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Toll-like receptor induced expression of epithelial cytokine receptors on hemopoietic progenitors is altered in allergic asthma.

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ABSTRACT:

Background: Hemopoietic progenitor cells (HPC) migrate to sites of allergic inflammation where, upon stimulation with epithelial cytokines, they produce Th2 cytokines and differentiate into mature eosinophils and basophils. They also express Toll-like receptors (TLR) involved in anti-microbial responses.

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Objective: The objective of this study was to compare TLR expression on peripheral blood HPC and TLR-induced responses, in particular changes in epithelial cytokine receptors, in healthy and asthmatic subjects at baseline and following allergen challenge.

Methods: Ten healthy and 11 allergic asthmatic subjects were studied. HPC-enriched cell populations were stimulated with TLR-2, TLR-4 or TLR-9 ligands. TLR expression by circulating HPC and interleukin (IL)-25 (IL-17RB), IL-33 (ST2) and thymic stromal lymphopoietin (TSLPR) receptor expression after TLR ligation, were examined by flow cytometry at baseline and, in asthmatics, following allergen challenge. The effects of dexamethasone (Dex) on TLR-induced responses were also assessed.

Results: Asthmatics had significantly lower circulating HPC expressing TLR-2 and TLR-9 with a similar trend for TLR-4. TLR-4 stimulation of HPC yielded higher number of TSLPR⁺ cells in asthmatics compared with healthy subjects. A similar trend was seen for TLR-9 ligation, an effect further augmented by allergen inhalation. Allergen challenge also enhanced TLR-induced ST2 expression on HPC. Treatment with Dex *in vitro* increased TLR-4-induced TSLPR expression but had no effect on other epithelial cytokine receptors.

Conclusions & Clinical Relevance: These data demonstrate an interaction between allergen and TLR ligand exposure in asthmatics. Allergen inhalation augments the TLR-induced inflammatory response by HPC, possibly leading to increased “*in situ hemopoiesis*” through upregulation of TSLPR. These findings show that HPC may be a part of the pro-inflammatory cascade in pathogen-induced asthma exacerbation through their increased responsiveness to TLR stimulation.

INTRODUCTION:

Hemopoietic progenitor cells (HPC,) constitute a stem cell population residing in the bone marrow (BM). However, even in the steady state, small numbers of HPC are released from the BM and migrate to peripheral organs. In response to allergen or danger signals, increased numbers of HPC are present in BM and peripheral blood of atopic subjects, and activated HPC producing interleukin (IL)-5 and IL-13 can be detected in the sputum of asthmatic subjects, with numbers increasing in response to specific allergen inhalation challenge (1-2).

At the site of allergic inflammation, a phenomenon known as “*in situ hemopoiesis*” takes place leading to the differentiation of HPC into mast cells, eosinophils or basophils, a process stimulated by hemopoietic cytokines, i.e., IL-3, IL-5 and GM-CSF. Our group has previously found a key role for thymic stromal lymphopoietin (TSLP) in human eosinophil and basophil (Eo/B) differentiation in allergic subjects, in that it enhances IL-3-induced HPC differentiation (3). Moreover, TSLP along with IL-33 is a potent activator of HPC (4). TSLP, IL-33 and IL-25 are a group of cytokines collectively named “alarmins” which are released by epithelial cells in response to danger signals such as allergens or viruses (5).

Toll-like receptors (TLR) are a class of pattern-recognition receptors that play a key role in the detection of and response to pathogens (6). In previous work, we demonstrated that cord blood human HPC from high allergic risk newborns express TLR less abundantly than low allergic risk infants (7). It remains unknown if this phenomenon occurs in adult allergic subjects.

