

Original article

Nasal endothelial interleukin-10 expression is negatively correlated with nasal symptoms after allergen provocation

Background: Despite major efforts, factors that predict or correspond to the level of allergic symptoms remain elusive. Given our previous observations of mucosal interleukin-10 (IL-10) expression by local tissue cells and its described role as immune modulator, we hypothesized that, in allergic rhinitis, nasal mucosal IL-10 expression could influence the severity of symptoms.

Methods: In this study, we investigated endothelial IL-10 expression in nasal mucosa of healthy- and house dust mite allergic patients, both before and after provocation, and under nasal steroid therapy. Nasal turbinate biopsies were taken from healthy individuals as well as from house dust mite allergic patients, both before and after provocation. Allergic patients received fluticasone propionate aqueous nasal spray or control treatment. In the allergic patients, endothelial IL-10 scores based on immunohistochemical stainings were correlated with allergic symptoms, measured by visual analog scores.

Results: At baseline, variable levels of endothelial IL-10 were detected in nasal biopsies. After nasal provocation, but not at baseline, endothelial IL-10 expression corresponded very closely to the allergic symptoms after allergen provocation. Low symptom scores were correlated with high endothelial IL-10 scores. This correlation disappeared after fluticasone propionate treatment.

Conclusions: There is a large variation in the level of endothelial IL-10 expression both in healthy individuals and in house dust mite allergic patients. Endothelial IL-10 expression may affect local immune reactions resulting in reduced levels of allergic symptoms.

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Despite the fact that many of the factors in the pathophysiological mechanism leading to the expression of allergic disease have been described quite accurately (1), it is still not clear which of these factors will predict the level of allergic symptoms. For the development of efficient treatment regimes for allergic disease, it is of vital interest to assess how allergic sensitization leads to symptoms. The manifestation of allergic symptoms occurs in two phases: an early phase of about 60 min with a peak in symptoms between 15 and 30 min after allergen exposure and a late phase response that starts after 60 min and can last for about 24 h after allergen exposure (1). In the early phase, allergen-induced cross-linking of IgE on mast cells will trigger the release of histamine, leukotrienes, and

other allergic mediators that contribute to the manifestation of rhinorrhoea, sneezing, and nasal blockage, while the influx of eosinophils and other inflammatory cells, with the subsequent release of toxic mediators will contribute to these symptoms in the late phase response (1). However, it has been difficult to find strong correlations between clinical symptoms and any of these factors.

Although allergen-specific IgE is required for the development of allergic symptoms, it is not sufficient. Some individuals do have allergen-specific IgE, but when exposed to the relevant antigen, they do not develop any allergic symptoms at all (2). Moreover, even in patients who develop allergic symptoms, the total level of serum IgE (3, 4) or allergen-specific IgE (5, 6) is not related to the severity of these symptoms. Some research suggests that the ratio of allergen-specific IgE to IgG4 could be important (7–9). High serum levels of mouse allergen-specific IgG or IgG4 may be markers for clinical tolerance among laboratory mouse workers with detectable mouse-specific IgE, but these findings need to be confirmed in larger, prospective studies (10). Furthermore, the composition of the inflammatory influx does not accurately

Abbreviations: SIT, allergen-specific immunotherapy; IL-10, interleukin-10; METC, Medical ethical commission; RAST, radioallergen sorbent test; FPANS, fluticasone propionate aqueous spray; PNIF, peak nasal inspiratory flow; HDM, house dust mite; VAS, visual analog scale; APES, amino-phosphate-ethylsilane; NGS, normal goat serum; TSA, tyramide signal amplification; AUC, area under the curve.

describe the level of allergic symptoms. Reports about tissue eosinophilia are contradictory, with some studies showing a relationship between numbers of eosinophils and symptoms, while others do not (11–14). The situation is similar for the influx of regulatory T lymphocytes. It has been shown that successful allergen-specific immunotherapy (SIT) is accompanied by a rise in T cells with a regulatory phenotype (15, 16). However, a direct correlation between the number of regulatory T cells and allergic symptoms has not been shown. A possible caveat in many of these approaches to find correspondences between biomarkers and allergic symptoms is that the quantification of mediator levels or cell numbers may not correctly reflect the biological activity of these mediators or cells.

