

ORIGINAL ARTICLE

TPI ASM8 reduces eosinophil progenitors in sputum after allergen challenge

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Summary

Background TPI ASM8 contains two modified phosphorothioate antisense oligonucleotides (AON), one targeting the common beta chain (βc) of the IL-3/IL-5/GM-CSF receptors and the other targeting the chemokine receptor CCR3. Inhalation of TPI ASM8 significantly improves lung function and sputum eosinophilia after allergen inhalation challenge in asthmatics.**Objective** This study assessed whether TPI ASM8 reduces airway levels of haemopoietic progenitor cells.**Methods** This open-label study was conducted in 14 stable, allergic mild asthmatic subjects with early- and late-phase allergen-induced bronchoconstriction. Subjects underwent allergen challenges after 4-day treatment with placebo, 4 mg b.i.d. and 8 mg o.d. of TPI ASM8. Sputum was induced before, 7 and 24 h after allergen challenges for progenitor measurements. Treatments were separated by 2–3 weeks.**Results** TPI ASM8 reduced allergen-induced sputum eosinophils, and the early and late asthmatic responses ($P < 0.05$). TPI ASM8 also reduced the number of $CD34^+CCR3^+$ cells ($P = 0.004$) and $CD34^+IL-5R\alpha^+$ cells ($P = 0.016$), and the proportion of $CD34^+$ cells expressing IL-5R α ($P = 0.036$).**Conclusions and Clinical Relevance** TPI ASM8 was safe and well tolerated. The results of this study demonstrate blocking of CCR3 and βc expression by TPI ASM8 significantly inhibits the accumulation of eosinophils and eosinophil progenitors in the airways after allergen challenge. Inhibition of airway progenitor cell accumulation presents a novel therapeutic target.**Keywords** airway inflammation, allergen inhalation, allergic asthma, antisense oligonucleotides, eosinophils, progenitor mobilization*Submitted 24 March 2011; revised 15 May 2011; accepted 31 May 2011*

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Introduction

Haemopoietic myeloid progenitors contribute to the ongoing recruitment of pro-inflammatory cells, such as eosinophils and basophils, to target tissue sites in allergic diseases. It is evident that the development of allergic inflammation is associated with the ability of the bone marrow and affected tissues to support the mobilization, proliferation and *in situ* differentiation of haemopoietic progenitors.

In humans, bone marrow-derived $CD34^+CD45^+$ haemopoietic progenitor cells (HPC) are higher in the circulation of subjects with allergic asthma compared with controls [1] and have been detected in different airway compartments of allergic individuals including mucosa of the upper and lower airways and airway secretions [2–7]. The number of HPC in sputum samples from asthmatic donors is further enhanced following allergic stimulation of the airways [4]. In mouse models of allergic asthma,

lung-extracted HPC differentiated into eosinophils thus demonstrating another mechanism for rapidly increasing the local level of mature eosinophils in tissue during an inflammatory response [8].

The chemokine eotaxin and the cytokine IL-5 are critical for mobilization and differentiation, respectively, of eosinophils [9]. Thus, expression of receptors for these mediators is another critical component of tissue infiltration. A thorough examination of eosinophils from bone marrow samples shows higher expression of CCR3 in subjects with asthma vs. control subjects, suggesting there is an increased pool of $CCR3^+$ mature and immature eosinophils in subjects with asthma which can be rapidly mobilized following appropriate stimulation [10]. Fluctuations in CCR3 expression on human bone marrow $CD34^+$ cells may also facilitate chemokine-mediated progenitor cell mobilization and differentiation to the peripheral circulation, as evidenced by increased levels of CCR3 and IL-5R α -subunit expression on

