

Antisense Therapy against CCR3 and the Common Beta Chain Attenuates Allergen-induced Eosinophilic Responses

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Rationale: The drug product TPI ASM8 contains two modified phosphorothioate antisense oligonucleotides designed to inhibit allergic inflammation by down-regulating human CCR3 and the common beta chain (β_c) of IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor receptors.

Objectives: This study examined the effects of inhaled TPI ASM8 on sputum cellular influx, CCR3 and β_c mRNA and protein levels, and the airway physiologic response after inhaled allergen.

Methods: Seventeen subjects with mild atopic asthma were randomized in a crossover study to inhale 1,500 μ g TPI ASM8 or placebo by nebulizer, once daily for 4 days. On Day 3, subjects underwent allergen inhalation challenge. Sputum samples were collected before and after allergen. CCR3 and β_c protein levels were measured by flow cytometry, mRNA was measured using real-time quantitative polymerase chain reaction, and the FEV₁ was measured over 7 hours after challenge.

Measurements and Main Results: Compared with placebo, TPI ASM8 inhibited sputum eosinophil influx by 46% ($P = 0.02$) and blunted the increase in total cells (63%) after allergen challenge. TPI ASM8 significantly reduced the early asthmatic response ($P = 0.04$) with a trend for the late asthmatic response ($P = 0.08$). The allergen-induced (Day 2 to Day 3) levels of β_c mRNA and CCR3 mRNA in sputum-derived cells were inhibited by TPI ASM8 ($P = 0.039$ and $P = 0.054$, respectively), with no significant effects on the cell surface protein expression of CCR3 and β_c ($P > 0.05$). No serious adverse events were reported.

Conclusions: TPI ASM8 attenuates the allergen-induced increase in target gene mRNA and airway responses in subjects with mild asthma. Clinical trial registered with www.clinicaltrials.gov (NCT 00264966).

Keywords: antisense oligonucleotides; eosinophils; allergen inhalation; airway inflammation

Asthma is characterized by reversible airway obstruction, airway hyperresponsiveness, and airway allergic cellular inflammation, with a predominance of eosinophils being a key feature of allergic asthma. Subjects with allergic asthma develop an immediate IgE-mediated early asthmatic response (EAR) after inhalation of a sufficient dose of an allergen to which they are sensitized. Up to 50% of these subjects also develop a late asthmatic response (LAR), which usually begins 3 to 4 hours after allergen inhalation challenge (1). The LAR is associated with

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Antisense oligonucleotide therapy targeting the receptors for eotaxin (CCR3) and IL-5, IL-3, and granulocyte-macrophage colony-stimulating factor (common β chain) has been successful in animal models of asthma. This has not been studied in humans.

What This Study Adds to the Field

This study demonstrates that antisense therapy inhibits allergen-induced asthmatic responses in humans.

elevated levels of eosinophils, as well as with other effector cells, including basophils and mast cells (2, 3). Migration and proliferation of these effector cells in the airways, including eosinophils, basophils, and mast cells, may depend on signaling of several chemokines acting through the chemokine receptor CCR3 (4, 5), and cytokines IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF), which stimulate their respective receptors composed of a common β chain (β_c) and an individual α chain (6).

Antisense oligonucleotides can be used as a therapeutic strategy to down-regulate the transcription of specific proteins. Phosphorothioate oligodeoxynucleotides (ODNs) are designed to impart resistance to nucleases, thereby increasing stability, and they can inhibit gene expression through formation of low-stability duplexes with complementary RNA. ODNs are largely defined by their polyanionic character, which has been known to interact with cationic sites on blood proteins. TPI ASM8 (Topigen Pharmaceuticals, Montreal, PQ, Canada) is a drug product containing two modified phosphorothioate antisense oligonucleotides: TOP004 directed against human β_c of IL-3, IL-5, and GM-CSF receptors, and TOP005 directed against human CCR3. ODNs directed against CCR3 and β_c have been shown to inhibit mRNA and protein expression (7) and to attenuate antigen-induced eosinophil efflux to the airways of rats (8–10). We hypothesized that TPI ASM8 would inhibit eosinophil migration to the airways by down-regulating expression of CCR3 and β_c , and that this would in turn affect the physiologic response after challenge. This study, therefore, examined the safety of four daily doses of nebulized TPI ASM8 in patients with mild asthma and the effects on allergen-induced airway inflammation, target gene expression, and physiologic changes. The primary outcome variable was the allergen-induced LAR, and secondary variables included safety and tolerability, gene expression and protein levels of CCR3 and β_c , allergen-induced EAR, airway inflammation, and hyper-

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responsiveness. Some of the results of these studies have been previously reported in the form of an abstract (11, 12).

METHODS

Subjects

Subjects were nonsmoking men and women (7 male/10 female), aged 19 to 57 years old (Table 1), with mild atopic stable asthma. FEV₁ was 70% or more of predicted and baseline methacholine PC₂₀ (the provocative concentration of methacholine causing a 20% fall in FEV₁) was 16 mg/ml or less. Subjects had no other lung disease, no lower respiratory tract infection or worsening of asthma for 6 weeks before screening, and avoided exposure to sensitizing allergens apart from house dust mite. Subjects were steroid naive, infrequently used inhaled β_2 -agonist for treatment of asthma, and refrained from β_2 -agonist and caffeinated beverages before laboratory visits.

