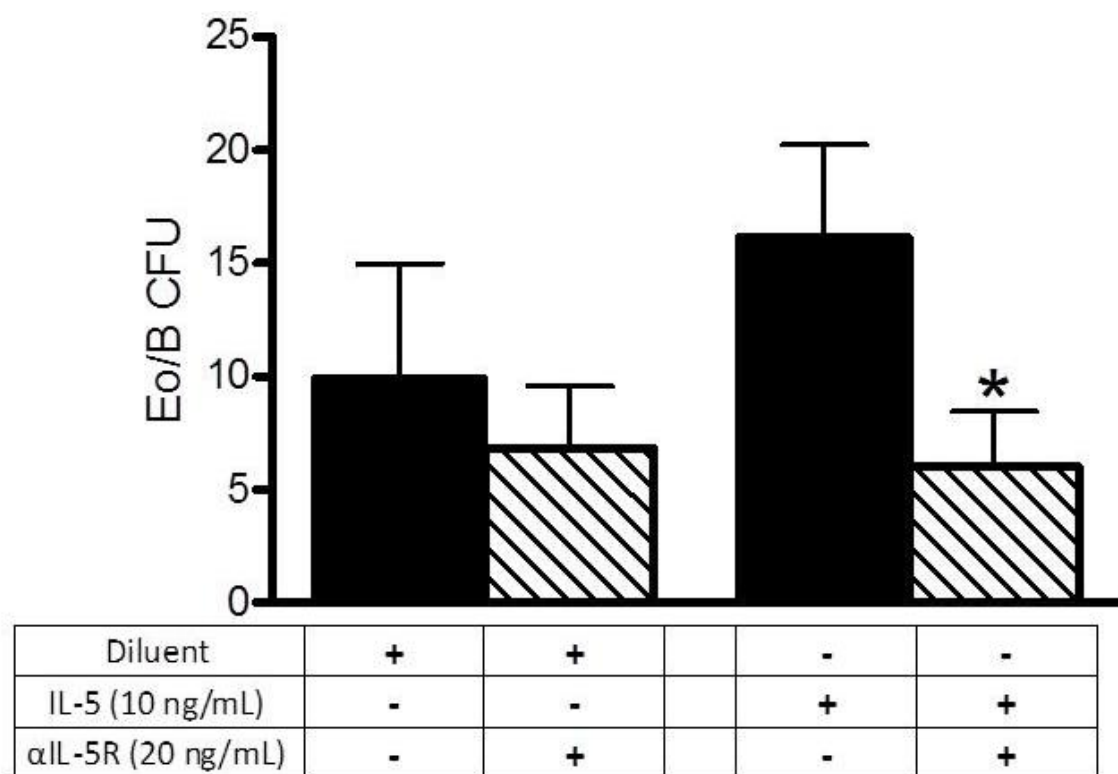


Figure 2