Table 1. Subject demographics collected during the baseline screening period

Subject	Age	Gender	Predicted FEV ₁ (%)	Methacholine PC ₂₀ (mg/mL)	EAR (% change)	LAR (% change)	Allergen extract	Cumulative dose (BAU)	Sputum eosinophils (%)
1	42	F	91.2	0.50	-26.0	-16.8	Cat	30.4	6.4
2	48	M	98.0	5.79	-28.6	-21.4	HDMDP	228.5	1.3
3	19	F	109.4	3.13	-36.8	-25.3	Cat	304.7	3.6
4	27	M	104.0	4.78	-38.9	-28.9	HDMDP	914.0	1.0
5	29	M	87.3	1.36	-31.1	-23.0	Alternaria	487.5	3.0
6	28	F	95.2	2.83	-39.7	-15.9	HDMDP	106.6	0.9
7	40	M	97.2	2.38	-42.2	-15.0	Grass	243.8	0.6
8	33	M	87.4	3.56	-29.5	-19.7	HDMDP	213.3	2.3
9	19	F	84.4	5.74	-46.6	-25.9	HDMDP	106.6	4.7
10	20	F	99.3	4.72	-20.0	-20.0	Cat	1137.5	0.0
11	19	M	101.0	19.4	-26.2	-19.0	Grass	1137.5	0.2
12	58	M	97.2	19.6	-34.1	-20.5	Cat	142.2	0.8

FEV₁, forced expiratory volume in 1 s; PC₂₀, provocative concentration causing a 20% fall in FEV₁; EAR, early asthmatic response; LAR, late asthmatic response; BAU, bioequivalent allergy unit.

bone marrow CD34⁺ cells from donors following the development of a late asthmatic response to inhaled allergen [11–13]. Local IL-5-dependent differentiation of progenitor cells has been observed in nasal mucosa; *ex vivo* culture with IL-5 or allergen showed a reduction in CD34⁺/IL-5R α mRNA⁺ cell numbers and a concurrent increase the number of MBP immunoreactive cells, demonstrating that a subset of eosinophils (and possibly MBP-positive basophils) can differentiate within the explanted tissue [3]. Similar observations have been reported in bronchial biopsies taken from asthmatics following IL-5 inhalation [14].

While it is evident that in asthma the lung tissue can provide a microenvironment suitable for extramedullary eosinophilopoiesis and basophilopoiesis, the effect of targeting critical cytokine and chemokine pathways to control tissue infiltration of progenitors in asthmatic subjects has not been investigated to date. TPI ASM8 is a drug product containing two modified phosphorothioate antisense oligonucleotides (AON): TOP004 directed against human β c of IL-3, IL-5 and GM-CSF receptors, and TOP005 directed against human CCR3 [15]. These AONs down-regulate the transcription of CCR3 and β c, and previously have been shown to inhibit allergen-induced eosinophilia and airway dysfunction in sensitized rats [16–18] and human asthmatics [19]. Here, we hypothesized TPI ASM8 could inhibit the migration of eosinophil progenitors into the airways. This was assessed by comparing the level of HPC in the sputum of asthmatic subjects after allergen challenge with placebo treatment and TPI ASM8 at 4 mg b.i.d. and 8 mg o.d.

Methods

Subjects

Fourteen subjects entered the baseline period. Subjects were non-smoking men and women (eight males/six females), aged 19–58 years old, with mild atopic stable asthma. Subjects has

a forced expiratory volume in 1 s (FEV₁) was $\geq 70\%$ of predicted and a measureable methacholine PC₂₀ (the provocative concentration of methacholine causing a 20% fall in FEV₁). Subjects had no other lung disease, no lower respiratory tract infection or worsening of asthma within 6 weeks, and avoided exposure to sensitizing allergens apart from house dust mite. Subjects were steroid naïve, infrequently used inhaled β_2 -agonist for treatment of asthma, and refrained from β_2 -agonist and caffeinated beverages before laboratory visits. Of those that met the inclusion/exclusion criteria, 12 subjects completed all of the study treatment periods. Subject demographics are shown in Table 1.