Study Design

This trial was a three-center, double-blind, randomized, placebo-controlled, crossover study, comparing 4 days of treatment with a nominal dose of 1,500 μ g inhaled TPI ASM8 with placebo (Figure 1). The primary outcome variable was the LAR, and the secondary outcome variables were the EAR, airway hyperresponsiveness, sputum eosinophils, and mRNA and protein levels of CCR3 and β_c . The study was performed from December 2005 to June 2006. Each site was given a block of kits containing drug and placebo. The sequence of treatment was computer generated, and eligible subjects were assigned the next available kit at the site by study staff. All subjects and trial staff remained blind to the random order until after locking the database.

The study was approved by the ethics research board of the respective institutions, and signed informed consent was obtained from subjects. Screening of subjects occurred over 3 consecutive days (Figure 1). Those who developed a fall in FEV₁ of at least 20% within 2 hours postallergen, and of at least 15% between 3–7 hours postallergen, were randomized to two treatment periods separated by 4 weeks. Each treatment period consisted of five consecutive morning visits with dosing on Days 1–4, followed by a sixth visit 4 weeks later. Day 1 methacholine PC₂₀ was required to be within one doubling dose, and FEV₁ within 10% of that measured during the screening period to demonstrate asthma stability. Days 1, 2, and 4 included predose measurements of lung function, then airway hyperresponsiveness by methacholine PC₂₀, followed by sputum induction, then drug administration. On Days 1 and 4, blood and sputum samples were collected at 2.5 or 4.5 hours, and 7.5 and 24 hours postdosing for pharmacokinetic (PK) assessment. On Day 3, the third dose was administered 30 minutes before allergen, and a sputum sample was induced 7 hours after allergen. Subjects returned

to the laboratory on Day 5 for safety labs, and Day 28 for immunogenicity skin test. Activation of the alternative complement system was monitored predose and postdose on Day 4, by measurement of the plasma levels of Bb (a split product of complement factor B).

Laboratory Procedures

Study medication. TPI ASM8 is a 1:1 (wt:wt) mixture of TOP004, a 19-mer phosphorothioate oligonucleotide directed against the β_c subunit of IL-3, IL-5, and GM-CSF receptors with the sequence 5'-GGGT CTGCDGCGGGDTGGT, and TOP005 a 21-mer phosphorothioate oligonucleotide directed against the chemokine receptor CCR3 with the sequence 5'-GTTDCTDCTTCCDCCTGCCTG, where D = 2-amino-2'-deoxyadenosine. The dose selected for this study was based on the results of the phase I trials demonstrating tolerability of TPI ASM8 at doses up to 6 mg. In order for the cumulative dose in this trial not to exceed the highest single dose administered in the phase I studies, a dose level of 1.5 mg of TPI ASM8 per day (equivalent to a cumulative dose of 6 mg over the 4-d dosing regimen) was selected.

TPI ASM8 inhalation. TPI ASM8 or placebo (100 mM phosphate-buffered saline, pH 7.4; Pyramid Laboratories, Inc., Costa Mesa, CA) was administered by inhaling 1 ml (1,500 μ g TPI ASM8) through a mouthpiece via an Aeroneb clinical nebulizer (Respironics, Murrysville, PA) with an output of 0.49 ml/minute, a mass median aerodynamic diameter (MMAD) of 2.4 μ m and volume median diameter of 4.5 to 5.0 μ m. Lung deposition was estimated to be approximately 6–15% (90–220 μ g) based on the MMAD and prior literature (13). Dosing had no effect on the FEV₁.

Methacholine inhalation test. Methacholine inhalation challenge was performed as described by Cockcroft (14), using tidal breathing, from a Wright nebulizer (Roxon Meditech, Montreal, PQ, Canada). The test was terminated when a fall in FEV₁ of at least 20% of the baseline value occurred, and the methacholine PC₂₀ was calculated.

Allergen inhalation challenge. The FEV₁ was required to be within 10% of baseline to proceed with the challenge. Allergen inhalation was performed as described by O'Byrne and colleagues (15). The concentration of allergen extract for inhalation was determined from a formula described by Cockcroft and coworkers (16), and doubling concentrations of allergen were given until a greater than 20% fall in FEV₁ at 10 minutes postallergen was reached. The FEV₁ was then measured at regular intervals until 7 hours after allergen inhalation. Subjects inhaled the same dose of allergen for the two treatment periods with three exceptions: two subjects failed to inhale the final allergen dose during the TPI ASM8 treatment period, and one subject failed to inhale the final allergen dose during placebo treatment for safety reasons. Data from all 17 subjects were included in the statistical analyses of the early and late responses.

Sputum analysis. Sputum was induced and processed using the method described by Pizzichini and coworkers (17). The total cell count was determined using a Neubauer hemocytometer chamber (Hausser Scientific, Blue Bell, PA) and expressed as the number of cells per milliliter of sputum. Cells were prepared on glass slides for differential counts and stained with DiffQuik (American Scientific Products, McGaw Park, IL).