In the present study, we sought to determine if TLR-2, -4 and -9 expression on peripheral blood HPC, as well as TLR-induced responses differ between allergic asthmatics and healthy controls. We also investigated TLR-mediated changes in IL-25- (IL-17RB), IL-33- (ST2) and TSLP- (TSLPR) receptors by HPC in healthy and allergic asthmatic subjects, and the effect of allergen

challenge on TLR-induced responses. Due to the fact that corticosteroids are the most potent and commonly used group of anti-inflammatory drugs used in the treatment of asthma and asthma exacerbations, we also evaluated the effects of dexamethasone (Dex) on TLR-induced responses by HPC *in vitro*.

MATERIALS AND METHODS:

Subjects:

Eleven subjects with mild allergic asthma, aged 18 to 65 years, were enrolled (Table 1). All were atopic, based on one or more positive skin prick tests to common aeroallergens, had a baseline FEV1 > 70% of predicted, and had previously documented dual airway responses to inhaled allergen. All asthmatic subjects used only intermittent (not daily) β 2-agonists. The control group comprised 10 non-atopic (negative results of skin prick test) and non-asthmatic (PC_{20} >16 mg/ml) subjects with no other significant disease. Subjects were excluded if pregnant, current smokers, or ex-smokers with more than 10 pack-years. All subjects gave written informed consent, and the study was approved by the Hamilton Integrated Research Ethics Board.

Study design:

Asthmatics underwent a randomized, cross-over, diluent-controlled allergen inhalation challenge. All subjects were screened on visit 1 and underwent a methacholine inhalation challenge, skin prick testing, sputum induction and blood collection (Pre-challenge time point). On visit 2, subjects were randomized to receive either a diluent or allergen challenge. Subjects returned for visit 3, 24 hours post-challenge for methacholine challenge, sputum induction and blood collection (Post-challenge time point). A repeat of the 3 visits, with the alternative diluent or allergen challenge, was conducted 2 to 4 weeks later. Healthy subjects underwent

methacholine challenge and skin prick testing to exclude asthma and atopy, respectively, and blood sampling at one visit (see Figure 1 for study flow chart). Baseline receptor expression and cytokine production for asthmatic subjects was based on the results obtained from experiments performed on samples collected at Visit 1.

Allergen challenge:

The allergen challenge was performed as previously described (9). Briefly, the challenge allergen was identified using results from skin prick testing. The concentration of allergen causing a 20% fall in FEV₁ was predicted using the methacholine PC₂₀, and the titration of allergen was determined from the skin prick testing as previously described (10). The early asthmatic response (EAR) was defined as a $\geq 20\%$ fall in FEV₁ between 0 and 3h after allergen, while the late asthmatic response (LAR) was defined as a $\geq 15\%$ fall in FEV₁ between 3h and 7h after allergen inhalation. All asthmatic subjects enrolled in the study had both EAR and LAR (dual responders).

Blood collection and processing:

One hundred mL of blood were collected through direct venipuncture into heparinized vacutainer tubes (Becton– Dickinson, Franklin Lakes, NJ, USA) at time points specified above. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation and subjected to staining for TLR and HPC enrichment and cultures. HPC were stimulated overnight with a TLR-2 agonist (lipoteichoic acid, LTA), a TLR-4 agonist (lipopolysaccharide, LPS) or a TLR-9 agonist (ODN2006). Following stimulation, cell free supernatant was collected for cytokine measurements and remaining cells were subjected to flow cytometry staining for epithelial cytokine receptors (for details regarding sample collection, processing, cultures and flow cytometry see the Online Data Supplement).

Statistical analysis:

Data were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). For clarity, all data are presented as means $\pm$ SEMs. Normality of data distribution was tested with Shapiro-Wilk test. Statistical analysis for the inhalation challenges was performed by using 2-way ANOVA and the post hoc Bonferroni test. The *in vitro* experiments with normally distributed data were analyzed by using 1-way ANOVA with a post hoc Tukey test, paired t-test and unpaired t-test, when appropriate. Not normally distributed data were analyzed with Friedman test with the post hoc Dunn's test, Wilcoxon signed rank test for paired data and U Mann-Whitney test for unpaired data, when appropriate.