Study design

This study was designed as a single center, placebo-controlled, open-label study comparing two doses of inhaled TPI ASM8 (Topigen Pharmaceuticals Inc., Montreal, QC, Canada) to placebo on efficacy outcomes and safety for the treatment of allergen-induced asthma. The study was approved by the McMaster Faculty of Health Sciences/Hamilton Health Sciences Research Ethics Board and signed informed consent was obtained from subjects. The study was divided into two periods. During the baseline period subjects underwent allergen challenges to document early- and late-phase bronchoconstriction. During this baseline period, subjects practised dosing by inhaling saline nebulized using the I-nebTM (Philips Respironics, Pittsburgh, PA, USA) for 4 days. Baseline allergen challenge was conducted on the morning of the fourth day of saline treatment, and thus served as a placebo control for the study (Fig. 1).

Subjects demonstrating early-phase ($> 20\%$ fall in FEV₁ 0–2 h post-allergen) and late-phase ($> 15\%$ fall in FEV₁ 3–7 h post-allergen) responses, and with sputum eosinophil levels $> 6\%$ at 7 h or 24 h post-allergen, met the criteria to receive active treatment with TPI ASM8. These subjects entered a 2-week washout period after

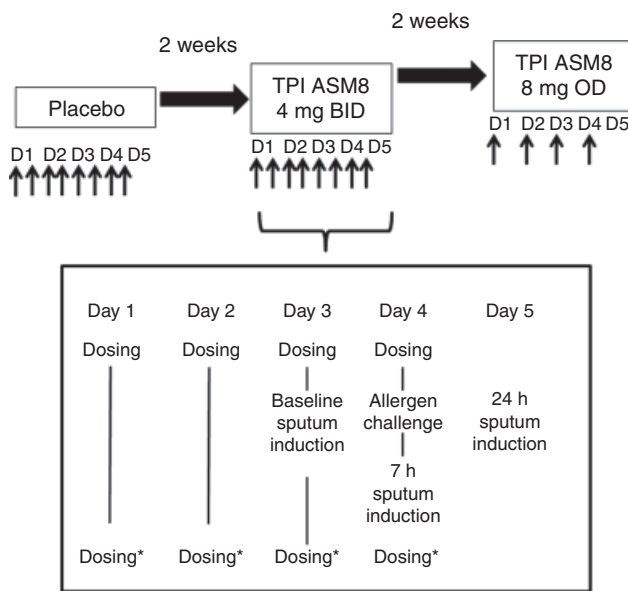


Fig. 1. Study design. Each open label treatment period consisted of 4 days dosing and allergen challenge on dosing Day 4.

which asthma stability criteria (FEV_1 within 10% of baseline and methacholine PC_{20} within one doubling dose of baseline) were required in order to proceed with the first dose of medication. Subjects inhaled 4 mg TPI ASM8 b.i.d. for four consecutive days and allergen challenge was conducted on Day 4. After a 2-week washout subjects inhaled 8 mg TPI ASM8 o.d. for four consecutive mornings and allergen challenge was conducted on Day 4. Morning doses on Days 1, 3 and 4 were administered in the laboratory. On Day 3, subjects underwent sputum induction 45 min after the morning dosing. On Day 4, subjects underwent allergen challenge 45 min after the morning dosing, and spirometry was measured at regular intervals until 7 h post-challenge when sputum was induced. Subjects returned the following morning on Day 5 for sputum induction (Fig. 1). The current study evaluated sputum eosinophil progenitors levels after allergen challenge at each dose of TPI ASM8 compared with placebo. Sputum differential cell counts and early- and late-phase bronchoconstriction were also compared with placebo.

Laboratory procedures

Study medication. TPI ASM8 contains two phosphorothioate AONs, TOP 004 and TOP 005 at a 1 : 1 ratio by weight. The nucleotide sequence of each of the two AONs in TPI ASM8 is as follows TOP 004: 5'-GGGTCTGCXGCGGGXTGGT and TOP 005: 5'-GTTXCTXCTTCCXCCTGCCTG) where X is 2-amino-2'-deoxyadenosine. TPI ASM8 solution was prepared in 1.2 mL vials of 4 mg/mL. The solution was administered using the I-nebTM nebulizer breath-actuated device with a 0.5 mL medication chamber. Inhalation of 4 mg was achieved by

filling the medication chamber twice with 0.5 mL, and 8 mg required the nebulizer chamber to be filled four times. Inhalation of 0.5 mL took approximately 5 min. Placebo was 0.5 mL normal saline.