Flow cytometry. Sputum cells were stained with antibodies to surface markers (CD45-phycoerythrin [PE] and CD16-PE-Cy5; Pharmingen, Mississauga, ON, Canada), followed by recombinant human IL-3 or eotaxin (R&D Systems, Minneapolis, MN) and corresponding negative controls. The level of surface staining was enumerated using FACSScan (BD, San Jose, CA) and expressed as percentage of cell population positive for receptor, and median fluorescent intensity (MFI) of the cell population.

Quantitative reverse transcriptase–polymerase chain reaction. Total RNA was extracted from sputum cells and reverse transcription using Superscript II RNase H- reverse transcriptase (Invitrogen, Burlington, ON, Canada) was performed. Target genes (CCR3 and β_c) and control housekeeping genes (β_2 -macroglobulin, PPIB) were amplified using specific primers by quantitative real-time polymerase chain reaction using the LC FastStart DNA Master SYBR Green (Roche, Mannheim, Germany) and the LightCycler Instrument version 2.0 (Roche). Expression of CCR3 and β_c was normalized to the expression of housekeeping genes.

PK assessments. To determine the concentrations of TOP004 and TOP005 in human sputum cells and plasma samples, a hybridization/ligation ELISA modified from the method described by Yu and

TABLE 1. SUBJECT DEMOGRAPHICS

Subject No. (n = 17)	Age (yr)	Sex	Predicted FEV ₁ (%)	FEV ₁ /FVC Ratio	Methacholine PC ₂₀ (mg/ml)	Allergen
1	19	F	95.1	0.87	15.6	HDM
2	25	F	99.0	0.83	10.3	HDM
3	25	M	97.0	0.81	8.4	Cat
4	22	F	93.0	0.97	1.1	Cat
5	40	F	88.0	0.86	0.4	Cat
6	25	F	120.0	0.85	5.5	HDM
7	38	M	82.0	0.71	5.9	Grass
8	24	F	101.0	0.82	2.6	HDM
9	41	M	95.0	0.69	2.1	HDM
10	28	F	89.0	0.67	0.4	Cat
11	27	F	101.7	0.85	1.8	Birch
12	37	M	81.0	0.69	2.5	Cat
13	34	M	72.1	0.74	0.6	Cat
14	44	F	74.6	0.69	2.4	Cat
17	21	F	109.0	0.77	6.2	Cat
19	57	M	96.0	0.82	1.0	Cat
20	45	M	98.0	0.68	11.7	HDM

Definition of abbreviations: F = female; M = male; HDM = house dust mite; PC₂₀ = provocative concentration of methacholine causing a 20% fall in FEV₁.

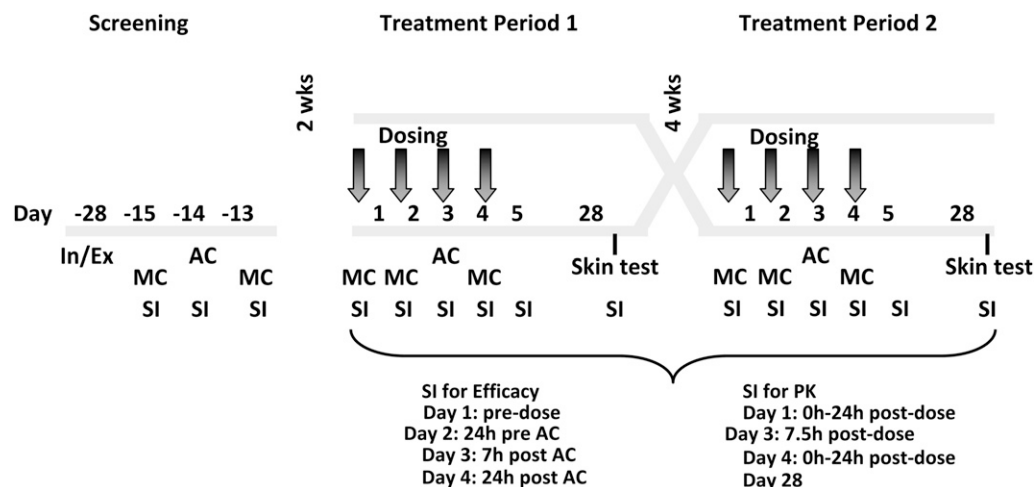


Figure 1. Randomized crossover study schematic. AC = allergen inhalation challenge; In/Ex = inclusion/exclusion criteria review; MC = methacholine inhalation challenge; PK = pharmacokinetics in sputum; SI = sputum induction.

colleagues was used (18). Briefly, complementary sequences to each ODN (Invitrogen, Burlington, ON, Canada) were hybridized to their targets in plasma or dithiothreitol-treated sputum and the hybridization complexes were captured onto neutravidin-coated plates (Pierce, Rockford, IL). Ligation probes containing 3' digoxigenin label (Invitrogen) were added and covalently bound to the hybridization complex. Anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Diagnostics, Laval, PQ, Canada) was added and the resulting fluorescence of the AttoPhos (Roche Diagnostics) or methylumbelliferyl phosphate substrate was detected using a Spectramax Gemini fluorescence plate reader (Molecular Devices Corporation, Sunnyvale, CA).