RESULTS:

All subjects demonstrated a dual response to allergen inhalation, with a mean maximum fall in FEV₁ of $36.87 \pm 3.02\%$ during the early response ($P < 0.001$) followed by a $24.09 \pm 3.62\%$ fall during the late response ($P < 0.01$). No significant change in FEV₁ occurred during the diluent challenge (see supplementary Figure S1). Allergen inhalation led to a significant increase in sputum eosinophils and decrease in methacholine PC₂₀ compared with diluent (Table 2). The percentage of circulating TLR-2+ and TLR-9+ HPCs were significantly lower in allergic asthmatics when compared with healthy subjects ($p < 0.05$) (Figure 2A). A similar trend was observed for TLR-4+ HPC but the difference did not reach statistical significance ($p = 0.057$). Allergen inhalation did not change the percentage of circulating TLR-2+, TLR-4+ and TLR-9+ HPC when compared to pre-challenge and diluent challenge values (Figure 2B).

Overnight stimulation with LPS, and ODN2006, but not LTA, led to a significant increase in TSLPR expression on HPC in both healthy and asthmatic subjects (Figure 3A). Stimulation of asthmatic HPC with LPS yielded significantly higher expression of TSLPR, compared with HPC from healthy controls ($22.51 \pm 4.24\%$ vs $11.6 \pm 1.34\%$, respectively; $p < 0.05$), with a similar trend for asthmatic HPC after stimulation with ODN2006 ($p = 0.054$). TLR ligation did not affect IL-17RB expression on HPC from healthy subjects (Figure 3B). By contrast, stimulation of HPC obtained from asthmatic subjects with ODN2006 led to a significant decrease in the percentage of IL-17RB expressing cells compared with unstimulated cultures ($44.63 \pm 6.08\%$ vs $65.87 \pm 7.45\%$, respectively; $p < 0.05$), with a similar trend for LPS-stimulated cells ($p = 0.06$). There was no significant difference between healthy and asthmatic subjects in the percentage of IL-17RB+ HPC in unstimulated and stimulated cultures (Figure 3B). TLR-ligation did not yield significant changes in ST2 expression on healthy and asthmatic HPC at baseline. In addition, there was no difference in ST2 expression between healthy and asthmatic subjects (Figure 3C). Allergen challenge led to a further increase in TSLPR expression by LPS-stimulated HPC, compared to diluent ($32.37 \pm 4.14\%$ vs $18.25 \pm 4.94\%$, respectively; $p < 0.05$) (Figure 4A). Stimulation with ODN2006 led to a significant overexpression of TSLPR post-allergen compared with pre-challenge ($18.86 \pm 3.06\%$ vs $12.81 \pm 2.01\%$, respectively; $p < 0.05$) but not diluent values. Furthermore, LPS stimulation induced a higher percentage of ST2+ HPC post allergen compared to pre-challenge ($2.78 \pm 0.64\%$ vs $2.02 \pm 0.39\%$, respectively; $p < 0.05$) but not diluent values. A similar effect was seen following ODN2006 stimulation ($1.55 \pm 0.36\%$ pre allergen vs $1.94 \pm 0.56\%$ post allergen; $p < 0.05$). There was no effect of allergen inhalation on TLR-induced IL-17RB (Figure 4C).

Previously, our group demonstrated that autocrine TNF- α upregulates TSLPR on HPC (3). Here, we showed that LPS-stimulated HPC from both healthy and asthmatic subjects produce substantial amounts of TNF- α (Figure 5A). Significantly higher concentrations of TNF- α were detected in LPS-stimulated asthmatic HPC cultures compared with healthy controls (247.9 $\pm$ 43.90 pg/ml vs 105.9 $\pm$ 23.02 pg/ml, respectively; p <0.05). There was a further significant increase in LPS-induced TNF- α release by HPC following allergen challenge (Figure 5B). When anti- TNF- α antibody was added to LPS-stimulated cultures, the yield of TSLPR+ HPC decreased, compared with cultures without the neutralizing antibody. However, TSLPR was still significantly overexpressed compared with unstimulated cells (Figure 5C).