Methacholine inhalation. Methacholine inhalation challenge was performed as described by Cockcroft [20], using tidal breathing, from a Wright nebulizer. The test was terminated when a fall in FEV_1 of at least 20% of the baseline value occurred, and the methacholine PC_{20} was calculated.

Allergen inhalation challenge. Allergen challenge was performed as described by O'Byrne and colleagues [21]. The concentration of allergen extract for inhalation was determined from a formula described by Cockcroft and colleagues [22]. The baseline allergen challenge administered doubling concentrations of allergen until a $\geq 20\%$ fall in FEV_1 at 10 min post-inhalation was reached. The FEV_1 was then measured at regular intervals until 7 h after allergen inhalation. The maximum percent fall in FEV_1 was recorded for the early (0–2 h post-allergen) and late (3–7 h post-allergen) phase responses. The same dose of allergen was used for all three allergen challenges.

Sputum induction. Sputum was induced and processed using the method described by Pizzichini and colleagues [23]. Cytospins were prepared on glass slides and stained with Diff Quik (American Scientific Products, McGaw Park, IL, USA) for differential counts, which were enumerated by an observer blinded to the treatment period. The remaining sputum cell suspension was used for measurements of HPC.

Flow cytometry. Sputum cells were stained with antibodies to surface markers [CD45-fluorescein isothiocyanate, CD34-allophycocyanin and CDw125 (IL-5R α)-phycoerythrin (PE): BD Biosciences, San Jose, CA, USA; CCR3-PE: MBL, Woburn, MA, USA]. The level of surface staining was enumerated using a FACS Calibur machine (BD Biosciences) by an operator blinded to the treatment period of the sample, and expressed as absolute cell counts per millilitre of sputum as described previously [4]. CD34⁺CD45⁺ are referred in the text as HPCs and receptor expression within this population are denoted as CD34⁺IL-5R α ⁺ and CD34⁺CCR3⁺ cells.

Statistical analysis

Results are expressed as mean $\pm$ standard error of the mean (SEM), with the exception of sputum HPC/mL, which are expressed as geometric mean $\pm$ GSEM. Sputum HPC/mL values were $\log_{10}$ transformed to fit a normal distribution before analysis. The early-phase (EAR) and late-phase asthmatic responses (LAR) and the number of sputum eosinophils were compared with the corresponding time-

point during placebo treatment using a Wilcoxon signed-rank test (two-sided). Sputum HPC were analysed using a two-way analysis of variance (Statistica v7.0), with main factors treatment, time, and treatment $\times$ time. *Post hoc* tests for pre-planned comparisons were carried out.

Results

Airway responses

The maximum percent fall in FEV₁ during the EAR with placebo treatment was $32.5 \pm 1.8\%$ and during the LAR was $22.8 \pm 1.8\%$. Treatment with 4 mg b.i.d. and 8 mg o.d., significantly attenuated the LAR to $12.8 \pm 1.8\%$ ($P = 0.0008$) and $10.6 \pm 2.0\%$ ($P = 0.00007$), respectively, compared with placebo. Although there was no significant inhibition of the EAR with 4 mg b.i.d., treatment with 8 mg o.d. of TPI ASM8 significantly attenuated the EAR to $21.5 \pm 3.6\%$ compared with placebo ($P = 0.006$).

Sputum eosinophils

Sputum eosinophils increased significantly from $0.15 \pm 0.07 \times 10^6/\text{mL}$ pre-allergen to $1.10 \pm 0.17 \times 10^6/\text{mL}$ and $1.24 \pm 0.28 \times 10^6/\text{mL}$ at 7 and 24 h post-allergen, respectively ($P < 0.05$) with placebo. Treatment with 8 mg o.d. of TPI ASM8 significantly inhibited allergen-induced sputum eosinophils to $0.46 \pm 0.13 \times 10^6/\text{mL}$ at 7 h

and $0.35 \pm 0.04 \times 10^6/\text{mL}$ 24 h after allergen inhalation challenge ($P = 0.005$ and $P = 0.04$, respectively). The numeric decrease in sputum eosinophils to $0.87 \pm 0.20 \times 10^6/\text{mL}$ at 7 h and $0.53 \pm 0.14 \times 10^6/\text{mL}$ at 24 h after allergen challenge during 4 mg b.i.d. treatment did not reach statistical significance ($P > 0.05$).