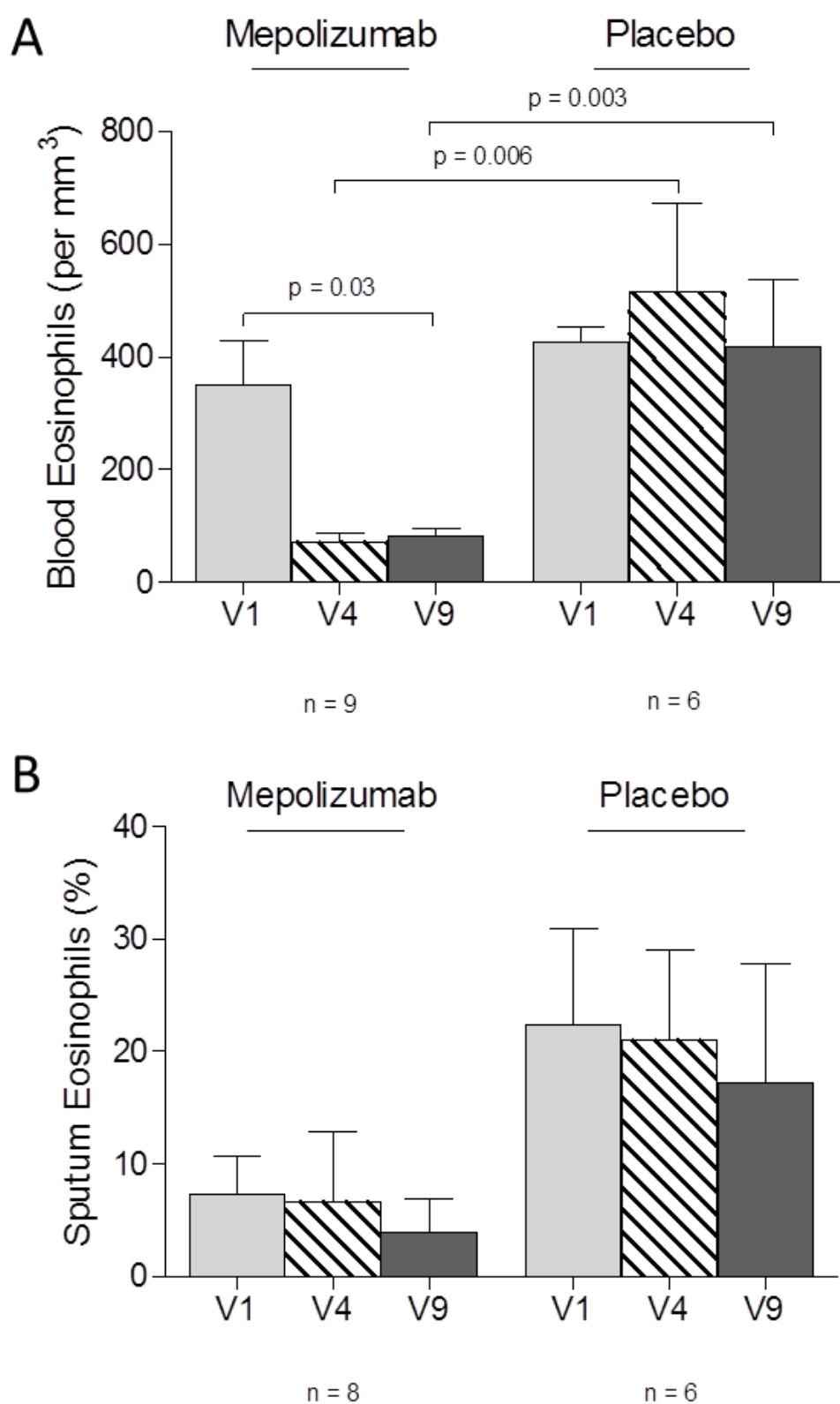


Fig 3

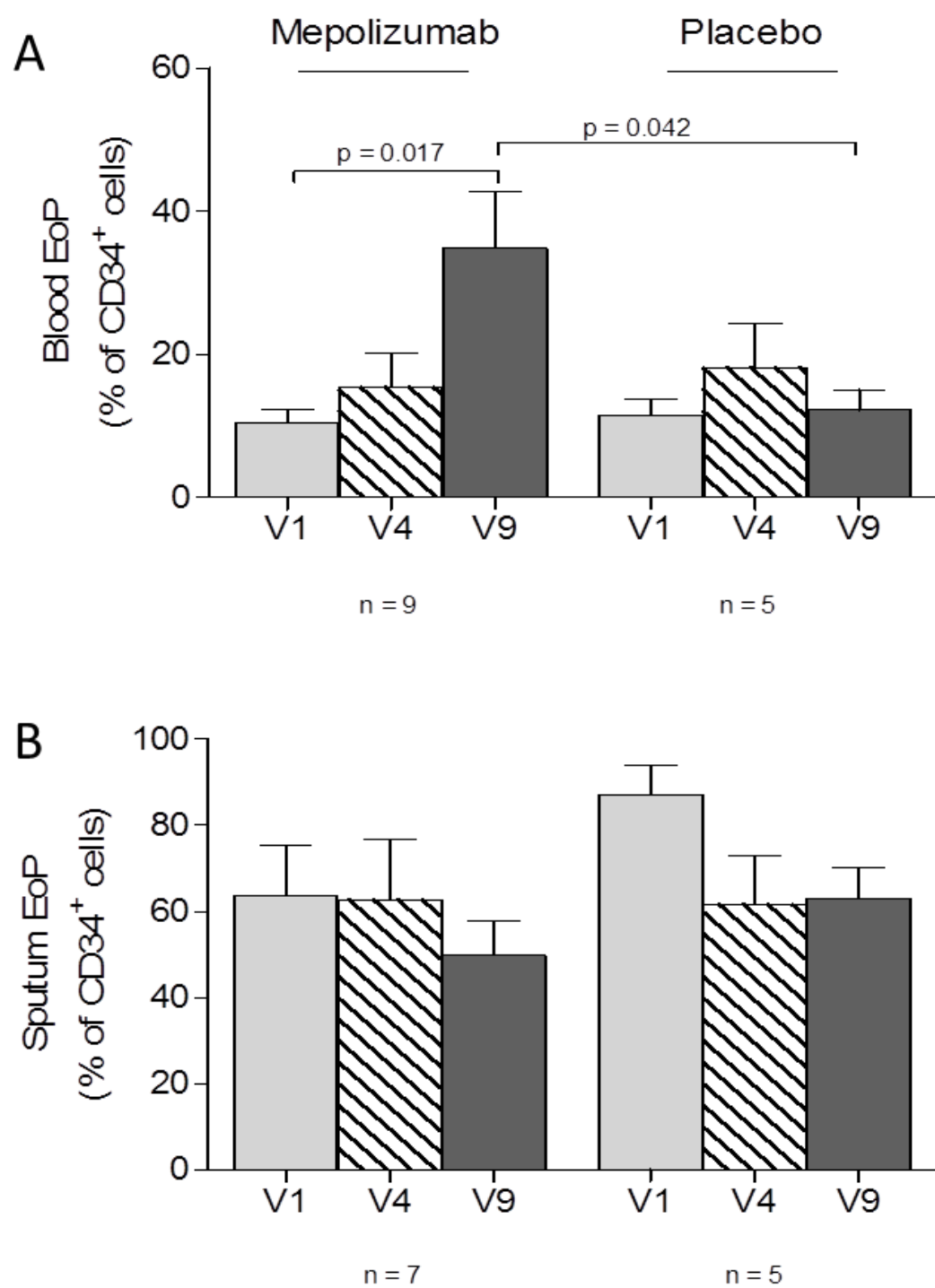