Overnight stimulation of HPC with LPS induced significantly higher concentrations of IL-5 and IL-13 compared with unstimulated cultures from both healthy and asthmatic subjects (see supplementary Figure S2A and S2C). There was a significant increase in LPS-induced IL-5 production following allergen inhalation compared to baseline (see supplementary Figure S2B). However, no changes were noted in LPS-induced IL-13 release following allergen challenge (see supplementary Figure S2D).

Overnight stimulation of HPC with Dex did not affect any of studied TLR expression (Figure 6A). However, overnight treatment with Dex alone yielded significantly higher percentages of TSLPR+ HPC and this effect was further augmented when HPC were co-cultured with Dex and LPS (Figure 6B). Dex has no effect on either IL-17RB or ST2 expression on HPC (see supplementary Figure S3A and S3B). Incubation with Dex alone did not affect production of TNF- α by HPC nor did it alter LPS-induced TNF- α production (Figure 6C). However, Dex abolished LPS-induced release of IL-5 and IL-13 by HPC (see supplementray Figure S4A and S4B).

DISCUSSION:

TLR are innate receptors involved in anti-microbial responses. Studies have shown that allergy status and the type of allergic disease may affect various TLR expression and function by different cells. For instance, TLR-9 expression on circulating dendritic cells was reported to be increased in asthma, but unchanged in allergic rhinitis, and decreased in patients with allergy to hymenoptera venom (11-13). Interestingly, even when TLR-9 expression in allergic subjects is not different compared with healthy controls, the response to TLR-9 ligation is altered, as indicated by decreased IFN- α production (12). Our group has previously shown that TLR expression and responses by cord blood HPC from “high allergy risk infants” are altered compared to “low allergy risk” ones (7). The current study showed that this phenomenon persists in adulthood in that TLR expression on HPC is decreased in the adult allergic asthmatic population. Additionally, the responses to TLR ligation were increased in subjects with mild allergic asthma.

These findings are in line with previous studies showing that TLR expression is not necessarily a good predictor of altered response to TLR stimulation, and that the response may differ between asthmatic and normal subjects. For example, Lee et al (14) showed that during asthma exacerbation, compared to stable disease, asthmatic PBMC secrete significantly higher levels of TNF- α and IL-6 after TLR-1/2 and TLR-7/8 stimulation, but lower after TLR-9 stimulation, despite unchanged TLR expression. Similarly, despite comparable TLR3 expression by asthmatic and normal bronchial epithelial cells (BEC), asthmatic BEC have reduced antiviral responses, most likely due to impaired signaling pathways leading to production of type I and III IFN (15).

Our results showed that activation of TLR-4 and TLR-9, but not TLR-2, increased TSLPR expression on HPC in both healthy and asthmatic subjects. Moreover, TLR-4 ligation significantly overexpressed TSLPR in asthmatics compared with healthy controls and a similar trend was seen for TLR-9 ligation. TLR stimulation did not affect IL-17RB expression in healthy subjects. However, ODN2006 had a small but significant downregulating effect on IL-17RB expression by allergic asthmatic HPC. Further studies are warranted to explore the significance of this finding. There was no effect at all of TLR ligands on ST2 expression on HPC. Thus, it seems that TLR-mediated effects on epithelial cytokine receptors are mainly associated with upregulation of TSLPR.

There is evidence that exposure to allergen may affect TLR expression and response. For example, TLR-4 expression increased in leukocytes in nasal fluid, peripheral blood and bone marrow during symptomatic allergic rhinitis (16). Furthermore, nasal challenge with allergen followed by LPS augmented the release of IL-4, IL-5, IL-10, IL-13, IFN- γ and TNF- α (16). In contrast, allergen challenge does not affect TLR-9 expression on blood and sputum plasmacytoid dendritic cells, but impairs IFN- α production by these cells (17-18). In our study, we did not notice any change in the expression of the TLR receptors that were examined. This might be due to the fact that we investigated circulating HPC only, and did not examine HPC in other compartments, i.e. bone marrow or airways. However, we detected augmented LPS and ODN2006-induced TSLPR expression on HPC following allergen challenge, indicating that TLR responses were altered by allergen exposure, despite unchanged receptor expression. In addition to TSLPR upregulation, we observed a significant increase in ST2 expression following allergen challenge compared to baseline in LPS and ODN2006 cultures.