Haemopoietic progenitor cells in sputum

HPC were enumerated in sputum samples using logical sequential multi-gating flow cytometric analyses (Fig. 2). Treatment with 4 mg TPI ASM8 b.i.d. had no effect on the numbers of total CD34⁺HPC within the sputum (Fig. 3). Although treatment with 8 mg TPI ASM8 o.d. showed a trend for reduction of the absolute number of sputum CD34⁺ cells at each time-point, this was not statistically significant.

Analyses of subpopulations of the HPC showed that there was a significant reduction in the absolute number of eosinophil progenitors (CD34⁺IL-5R α ⁺ cells) with 8 mg o.d. compared with placebo ($P = 0.008$, Fig. 4a). Pre-planned comparisons of CD34⁺IL-5R α ⁺ cells at baseline and 24 h post-allergen were significantly lower with 8 mg o.d. treatment compared with placebo; placebo baseline 302 cells/mL (GSEM 102–155) vs. 8 mg o.d. baseline 159 cells/mL (GSEM 38–50) ($P = 0.035$), placebo 24 h post-allergen 437 cells/mL (GSEM 148–224) vs. 8 mg o.d. 24 h post-allergen 138 cells/mL (GSEM 33–44) ($P = 0.0499$). There was no effect of 4 mg b.i.d. on the absolute number of CD34⁺IL-5R α ⁺ cells ($P = 0.91$) (Fig. 4a).