Fig. 4

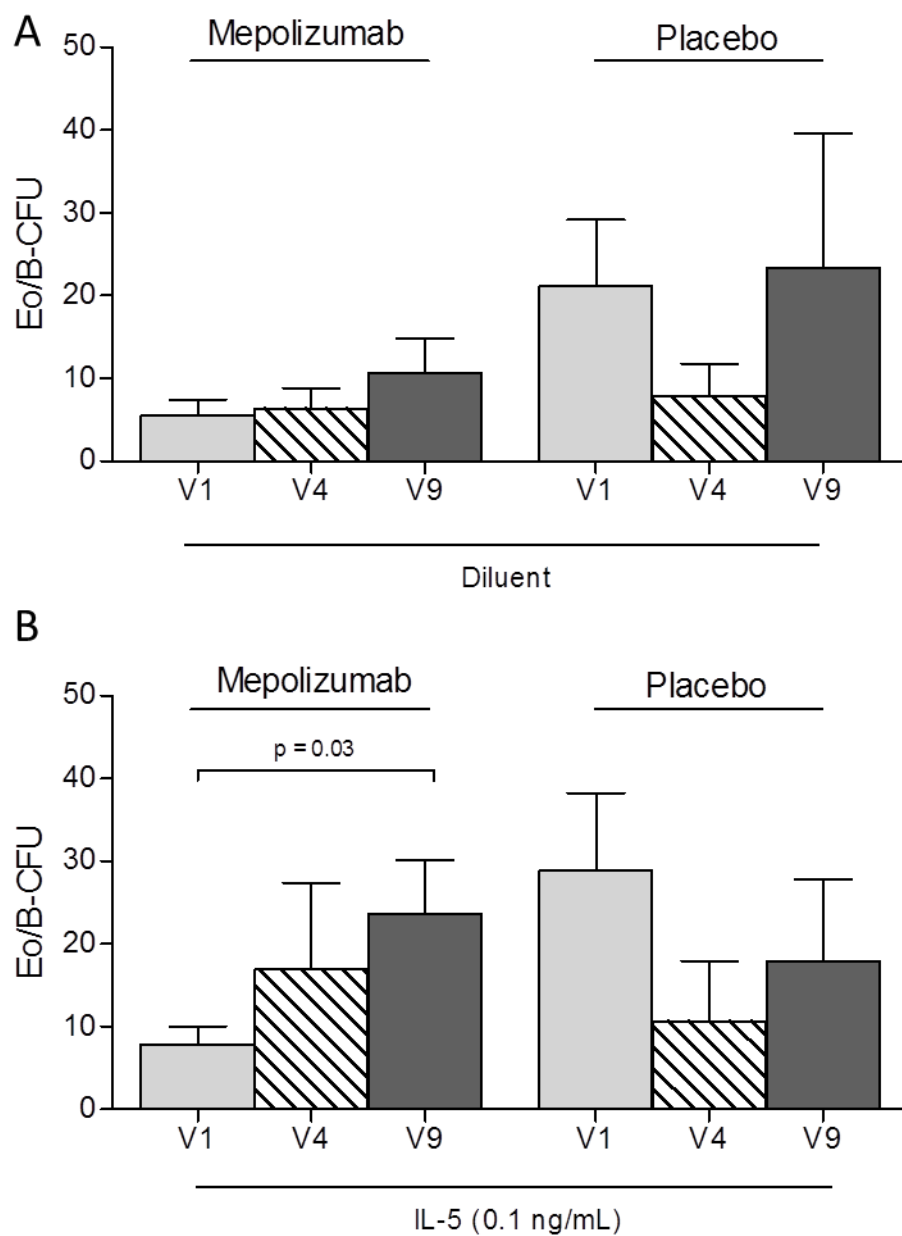


Fig 5