Both TSLP and IL-33 are potent activators of HPC. For instance, they induce production of IL-5 and IL-13 and modulate HPC migration in subjects with allergic asthma (1,4). Our group has shown that TSLP signaling upregulates IL-3R α on peripheral blood CD34+ cells, rendering them more responsive to the effects of IL-3, with concomitant functional Eo/B colony forming unit differentiation (3). Moreover, there is evidence that IL-33 and TSLP are produced in large amounts during rhinovirus infection (19-20). Given these facts, it is plausible that concomitant exposure to allergen and infectious factors augments HPC pro-inflammatory responses. Our previously published study showed that autocrine TNF- α upregulates TSLPR on HPC (3). Current findings demonstrated that TLR stimulation leads to a robust release of TNF- α by HPC and asthmatic progenitors produce significantly more TNF- α upon stimulation with LPS compared with cells obtained from healthy donors. Moreover, in asthmatic subjects LPS-dependent TNF- α release is further augmented by allergen inhalation. This suggests that increased TLR-induced TNF- α production by HPC following allergen challenge may be responsible for TSLPR overexpression. Unfortunately, due to limited numbers of HPC, we were unable to perform *ex vivo* TNF- α neutralizing experiments following allergen challenge, to test this hypothesis. Nonetheless, our data show that neutralizing TNF- α does not abolish TLR-induced TSLPR, suggesting that TLR ligation has a direct effect on TSLPR, and not solely through autocrine TNF- α release. Nevertheless, these results support the importance of TNF- α in pathogen-induced asthma exacerbations as previously shown in a murine model (21). Indeed, infliximab, a recombinant anti-TNF- α antibody, and etanercept, a soluble TNF- α receptor fusion protein, demonstrated clinical benefit in reducing asthma exacerbations (22-23). Of note, however, studies have not demonstrated consistent benefit, and serious concerns have been

raised with regard to the safety of TNF- α blockade in asthma, making it unlikely that this approach will be used for the management of asthma (24).

TSLP blockade, which was previously shown to ameliorate allergen-induced responses in mild asthmatics, may be also a promising agent to inhibit pro-inflammatory responses mediated by both TNF- α - and TLR-induced TSLPR overexpression underlying pathogen-induced asthma exacerbation (25). In addition, allergen challenge increased LPS-induced production of IL-5 compared with pre-allergen values whereas there was no change in IL-13 release. IL-5 is one of the cytokines regulating differentiation of committed progenitors towards the Eo/B lineage and its increased autocrine production may boost this process at sites of inflammation (26).

Collectively, these findings suggest that HPC may be a part of the pro-inflammatory cascade in pathogen-induced asthma exacerbation through their increased responsiveness to TLR stimulation which is further augmented by allergen exposure.

It is well established that corticosteroids are the most potent anti-inflammatory drugs used in the treatment of asthma and asthma exacerbations. There is also evidence that corticosteroids may affect TLR expression and function by various cell types. For example, Dex enhanced bronchial epithelial cell TLR-2 expression (27). It also inhibited cytokine- and ligand-induced TLR-2, TLR-3, and TLR-4 expression and chemokine release by human airway smooth muscles but potentiated TLR-2 expression induced by combined IFN- γ and TNF- α stimulation (28). In addition, steroids increased expression of TLR-2, -3 and -4 on dendritic cells yet their stimulation with TLR-derived signals did not induce maturation (29). In the current study we examined the effects of Dex on TLR expression and TLR-induced responses by HPC *in vitro*. We showed that treatment with Dex did not affect expression of any of the studied TLR. However, Dex augmented LPS-induced TSLPR expression on HPC, most likely through