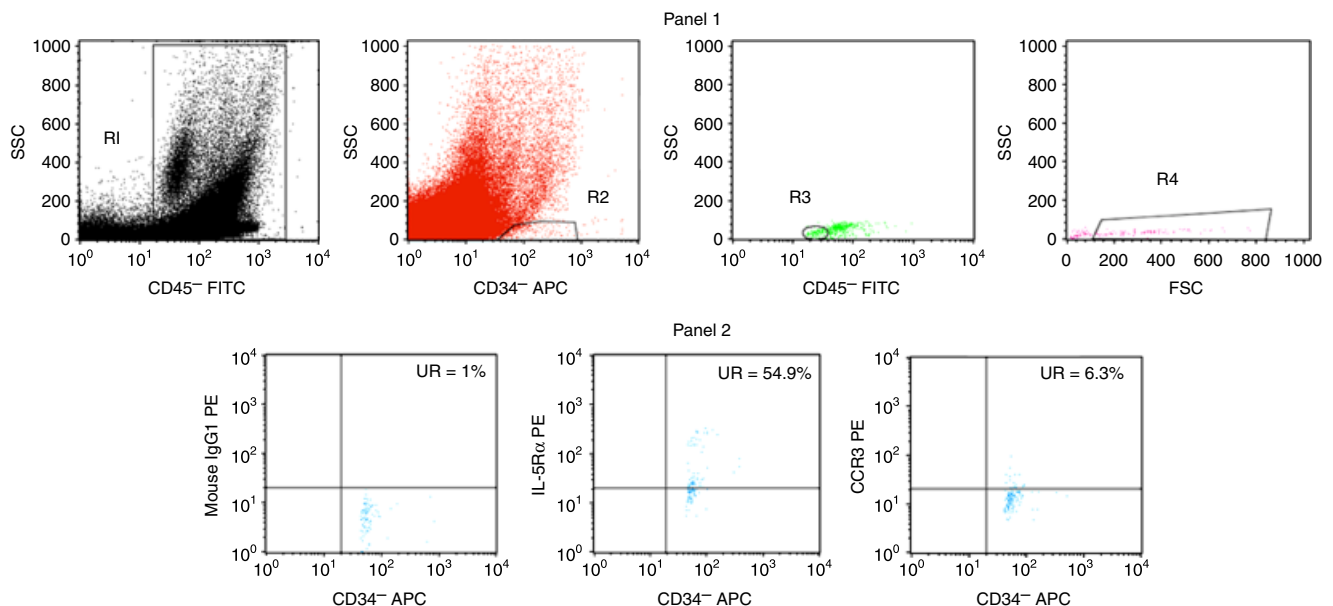


Fig. 2. The sequential multi-gating flow cytometric analyses of sputum extracted cells. Panel 1: cells were first gated on CD45-positive events (region R1) and then on CD34^{high} events (region R2). These were then back-gated on CD45^{low} (region R3) and true progenitors were assessed as events that fell in the lymphomononuclear region: SSC^{low} and FSC^{low} (region R4). The gate statistics show that for a total of 792940 CD45⁺ cells we are able to detect 93 CD34⁺ cells. Panel 2: Isotype control vs. the specific staining for IL-5R α or CCR3 in the CD34⁺CD45⁺ population gated in R4. The quadrant statistics show that <1% of total CD34⁺ cells are stained with the isotype control. Using a 99% confidence limit for the CD34⁺CD45⁺ cells referred to as haemopoietic cells (HPC), we found 54.9% positive staining with IL-5R α antibody and 6.3% positive staining for CCR3 antibody. APC, allophycocyanin; FITC, fluorescein isothiocyanate.

There was a significant effect of 4 mg b.i.d. on the proportion of sputum CD34⁺ cells expressing IL-5R α ($P=0.015$) and a trend for an effect of 8 mg o.d. ($P=0.065$, Fig. 4b). *Post hoc* analyses of pre-planned comparisons between 4 mg b.i.d.

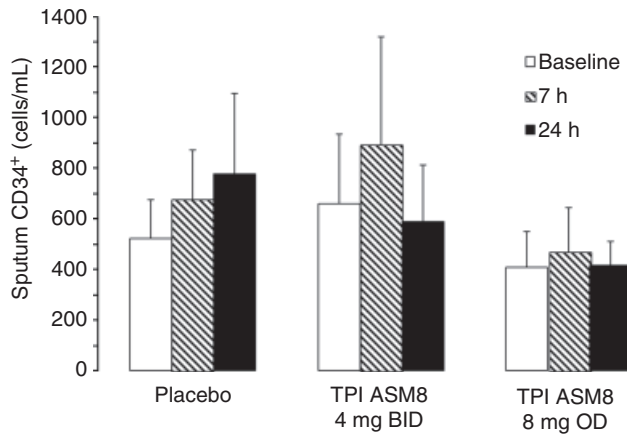


Fig. 3. The absolute number of CD34⁺ cells were enumerated in sputum samples at baseline, 7 and 24 h post-allergen inhalation challenge following treatment with placebo, 4 mg b.i.d. and 8 mg o.d. of TPI ASM8. Data are presented as geometric mean $\pm$ GSEM. Treatment with TPI ASM8 had no effect on total haemopoietic cell (HPC) numbers in the sputum.

and placebo showed TPI ASM8 reduced the percentage of sputum CD34⁺ cells expressing IL-5R α at 7 h ($P=0.014$) and a trend for reduction at baseline ($P=0.07$, Fig. 4b).

Up-regulation of CCR3 on CD34⁺ is associated with increased migrational responsiveness of progenitor cells [11]. In this study, there was a significant reduction in the absolute number of sputum-derived CD34⁺CCR3⁺ cells with 8 mg o.d. compared with placebo ($P=0.002$, Fig. 4c). Pre-planned comparisons of CD34⁺CCR3⁺ cells at baseline and 24 h post-allergen were significantly lower with 8 mg o.d. treatment compared with placebo; placebo baseline 251 cells/mL (GSEM 77–112) vs. 8 mg o.d. baseline 71 cells/mL (GSEM 36–74) ($P=0.007$), placebo 24 h post-allergen 132 cells/mL (GSEM 67–137) vs. 8 mg o.d. 24 h post-allergen 25 cells/mL (GSEM 13–29) ($P=0.013$). There was no effect of 4 mg b.i.d. on the absolute number of CD34⁺CCR3⁺ cells ($P=0.84$). There was no effect of 4 mg b.i.d. or 8 mg o.d. TPI ASM8 on the percentage of sputum CD34 cells expressing CCR3 ($P=0.13$ and $P=0.24$, respectively, Fig. 4d).