The vast majority of the numerous different immunogenic environmental factors to which the nasal mucosa is constantly exposed do not elicit innate or adaptive defense mechanisms. We hypothesized that this could be explained by local interleukin-10 (IL-10) production establishing a tolerogenic environment. Part of our research interest is therefore the expression profile of the immune suppressive cytokine IL-10 in the nasal mucosa. The first aspect we investigated in this context was the expression of IL-10 in the epithelium of the nasal mucosa (17). We previously showed that in individuals with house dust mite allergy, this expression correlated well with allergic symptoms at baseline, but not with allergic symptoms after allergen provocation. In this study, we extend our observations and show that IL-10 is expressed in the endothelial cells of the blood vessels present in the nasal mucosa of both healthy individuals and allergic patients. After nasal provocation, but not at baseline, we describe a strong, inverse correlation between endothelial IL-10 expression and symptoms of allergic rhinitis. Our data suggest that local expression of IL-10, possibly through its effect on the activity of inflammatory cells or mediators, regulates the extent of the local immune response and its consequences for the severity of allergic symptoms.

Material and methods

Healthy individuals

After informed consent and approval from Medical Ethical Commission (METC) had been obtained, five healthy nonallergic subjects listed for rhinoplasty or septum correction were recruited. The subjects had no history of allergic disease or prior use of nasal medication did not have concurrent respiratory tract infections and had their nonallergic status confirmed with a skin prick test for common aeroallergens (ALKAbello, Nieuwegein, The Netherlands). The screening panel included tree mixture, grass, weed mixture, dust mite (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), cat, and dog. Before surgery, two biopsies were taken from the inferior turbinate with a Gerritsma/Fokkens forceps (Explorent, Tubingen, Germany), embedded in TissueTek (Sakura, Zoeterwoude, the Netherlands) snap frozen in liquid N₂, and stored at –80°C until further use.

Allergic patients

Twenty-one persistent rhinitis patients (median age 42, range 17–63) with positive HDM skin-prick tests or radioallergosorbent tests (RAST), and symptoms for more than 1 year, were included (Table 1). Patients with positive skin-prick test for other allergens besides dust mite were not excluded as long as these sensitizations were not clinically relevant. The medical ethics committee of the Erasmus MD approved the study and all patients gave their written informed consent.

At visit 1, a baseline nasal mucosa biopsy was obtained and baseline symptom scores were recorded. After randomization, 10 patients received fluticasone propionate aqueous spray (FPANS) (200 µg in two actuations per nostril daily for 7 days) and 10 received a placebo (Table 2). Following treatment on day 6, patients reported to the clinic for visit 2, and symptom scores and peak nasal inspiratory flow (PNIF) were measured.

After bilateral nasal provocation using a nasal spray delivering a fixed volume of 0.089 ml of 1000 biological units (BU)/ml HDM (total amount 2 × 89 BU) [ALK-Abellø, Nieuwegein, the Netherlands], symptom scores and PNIF were recorded every 15 min. After 90 min, patients left the clinic and recorded symptoms and PNIF on an hourly basis for up to 8 h at home. After administration of the study drug at home on day 7, patients re-entered the clinic for visit 3. After an assessment of symptom scores and PNIF, and after the application of local anesthesia to the inferior turbinate, a nasal biopsy was obtained. All the visits took place between February and May 2000, outside the Dutch grass pollen season. Neither vehicle provocation nor local anesthesia led to the induction of clinical symptoms. All biopsy specimens of nasal mucosa were taken from the inferior turbinate by the same investigator using the method described above (11, 15, 18). Unfortunately, one patient from the FPANS group was withdrawn from the study for noncompliance with the protocol.

Assessment of symptoms

Visual analog scale (VAS) scores for rhinorrhoea, sneezing, and nasal blockage were recorded by placing a vertical mark on a horizontal 100-mm line. Total VAS was calculated as the sum of the three VAS symptoms scores (total range 0–300) (19).

Peak nasal inspiratory flow

Peak nasal inspiratory flow (in-check inspiratory meter with face-mask; Clement Clarke, Harlow, UK) was used to measure nasal

Table 1. Overview of the inclusion and the exclusion criteria for allergic rhinitis patients

Inclusion	Exclusion
House dust mite allergy	Nasal surgery 6 months prior to the study
Age between 16 and 65 years	Nasal polyps
Non-smoking	Significant septum deviation
Healthy	Immunotherapy for house dust mite
Informed consent	Asthma requiring inhaled corticosteroids
	Pregnancy/lactation
	Systemic corticosteroids 2 months prior to the study
	Intranasal corticosteroids, antihistamines or other anti-allergic medication 1 month prior to the study