Statistical Analysis

The statistical model used crossover analysis of variance testing for sequence group, period, and treatment effects on the per-protocol population for all variables with the exception of mRNA, which applied the Wilcoxon test. All tests of hypotheses were two-sided, with statistical significance defined as $P \leq 0.05$. Data are analyzed from $n = 17$ subjects and presented as mean $\pm$ SEM unless otherwise noted.

RESULTS

Seventeen subjects completed the study protocol, and the sequence groups were not statistically different for number, sex, ethnicity, race, age, weight, and height.

Safety

Hematology, chemistry, urinalysis, and complement pathway activation were similar across the two groups. Adverse events were reported by five subjects while receiving TPI ASM8 and by four subjects while receiving placebo. The only adverse event rated as severe was a headache that occurred while receiving placebo, and only one mild adverse event was considered treatment related (pustular rash on the knees at Day 25) while receiving TPI ASM8. There were no serious adverse events reported in this study. Immunogenicity to TPI ASM8 was tested using a skin prick test and intradermal skin testing. A test was considered positive if subjects developed erythema with a wheal that increased by greater than 3 mm compared with phosphate-buffered saline. Skin tests were positive in 1 of 17 subjects receiving TPI ASM8, and 0 of 17 receiving placebo. Duplicate intradermal tests demonstrated that 2 of 15 subjects and 0 of 12 subjects tested positive with TPI ASM8 treatment, and 3 of 17 subjects and 1 of 14 subjects tested positive with placebo treatment.

TPI ASM8 PK in Sputum and Plasma

Peak sputum drug concentrations were generally attained by 2.5 or 4.5 hours postdose, and these levels dropped steadily over the

24 hours during the postdosing period (Figure 2). Terminal elimination half-life ($t_{1/2}$) ranged between 5.86 and 6.94 hours for both TOP004 and TOP005. Maximum plasma concentration (C_{max}) and area under the curve $AUC_{0-24 h}$ values were higher for TOP005 when compared with TOP004. Although the administered ratio was 1:1, TOP004:TOP005 ratios were 1:3.30 and 1:1.47 for C_{max} on Days 1 and 4, respectively, and 1:2.99 and 1:1.57 for $AUC_{0-24 h}$ on Days 1 and 4, respectively. There was therefore no evidence of accumulation because the accumulation ratio was 0.992 ± 0.314 for TOP004 ($n = 5$) and 1.41 ± 0.656 for TOP005 ($n = 5$). Only one subject had undetectable drug levels in sputum after TPI ASM8 treatment, and this subject had no attenuation of LAR with TPI ASM8 treatment. TOP004 and TOP005 were not measurable in the plasma of any subjects at any time after administration, confirming the low systemic exposure observed in phase 1 studies.

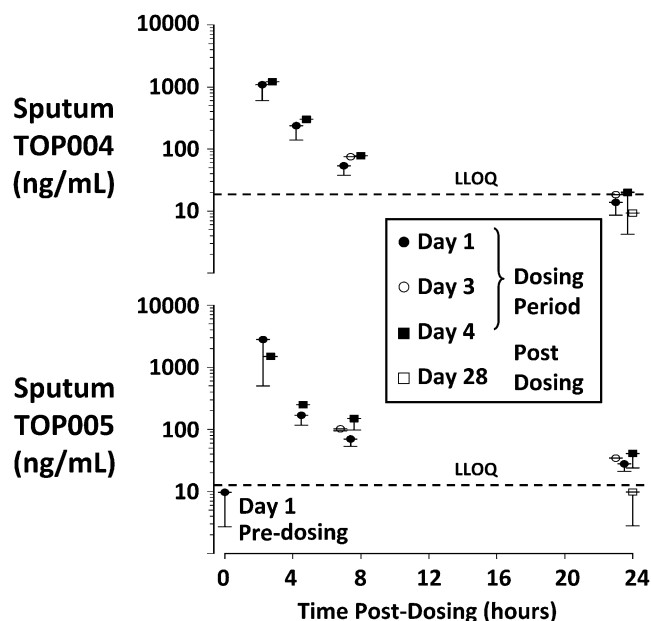


Figure 2. Levels of TOP004 and TOP005 in sputum collected on Days 1 and 4 predosing, 2.5 or 4.5 hours post-TPI ASM8 dosing and 7.5 hours post-TPI ASM8 dosing, Day 3 at 7.5 post-TPI ASM8 dosing, and Day 28 post-TPI ASM8 dosing. LLOQ = lower level of quantification.

Drug Effect on FEV₁

The FEV₁ did not change after TPI ASM8 treatment (3.43 ± 0.13 L/s on Day 1 pre-TPI ASM8 vs. 3.4 ± 0.13 L/s on Day 2 after one dose of TPI ASM8), or after placebo treatment (3.45 ± 0.14 L/s on Day 1 preplacebo vs. 3.44 ± 0.14 L/s on Day 2 after one dose of placebo) ($P > 0.05$).