increasing TLR responsiveness, as Dex did not affect TNF- α production. The data on the effects of corticosteroids on HPC are ambiguous. For example, Gauvreau et al showed that an 8 day treatment with inhaled budesonide (BUD) suppresses allergen-induced increases in Eo/B colony forming units (CFU) grown in the presence of GM-CSF, but not IL-5 or IL-3 (30). In a different study, BUD treatment reduced baseline levels of bone marrow Eo/B progenitors and CD34+IL-5R α + cells in atopic asthmatics but failed to inhibit allergen-induced increases in bone marrow CD34+IL-5R α + progenitors and Eo/B CFU growth in the presence of hemopoietic cytokines (31). Our findings regarding TSLPR upregulation by Dex are in line with other studies showing the pro-inflammatory effects of corticosteroids on HPC, such as enhancement of IL-5-mediated bone marrow and cord blood Eo/B progenitor differentiation (32). However, in our study Dex also demonstrated anti-inflammatory properties *in vitro* by abolishing LPS-induced production of IL-5 and IL-13 by HPC.

In summary, these data demonstrate an interaction between allergen and TLR ligand exposure in asthmatic subjects, suggesting that a prior allergen inhalation significantly augments the TLR-induced inflammatory response by HPC, possibly leading to increased “in situ hemopoiesis” through upregulation of TSLPR. Moreover, whereas *in vitro* treatment with a corticosteroid abolished TLR-induced IL-5 and IL-13 production by HPC it had an opposite effect on TSLPR expression. This phenomenon supports the involvement of HPC in the initiation and persistence of airways inflammation during infection-driven asthma exacerbations through a TSLP-dependent pathway.

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CONFLICT OF INTERESTS:

POB received consultancy fees from AstraZeneca and Chiesi, research funding from AstraZeneca, Novartis, Amgen, Genetech, Axican and Alakos, and participated in advisory boards for Boehringer Ingelheim, GSK, MedImmune, Merck, Takeda, Abbott.

All other authors declare no conflicts of interest.

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FIGURE LEGENDS:

Figure 1. Study design. PBMC – peripheral blood mononuclear cells; TLR – Toll-like receptors; LTA- lipoteichoic acid; LPS – lipopolysaccharide. Pre – before either allergen or diluent challenge; Post – 24 hours following either allergen or diluent challenge.

Figure 2. Toll-like receptor (TLR) expression on peripheral blood hemopoietic progenitor cells (HPC) in healthy (n=10) and asthmatic (n=11) subjects at baseline (A) and following diluent (white bars) and allergen challenge (black bars) (B). * p<0.05.

Figure 3. Effect of Toll-like receptor ligation on thymic stromal lymphopoietin (A), interleukin-25 (B) and interleukin-33 (C) receptor expression on peripheral blood hemopoietic progenitor cells in healthy (n=8) and asthmatic (n=10) subjects at baseline. LTA- lipoteichoic acid; LPS – lipopolysaccharide; TSLPR – thymic stromal lymphopoietin receptor; IL-17RB – interleukin-25 receptor B; ST2- interleukin-33 receptor; Iso – isotype antibody; Ab – specific antibody.

Figure 4. Toll-like receptor induced changes in thymic stromal lymphopoietin receptor (A), IL-17RB (B) and ST2 (C) expression on hemopoietic progenitor cells following diluent (white bars) and allergen challenge (black bars). LTA- lipoteichoic acid; LPS – lipopolysaccharide; TSLPR – thymic stromal lymphopoietin receptor; IL-17RB – interleukin 25 receptor; ST2- interleukin 33 receptor. *p<0.05.