Table 2. Characteristics and baseline values of the patients, median (with minimum and maximum values)

	Placebo	FPANS
Characteristics		
Number of patients	10	10
Age	30.5 (17–45)	44.0 (19–62)
Sex (male/female)	5/5	6/4
Wheal size HDM (mm)	5 (4–20)	5 (4–9)
Number of patients sensitized for tree	0	2
Number of patients sensitized for grass	2	5
Number of patients sensitized for cat	3	5
Number of patients sensitized for dog	4	6
Baseline values		
Total VAS (mm)	55 (6–184)	52.5 (13–155)
VAS nasal blockage (mm)	19 (6–74)	25 (0–86)
VAS rhinorrhoea (mm)	17 (0–60)	21.5 (0–54)
VAS sneezing (mm)	13.5 (0–50)	0 (0–51)
Maximal PNIF	137.5 (70–260)	125 (60–210)
Percentage of blood vessels expressing IL-10	53 (17–100)	73 (25–100)
Values after provocation		
AUC total VAS early response	43 (2–141)	17 (0–66)
AUC total VAS late response	1357 (91–3124)	267 (0–707)
AUC VAS rhinorrhoea early response	16 (2–43)	6 (0–25)
AUC VAS rhinorrhoea late response	461 (5–1216)	44 (0–335)
AUC VAS nasal blockage early response	18 (0–78)	6 (0–60)
AUC total nasal blockage late response	568 (30–1579)	94 (0–623)
AUC VAS sneezing early response	12 (0–22)	0 (0–4)
AUC VAS sneezing late response	87 (10–502)	4 (0–62)
Maximal PNIF 15 min after provocation	95 (30–220)	130 (80–210)
Maximal PNIF 30 min after provocation	97.5 (50–230)	142.5 (90–190)
Percentage of blood vessels expressing IL-10	50 (0–100)	42 (0–100)

airflow as described previously (20). After initial instruction and training, the highest value of three repeat measurements for each assessment was used.

Immunohistochemistry

Specimens were snap frozen and stored for immunohistochemistry. Briefly, each tissue specimen was cut into serial, 5- μ m-thick sections on a Micron HM 560 Frigocut and transferred onto microscope slides (Sigma Chemical Co., St Louis, MO, USA) coated with APES (amino-phosphate-ethylsilane), dried and stored at -80°C . For staining, slides were brought up to room temperature, air dried, and fixed in acetone for 10 min at room temperature. The slides were then rinsed in phosphate buffer saline (PBS; pH 7.8) and placed in a semi-automatic strainer [Sequenza; Shandon, Sewickley, PA, USA] and incubated with 10% (v/v) normal goat serum (NGS) [CLB, Amsterdam, The Netherlands] for 10 min. To block endogenous avidine and biotin, antibodies were diluted in 1% (v/v) blocking reagent [Roche, Basel, Switzerland]. The sections were then incubated for 60 min with mouse anti-human monoclonal antibodies directed against CD 34, or appropriate isotype control at room temperature. The sections were then rinsed with PBS for 5 min and incubated for 30 min with a biotinylated goat anti-mouse (1 : 50) immunoglobulin antiserum [Biogenics, Klinipath, Duiven, The Netherlands] for 30 min at room temperature. Subsequently, the sections were rinsed with PBS for 5 min and incubated for 30 min with alkaline-phosphatase conjugated goat anti-biotin. Slides were then rinsed with PBS and Tris buffer (0.2 mol/l, pH 8.5) and incubated for 30 min with New Fuchsin [Chroma, Kongen,

Germany] substrate (containing levamisole to block endogenous AP enzyme activity). Finally, the sections were then rinsed in distilled water, counterstained with Gill's haematoxylin, and mounted in glycerine gelatine [Vectamount, Vector Laboratories, Peterborough, UK].

Staining with anti IL-10 was performed using tyramide signal amplification (TSA). After incubation with biotinylated goat anti-mouse Ig serum, endogenous peroxidase was blocked using 0.2% (w/v) azide, 0.02% (v/v) hydrogen peroxide and 50% (v/v) methanol in PBS. Slides were then incubated with streptavidin conjugated peroxidase [NEN, Boston, MA, USA] for 30 min, biotinyl tyramide in Tris-HCL buffer for 10 min for amplification of the signal, alkaline-phosphatase conjugated goat anti-biotin, and New Fuchsin substrate for 20 min.