Allergen-induced Airway Responses

The maximum fall in FEV₁ within 2 hours after allergen (EAR) at screening was $34.4 \pm 2.5\%$. TPI ASM8 treatment significantly attenuated the EAR, with a $29.3 \pm 4.1\%$ fall in FEV₁ compared with $35.0 \pm 3.9\%$ with placebo ($P = 0.04$; Figure 3). The early AUC (AUC_{0–2 h}) with TPI ASM8 was $29.9 \pm 5.3\%$ fall · h compared with $37.3 \pm 4.8\%$ fall · h with placebo ($P = 0.14$). The maximum percentage fall in FEV₁ 3 to 7 hours after allergen (LAR) was $25.2 \pm 2.4\%$ at screening. TPI ASM8 attenuated the LAR, but this effect was not statistically significant. The LAR was $19.6 \pm 3.6\%$ with TPI ASM8 and $23.5 \pm 3.3\%$ with placebo ($P = 0.08$; Figure 3). The late AUC (AUC_{3–7 h}) was $46.4 \pm 9.7\%$ fall · h with TPI ASM8 and $55.6 \pm 8.8\%$ fall · h with placebo treatment ($P = 0.19$). There was a significant order effect, with greater protection of TPI ASM8 on the LAR when active drug was randomized to the second sequence ($P = 0.008$ and $P = 0.011$ for LAR and AUC_{3–7 h}, respectively). The subject with no detectable TPI ASM8 on all sputum PK measurements after treatment also had no attenuation of the LAR. With this subject removed from the analysis, the maximum fall in FEV₁ with TPI ASM8 was significantly attenuated compared with placebo ($P = 0.02$).

Airway Hyperresponsiveness

The geometric mean methacholine PC₂₀ of all subjects was 4.63 mg/ml (range, 0.4–15.6 mg/ml) at study entry, and remained within one doubling dose of that measured at screening when remeasured at Day 1 of each treatment period. The FEV₁ was also within 10% of screening values at Day 1 of each treatment period. Airway hyperresponsiveness before allergen was 4.48 mg/ml (range, 0.2–20.8 mg/ml) at Day 1 and 4.95 mg/ml (range, 0.4–19.0 mg/ml) at Day 2 with placebo, and 4.47 mg/ml (range, 0.3–17.9 mg/ml) at Day 1 and 4.39 mg/ml (range, 0.4–26.4 mg/ml) at Day 2 with TPI ASM8 treatment, with no significant difference between treatments ($P > 0.05$). At 24 hours after allergen, the methacholine PC₂₀ fell to 1.80 mg/ml (range, 0.1–8.7 mg/ml) with placebo, and to 1.33 mg/ml (range, 0.07–8.3 mg/ml) with TPI ASM8 treatment, with no difference in allergen-induced

changes in airway hyperresponsiveness between treatments ($P > 0.05$).

Sputum Inflammatory Cells

TPI ASM8 significantly inhibited the increase in sputum eosinophils that occurs 7 hours after allergen (Figure 4). During placebo treatment, sputum eosinophils increased from $3.7 \pm 1.2\%$ the day before allergen, to $24.4 \pm 5.1\%$ 7 hours after allergen. During TPI ASM8 treatment, sputum eosinophils increased from $3.2 \pm 4.5\%$ the day before allergen to only $14.4 \pm 4.0\%$ 7 hours after allergen (46% inhibition, $P = 0.02$). Twenty-four hours after allergen, sputum eosinophil levels were $15.7 \pm 3.1\%$ and $13.4 \pm 2.9\%$ after placebo and TPI ASM8 treatment, respectively ($P = 0.69$).

Seven hours after allergen, a trend was found for TPI ASM8 to inhibit the increase in total cell numbers (63% inhibition of increase compared with placebo, $P > 0.05$) and in total neutrophil numbers (32% inhibition of increase compared with placebo, $P > 0.05$). There was no difference between TPI ASM8 and placebo with respect to total macrophage numbers in sputum at any time points ($P > 0.05$; Figure 5).

mRNA Expression of β_c and CCR3 in Sputum Cells

Assessment of mRNA was available at all time points for 8 of the 17 subjects. Allergen induced higher levels of β_c and CCR3 mRNA in sputum cells, with median values 6.0-fold and 4.5-fold higher, respectively, at 7 hours after challenge when compared with Day 2 baseline. Treatment with TPI ASM8 significantly attenuated the increase in β_c mRNA ($P = 0.039$), and attenuated the increase in CCR3 mRNA in six of the eight samples assessed ($P = 0.054$; Figure 6). The results were similar when gene expression was normalized to either of the housekeeping genes.