Safety

There were no serious adverse events (AEs) in this study. Seventeen AEs were reported; three AEs occurred during the placebo/screening period, one during the 4 mg b.i.d.

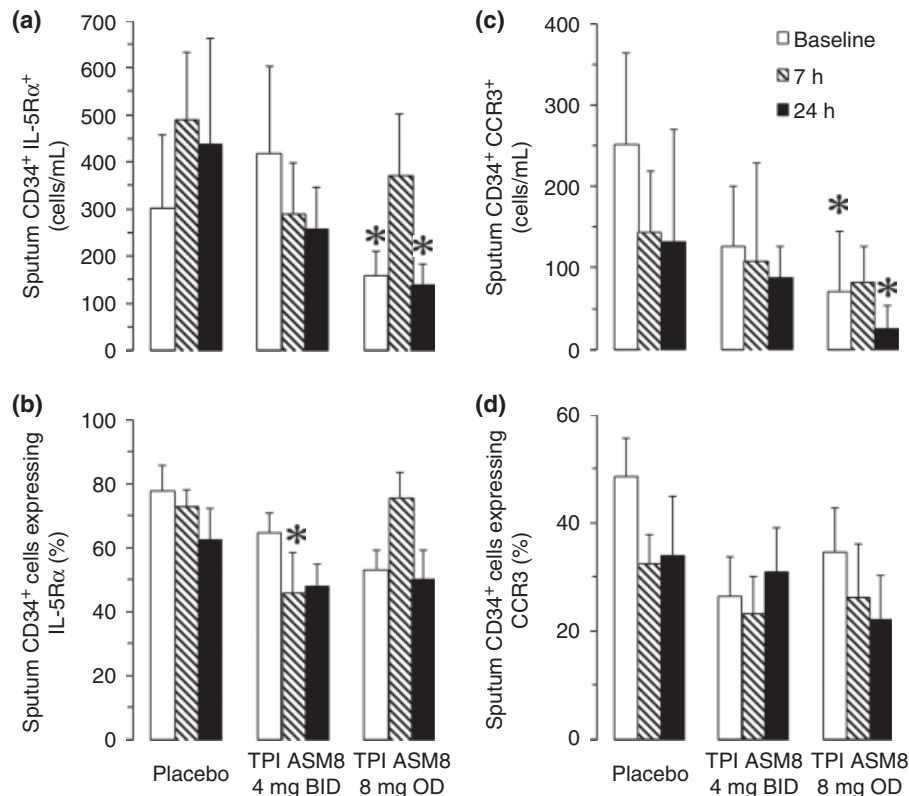


Fig. 4. (a) The absolute number of CD34⁺IL-5R α ⁺ cells; (b) the percentage of CD34⁺ cells expressing IL-5R α ; (c) the absolute number of CD34⁺CCR3⁺ cells; and (d) the percentage of CD34⁺ cells expressing CCR3 in sputum samples at baseline, 7 and 24 h post-allergen inhalation challenge following treatment with placebo, 4 mg b.i.d. and 8 mg o.d. of TPI ASM8. Percentages are presented as mean $\pm$ SEM and cells/ml are presented as geometric mean $\pm$ GSEM. * $P<0.05$ for pre-planned comparisons between placebo and TPI ASM8 treatment.

period, and four during the 8 mg o.d. period. The most frequently reported AE was headache, and only one AE, shortness of breath during the 8 mg o.d. dose was deemed to be possibly drug.