Microscopical assessment of histochemical staining

Two samples of each biopsy were coded and scored blind by three independent observers. The total number of blood vessels as well as the number of IL-10 positive blood vessels was counted. The mean number of blood vessels staining positive or negative for IL-10 was used to calculate the percentage of blood vessels staining positively.

Statistical analysis

We used the Mann–Whitney test to assess statistical differences between medians of different groups. Spearman's correlation test was performed to test correlations between the ranks of IL-10 expression and the ranks of symptom scores. The areas under the curve (AUCs) were calculated for the first-hour postprovocation (early phase) and for the remaining 23 h after provocation (late phase). *P*-values < 0.05 were considered statistically significant.

Results

Endothelial cells lining the blood vessels of the nasal mucosa express IL-10

Immunohistochemical staining for IL-10 revealed a distinctive pattern associated with the blood vessels in the lamina propria of the nasal mucosa. In both healthy individuals (Fig. 1A) and allergic rhinitis patients (Fig. 1B), there were morphological indications that the endothelium of some, but not all, blood vessels stained positive for IL-10. To confirm the identity of the endothelium and to see what percentage of blood vessels expressed IL-10, we checked for co-localization of IL-10 (Fig. 1B) and CD34 (Fig. 1C), a surface marker present on the endothelium of blood vessels (21, 22). In all individuals, a portion of blood vessels stained positive for IL-10.

Endothelial IL-10 scores correlate with allergic symptoms after allergen provocation

Expression of IL-10 was highly variable in the endothelium of the blood vessels (Fig. 2). We therefore investigated a potential correlation between these endothelial IL-10 levels and the levels of allergic symptoms. Prior to allergen provocation, however, none of the allergic