Protein Expression of β_c and CCR3 in Sputum Cells

Sputum eosinophils, neutrophils, lymphocytes, and macrophages demonstrated binding of IL-3 and eotaxin. At 24 hours postadministration of TPI ASM8, we observed no change in the percentage of sputum eosinophil, neutrophil, lymphocyte, or macrophage binding of IL-3 or eotaxin ($P > 0.05$). Also, the MFI remained unchanged in all cell types studied at 24 hours postdosing ($P > 0.05$). Likewise, there was no effect of TPI

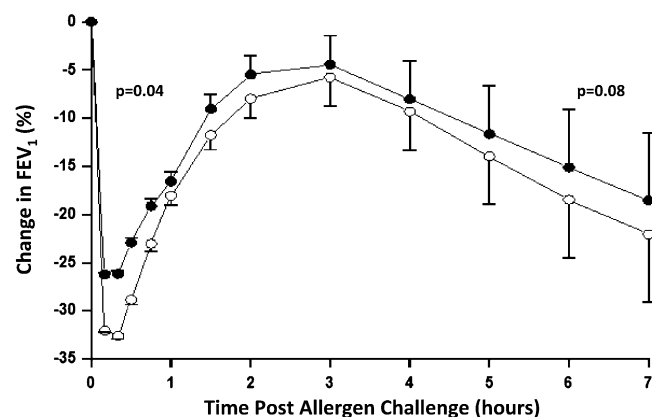


Figure 3. Allergen-induced maximum percentage fall in mean FEV₁ measured during the early (0–2 h) and late (3–7 h) airway responses during treatment with placebo and TPI ASM8.

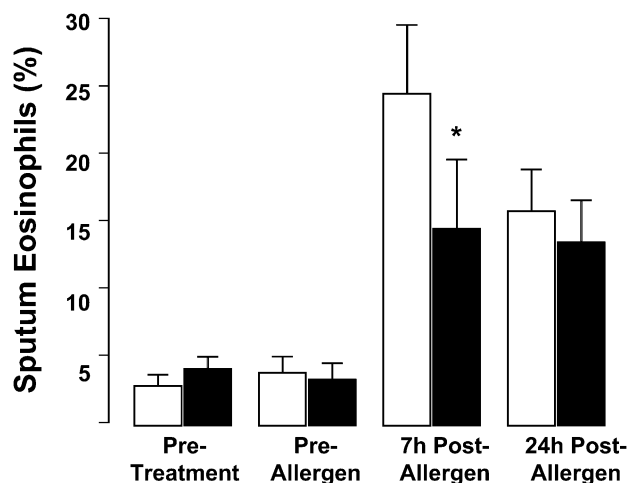


Figure 4. Percentage of sputum eosinophils (mean $\pm$ SEM) measured before and after allergen inhalation challenge during treatment with placebo (open bars) and TPI ASM8 (solid bars). * $P < 0.05$ compared with placebo.

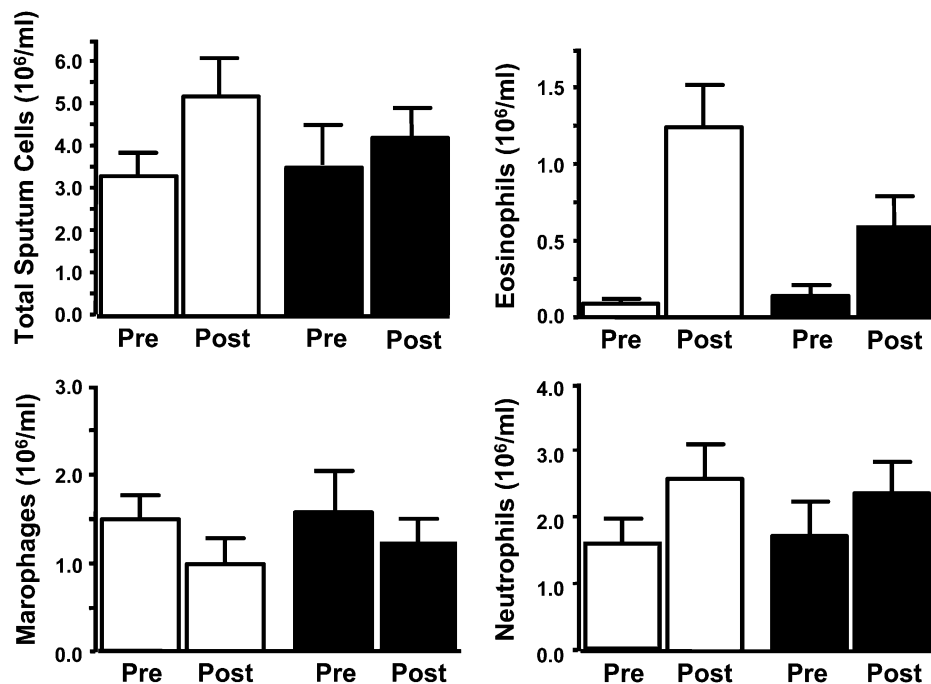


Figure 5. Leukocyte number per milliliter of sputum (mean $\pm$ SEM) measured before and 7 hours after allergen inhalation challenge during treatment with placebo (open bars) and TPI ASM8 (solid bars).

ASM8 on the percentage of cells expressing β_c , CCR3, or the level of β_c or CCR3 measured by MFI 7 hours after allergen ($P > 0.05$).

DISCUSSION

There is a lot of interest in the use of specific gene silencing to treat diseases. Because systemic delivery results in oligonucleotide accumulation mostly in the liver, spleen, and kidneys, it is possible that local delivery of oligonucleotides to the lungs would result in better therapeutic effects. This is the first report of RNA-targeted topical therapy having an effect in asthma.