Figure 5. TNF- α production by hemopoietic progenitor cells upon overnight stimulation with lipopolysaccharide in healthy (n=8) and asthmatic subjects (n=10) at baseline (A) and following diluent (white bars) and allergen challenge (black bars) (B). (C)- Effect of TNF- α neutralizing antibody on LPS-induced TSLPR expression (n=8). LPS – lipopolysaccharide; TSLPR – thymic stromal lymphopoietin receptor, α TNF – TNF- α neutralizing antibody. *p<0.05; **p<0.01;

***p<0.001

Figure 6. The effect of dexamethasone on Toll-like receptor (A) and thymic stromal lymphopoietin receptor (B) expression and TNF- α production by peripheral blood hematopoietic progenitor cells (n=8). TSLPR – thymic stromal lymphopoietin receptor. *p<0.05; **p<0.01; ***p<0.001.

TABLES:

Table 1. Subject demographics. FEV₁ - forced expiratory volume in 1 second; Meth PC20 - methacholine concentration causing a 20% fall in FEV₁; Ag- allergen; EAR – early asthmatic response; LAR – late asthmatic response; HDM – house dust mites.

Sex	Age (years)	Atopy	FEV ₁ (% of Predicted)	Meth PC ₂₀ (mg/ml)	Ag Inhaled	Max % Fall in FEV ₁ (EAR and LAR)	
Asthmatic Subjects							
M	28	+	85	5.52	HDM	-39.54	-19.89
F	47	+	96	0.34	CAT	-35.68	-15.0
M	36	+	88	13.6	GRASS	-41.58	-15.88
M	23	+	83	1.36	HDM	-40.94	-16.53
M	53	+	96	6.15	HDM	-26.32	-15.1
F	25	+	93	0.3	CAT	-32.75	-20.68
F	18	+	83	1.21	HDM	-42.07	-51.52
M	34	+	79	1.92	HDM	-22.61	-20.71
M	64	+	98	2.97	CAT	-60.30	-20.60
F	23	+	93	3.62	HORSE	-32.02	-42.59
M	27	+	103	14.2	HDM	-31.74	-26.53
Healthy Subjects							
F	50	-	99	>16	N/A	N/A	
F	26	-	101	>16	N/A	N/A	
M	34	-	85	>16	N/A	N/A	
M	40	-	95	>16	N/A	N/A	
M	28	-	114	>16	N/A	N/A	
M	34	-	97	>16	N/A	N/A	
M	41	-	107	>16	N/A	N/A	
F	37	-	84	>16	N/A	N/A	
F	64	-	87	>16	N/A	N/A	
M	37	-	80	>16	N/A	N/A	

Table 2. Airway inflammatory response following diluent and allergen challenge. *Diluent vs allergen, $P<0.05$; #Time point vs baseline, $P<0.05$. Meth PC₂₀ - methacholine concentration causing a 20% fall in forced expiratory volume in 1 second.

Inflammatory cells in sputum (10^6 cells/g sputum)						
	Eosinophils		Neutrophils		Macrophages	
Time (h)	Diluent	Allergen	Diluent	Allergen	Diluent	Allergen
0	0.007 $\pm$ 0.004	0.009 $\pm$ 0.004	1.17 $\pm$ 0.55	0.96 $\pm$ 0.44	2.26 $\pm$ 0.58	1.80 $\pm$ 0.39
7	0.019 $\pm$ 0.008	0.243 $\pm$ 0.097 [#]	0.51 $\pm$ 0.19	2.86 $\pm$ 1.08	1.11 $\pm$ 0.16	2.09 $\pm$ 0.49
24	0.017 $\pm$ 0.013	0.372 $\pm$ 0.158 ^{*#}	1.97 $\pm$ 0.82	2.82 $\pm$ 1.07	2.90 $\pm$ 0.88	2.89 $\pm$ 0.73
Meth PC ₂₀ (mg/ml)						
Time	Diluent			Allergen		
0	4.31 $\pm$ 1.36			3.57 $\pm$ 0.91		
24	6.54 $\pm$ 2.26			1.69 $\pm$ 0.35 [*]		