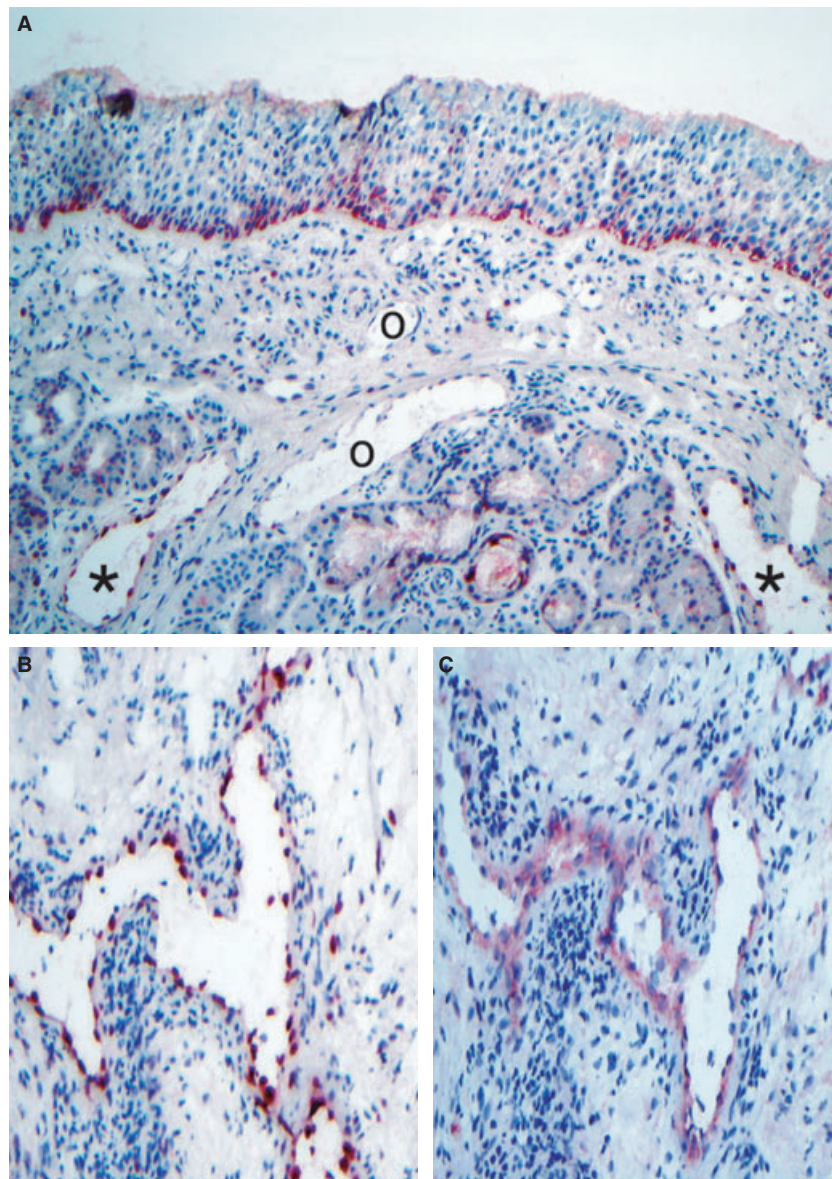


Figure 1. (A) Expression of IL-10 in blood vessels in the lamina propria in nasal biopsies of a healthy individual. All endothelial cells in positive blood vessels express IL-10. However, not all the blood vessels present in the lamina propria stained positive for IL-10. (*IL-10 positive blood vessel, °IL-10 negative blood vessel). (B) Close up of a blood vessel with endothelial IL-10 expression. Nasal biopsy of an allergic patient. (C) Same biopsy, consecutive slide. Anti-CD34 staining.

symptoms [Total VAS ($r = 0.125$, $P = 0.600$), VAS rhinorrhoea ($r = 0.334$, $P = 0.150$), VAS nasal blockage ($r = 0.302$, $P = 0.195$), VAS sneezing ($r = -0.060$, $P = 0.802$), and PNIF scores ($r = -0.202$, $P = 0.392$)] were linked to the endothelial expression level of IL-10.

After allergen provocation, this picture changes completely. There is a very high correspondence between the level of IL-10 expression in the endothelium and the extent of allergic symptoms in the placebo group (Table 3). This correlation was observed for nearly all allergic symptoms and in both the early and the late phase response. Not only the total VAS score (Figs 3A and 3B),

but also the individual VAS scores for rhinorrhoea (Figs 3C and 3D), nasal blockage (Figs 3E and 3F), and the late phase VAS score for sneezing (Fig. 3H) were closely related to endothelial IL-10. The early phase VAS score for sneezing was the only score to differ from the other parameters and was not correlated to the percentage of blood vessels expressing IL-10 (Fig. 3G).

Interestingly, all the measures for clinical symptoms after allergen challenge were inversely related to the percentage of blood vessels expressing IL-10 (Table 3). A high percentage of blood vessels expressing IL-10 corresponds to a relatively low level of symptoms after allergen