In asthma, there is an increase in the number of inflammatory cells within the airways. It is believed that, through the

release of their mediators and enzymes (4–6), these cells contribute to the persistence of inflammation and airway hyper-responsiveness (1–3). TPI ASM8 contains two antisense oligonucleotides (TOP004 and TOP005), which down-regulate mRNA transcripts of β_c and CCR3 (7–9), leading to a reduction of these receptors on the cell surface. In theory, cells with reduced levels of CCR3 and β_c will be less likely to signal through these receptors, and therefore their response to eotaxins 1–3, RANTES (regulated upon activation, normal T-cell expressed and secreted), monocyte chemoattractant protein (MCP)-3 and 4, lymphotactin, GM-CSF, IL-5, and IL-3 will be impaired. The eosinophil should be particularly susceptible to TPI ASM8, as many cell functions, including chemotaxis, survival, and activation, are mediated through CCR3 and β_c , respectively (5, 19–21), and recent data suggest that activation of both the CCR3 and β_c pathways may be important in severe asthma (22). Allergen challenge is a validated exacerbation model of allergic airway inflammation, resulting in airway eosinophilia.

We hypothesized that inhaled TPI ASM8 would suppress the movement, activation, and viability of cells (primarily eosinophils) involved in the allergic immune response in the airways after allergen inhalation challenge, and thus reduce the physiologic response. This study demonstrates that inhaled TPI ASM8 effectively attenuates allergen-induced airway eosinophilia and physiologic responses in subjects with mild allergic asthma, through down-regulation of CCR3 and β_c mRNA.

We observed a significant reduction in the EAR, and a trend for a reduction in the LAR, demonstrating that TPI ASM8 treatment is effective in attenuating allergen-induced bronchoconstriction. The EAR is dependent on IgE-mediated activation of resident mast cells and basophils that have migrated to airway tissue (23). A reduction in EAR by TPI ASM8 may be due to reduced trafficking, differentiation, or activation of CCR3-positive and β_c -positive mast cells, basophils, and their progenitors to the airways, although it is less likely that mast cell turnover would be affected with only 2 days of treatment before allergen challenges. Basophils, however, have been reported to be present at higher levels in the airways of subjects with asthma (24), and respond rapidly and vigorously to antigenic

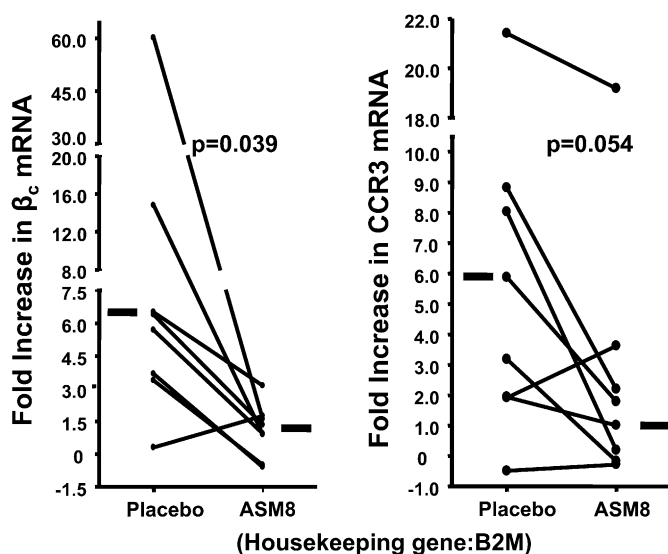


Figure 6. Increase in β_c mRNA and CCR3 mRNA (median) from preallergen baseline to 7 hours after allergen inhalation challenge during treatment with placebo and TPI ASM8.

stimulation (24). Although the EAR develops by constriction of airway smooth muscle in response to the mast cell and basophil products, histamine and cysteinyl leukotrienes (23, 25), it has also been shown that cytokines such as IL-5 may actually increase the contractile response of airway smooth muscle, indicating that smooth muscle on its own may be responsive to TPI ASM8 (26).

The reduction of the LAR by TPI ASM8 is believed to be mediated through attenuation of inflammatory cells. Although the eosinophil is a main target of this therapy, there are other cells that bear CCR3 receptors and signal through β_c and are known contributors to the inflammatory response. By flow cytometry, our data show that neutrophils, lymphocytes, and macrophages can bind eotaxin and IL-3 through CCR3 and β_c , respectively. The function of CCR3 and β_c on these cell types has not been fully elucidated. We observed no difference in the percentage of cellular surface receptors binding IL-3 or eotaxin, or a difference in the MFI of IL-3 or eotaxin binding, after TPI ASM8 treatment. However, these cells were expressing less RNA message for CCR3 and β_c as shown by real-time polymerase chain reaction. That we observed a reduction in sputum eosinophils with TPI ASM8 treatment supports the concept of reduced trafficking of eosinophils to the airways. Our interpretation is that only those cells expressing CCR3 and β_c would be capable of migrating to, surviving in, and being activated in the airways.