Discussion

In this study, we report the effect of RNA-targeted topical therapy on mature eosinophils and HPC in sputum samples from dual responder asthmatics. TPI ASM8 contains two AONs (TOP004 and TOP005), which down-regulate mRNA transcripts for βc and CCR3 [15–17] leading to a reduction of these receptors on the cell surface. A previous study has reported that treatment with TPI ASM8 attenuated allergen induced airway responses and airway eosinophilia [19]. In the current study, we report that treatment with TPI ASM8 had a significant effect on lowering not only mature eosinophils but also eosinophil/basophil progenitor cell populations in sputum. Analyses of data from 12 asthmatic subjects showed that the drug had no effect on the total number of HPC within the sputum, but there was a significant reduction in CD34⁺IL-5R α ⁺ cells numbers ($P=0.016$) and CD34⁺CCR3⁺ cells numbers ($P=0.004$). In addition, when the data were expressed as a percentage of the total HPC population, we detected a reduction in the proportion of CD34⁺ cells expressing IL-5R α ($P=0.036$) following pre-treatment with by TPI ASM8. There were trends for reduction of the proportion of CD34⁺ cells expressing CCR3 ($P=0.06$). The current data show that TPI ASM8 has effects that are specific for progenitors of eosinophil/basophil lineage. The effects observed with TPI ASM8 administered at a dose of 8 mg o.d. but not 4 mg b.i.d. may be due to long-term carryover effects of drug into the next treatment period, even though the washout period between periods was sufficient for complete drug washout from the airways.

Dorman *et al.* [4] have previously reported increased HPC and eosinophil progenitors (CD34⁺IL-5R α ⁺) in sputum after allergen inhalation challenge in dual responder asthmatics. Our current findings demonstrate a trend for an increase in the absolute number of CD34⁺ cells and CD34⁺IL-5R α ⁺ cells following allergen challenge in the placebo arm of the study, but these changes were not significant. This may reflect the increased variability within this population of asthmatics and a larger sample size may have shown a significant allergen-induced effect, although this was not a primary outcome of this study.

Expression of CCR3 and the common βc of IL-5, IL-3 and GM-CSF are critical for eosinophil and basophil

progenitor cells to migrate and proliferate in tissue. Studies have previously shown that up-regulation of CCR3 on CD34⁺ cells was detected on bone marrow progenitor cells following allergen challenge and that this was associated with migrational increased responsiveness to eotaxin, *in vitro* [11, 24]. As expected, treatment with TPI ASM8 reduced the level of CCR3 expression on CD34⁺ cells in the sputum as this was one of the drug targets. However, although the drug also targets βc mRNA, we found a reduction in the number of CD34⁺ cells expressing the α -chain of the IL-5 receptor. The IL-5 receptor is a heterodimeric receptor composed of a specific binding α -chain and the β -chain that is the signaling component shared with IL-3 and GM-CSF [25]. Studies have shown that IL-5 itself can regulate the expression of its own α -chain expression receptor on CD34⁺ cells [26]. Thus down-regulation of the signalling component of the IL-5R (i.e. βc chain) is likely to directly impact the reduction in IL-5R α -chain expression on CD34⁺ cells as has been shown in this study. We believe the decreased number of eosinophil progenitor cells in the airways is due to the effects of this drug on the cells that have already been recruited to the airways, because there is no detectable level of drug present in the circulation of these subjects post-dosing (data not shown).

Studies with anti-IL-5 have shown no effects on baseline levels of CD34⁺IL-5R α ⁺ cells in bone marrow and blood and only a partial reduction in CD34⁺IL-5R α mRNA⁺ cells in lung biopsies following intravenous infusions of mepolizumab [27]. No studies in humans have looked at the effect of modifying either IL-5- or CCR3-mediated pathways on the lung homing of HPC following allergen challenge. In the current study, we show for the first time that TPI ASM8 can selectively modulate the lung levels of specific HPC phenotype and that this may contribute to the observed marked reduction in lung eosinophilia.

In summary, we conclude that in addition to directly targeting mature granulocytes, TPI ASM8 may regulate allergen-induced lung eosinophilia and basophilia through a reduction in expression of CCR3 and βc -chain on HPC within the airways.

Acknowledgements

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