Perhaps it should not be surprising that sputum cells continued to express CCR3 and β_c protein on the cell surface after TPI ASM8 treatment, because these receptors enabled cells to traffic to the lumen where sampling occurred. By contrast, those cells that did not migrate to the airway lumen (i.e., eosinophils 7 h postallergen) may have demonstrated reduced protein expression. This was not measured because it would have required collection of biopsy tissue within this complex study design. Reduced levels of mRNA without a change in protein expression, as we observed in sputum cells, are not necessarily a contradiction because degradation of mRNA occurs more rapidly than changes in protein synthesis. That we observed down-regulation of target mRNA confirmed TPI ASM8 had an effect on target genes.

We also found a trend for a reduction in total cells and neutrophils (63 and 32% reduction compared with placebo, respectively; $P < 0.05$) and no difference in macrophages in the sputum 7 hours after antigen challenge in TPI ASM8-treated subjects. Additional experiments are necessary to assess whether these effects represent additional advantages of therapy with TPI ASM8. Importantly, it is clear that TPI ASM8 does not have proinflammatory effects at doses that decrease the allergic airway response in humans.

We observed no attenuation of sputum eosinophils at 24 hours postallergen. This lack of effect needs to be interpreted with caution because drug levels measured were lowest in the sputum at 24 hours postdosing. Optimum delivery of TPI ASM8 to maximize effect has yet to be determined. There was a significant order effect in the analyses of the LAR, showing that subjects receiving TPI ASM8 in the second sequence had greater protection afforded on the LAR (35% reduction compared with placebo). Furthermore, the levels of TOP004 and TOP005 were more consistent in subjects receiving TPI ASM8 in the second sequence ($C_{\max}$ SD 9.2-fold higher in sequence 1 than in sequence 2). This suggests that drug levels in some subjects may not have been sufficient for blocking allergen-induced effects; however, with the relatively small numbers of subjects and potential variability introduced using a multicenter approach, we were unable to show a relationship between inhibition of the LAR and the level of TOP004 or TOP005 in the airways. Because this is the

first study to successfully measure TPI ASM8 levels in airway samples, an assessment of drug delivery efficiency was not previously possible. It is estimated that, for the non-breath-actuated nebulizer used in the study, only a small proportion ($\sim 100 \mu\text{g}$) of the metered dose (1.5 mg) would have been delivered to the lung (27). The variable drug levels measured in airway samples suggests that optimal dosing conditions were not reached. Indeed, the post-treatment allergen challenge was performed on the third day or the equivalent of only two half-lives of phosphorothioate oligonucleotides within lung tissue (28, 29). On the basis of the results presented, it is likely that administration for longer periods, combined with better delivery to the lungs, will provide better efficacy in future studies.

The primary endpoint of the trial (LAR) showed a non-significant trend in favor of TPI ASM8. We conclude that TPI ASM8 has a protective effect on some allergen-induced airway responses, as evidenced by a reduced early-phase response and eosinophilic airway inflammation, and as a result of the pharmacologic action to down-regulate expression of CCR3 and β_c on inflammatory cells in the airways. Further studies will be needed to determine the optimal dosing of TPI ASM8 to enable a consistent protective effect in allergic asthma.

Conflict of Interest Statement: G.M.G. has been reimbursed \$5,000 by Topigen for protocol development for this clinical trial, and has received research funding for participating in clinical trials from Topigen, MedImmune, Altana Pharma, GlaxoSmithKline, and Boehringer Ingelheim; has participated as a speaker for Merck Canada; and has participated on advisory boards for MedImmune, Tannox, and Novartis, for which fees less than \$5,000 were received. L.P.B. has served on advisory boards for AstraZeneca, Altana, GlaxoSmithKline, Merck Frosst, and Novartis; received lecture fees from 3M, Altana, AstraZeneca, GlaxoSmithKline, Merck Frosst, and Novartis; received sponsorship for investigator-generated research from AstraZeneca, GlaxoSmithKline, Merck Frosst, and Schering. L.P.B. received research funding for participating in multicenter studies from 3M, Altana, AsthmaTx, AstraZeneca, Boehringer Ingelheim, Dynavax, Genentech, GlaxoSmithKline, IVAX, MedImmune, Merck Frosst, Novartis, Roche, Schering, Topigen, and Wyeth. L.P.B. received support for the production of educational materials from AstraZeneca, GlaxoSmithKline, and Merck Frosst; is a member of the Quebec Workmen Compensation Board Respiratory Committee. L.P.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.W.C. has participated as a speaker at scientific meetings organized by pharmaceutical companies (AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, and Methapharm) and participated on advisory boards (AstraZeneca Canada and Merck Canada) for which fees less than \$10,000 were received. A.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. F.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.M.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.M.R. is the inventor of ASM8 and founder of Topigen Pharmaceuticals, Inc. P.M.R. invested \$2,200,000 in Topigen and presently owns 4% of the stock in the company. P.M.R. is a consultant for Topigen. P.M.O. has been on advisory boards for AstraZeneca, Biolipox, GlaxoSmithKline, Topigen, and Resistentia, and has received lecture fees from these and other pharmaceutical companies including Chiesi and Ono Pharma. In addition, he received grants-in-aid for research studies from AstraZeneca, Altana, Biolipox, Boehringer, GlaxoSmithKline, MedImmune, Merck, Pfizer, and Wyeth.

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