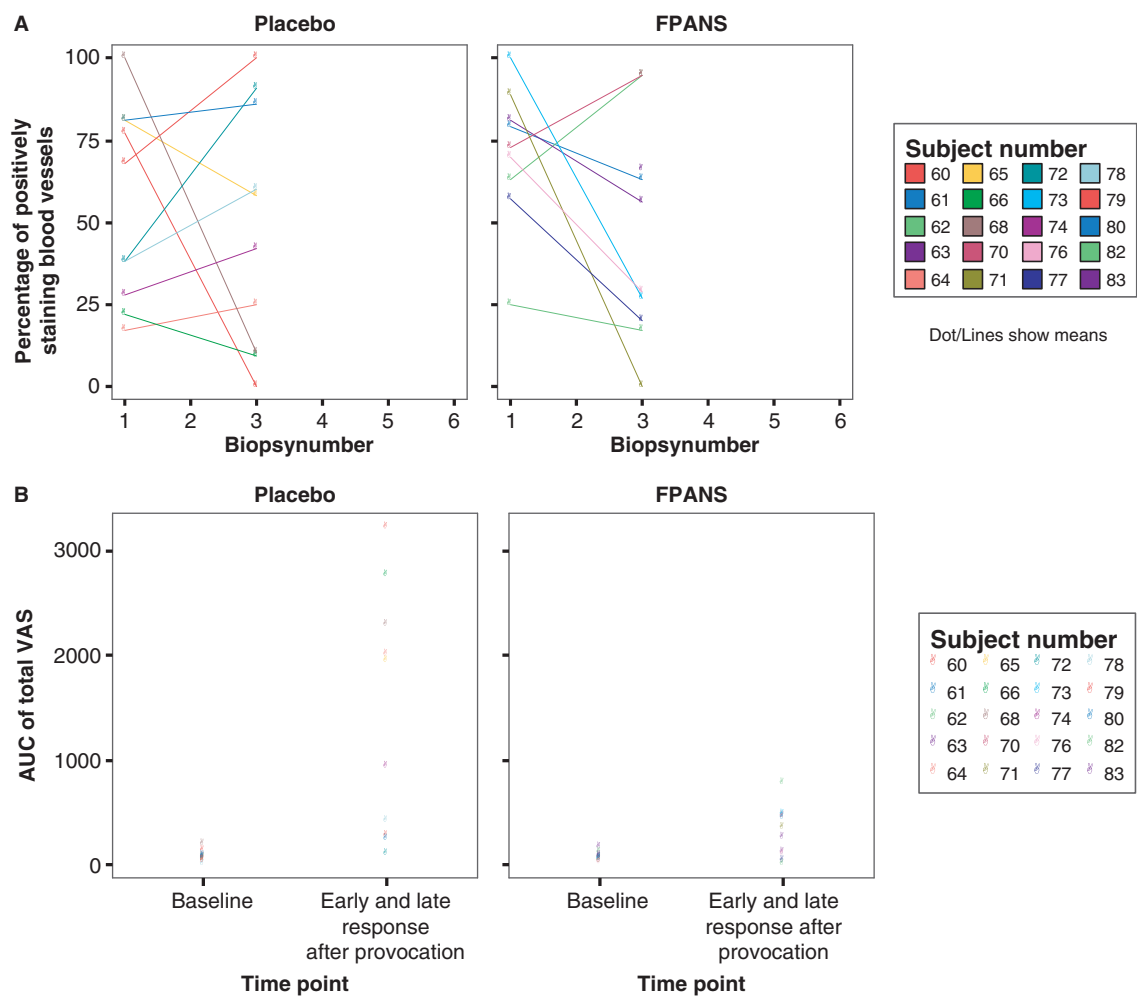


Figure 2. (A) Each patient's endothelial IL-10 expression before and after provocation, with the values connected by lines for individual patients. Each color symbolizes a different patient. Although endothelial IL-10 scores vary between individuals both before and after provocation, mean endothelial IL-10 scores remain unchanged after provocation (placebo group Wilcoxon Signed-Rank Test $P = 0.959$, FPANS group Wilcoxon Signed-Rank Test $P = 0.086$). (B) AUC of total VAS at baseline and after provocation. Colors symbolize patients as in Fig. 2A. Note that the patients with a distinct drop in the percentage of IL-10 positive blood vessels are the patients with the highest symptoms after provocation (patient numbers 60, 66, 68, 65).

challenge and, vice-versa, a low percentage of blood vessels expressing IL-10 corresponds to a relatively high level of symptoms after allergen challenge.

In subjects treated with FPANS, endothelial IL-10 scores do not correlate with allergic symptoms after allergen provocation

Given the results described above, we wondered whether how postprovocation endothelial IL-10 scores would relate to symptom scores in the actively treated patient group. In the group treated with FPANS, as with the placebo group, none of the baseline VAS scores correlated with endothelial IL-10 scores. By contrast with the placebo group, symptoms and IL-10 scores did not correlate after allergen provocation (Table 3). The correspondence between the level of expression of IL-10 in the

endothelium and the severity of allergic symptoms witnessed in the placebo group totally disappeared in the group treated with steroids.

Discussion

Considering the role attributed to the cytokine IL-10 as immune regulator, IL-10 expression in the endothelium of the lamina propria could be very important for the activity of resident cells or for the activity of the inflammatory cells that enter the mucosal tissue from the blood stream. This might be reflected by the high level of correspondence shown here (up to 90%) between allergic symptoms and the level of IL-10 in the endothelium.

Table 3. Correlation between allergic symptoms after allergen challenge and percentage of blood vessels expressing IL-10. Overview of correlation coefficient (r), the percentage of variation in complaints that can be explained by the variation in IL-10 levels (VAR = 100 times r^2), and the significance level (P)

Symptoms after allergen challenge	Placebo			FPANS		
	r	VAR	P	r	VAR	P
VAS total						
Early response	-0.770	59%	0.009	-0.225	6%	0.532
Late response	-0.952	91%	0.000	-0.298	9%	0.403
VAS rhinorrhoea						
Early response	-0.709	50%	0.022	0.210	4%	0.560
Late response	-0.806	65%	0.005	0.314	10%	0.377
VAS nasal blockage						
Early response	-0.709	50%	0.022	0.210	4%	0.560
Late response	-0.806	65%	0.005	0.314	10%	0.377
VAS sneezing						
Early response	-0.276	8%	0.382	-0.188	4%	0.602
Late response	-0.733	54%	0.016	0.066	0%	0.986

The first point we need to make is that this correspondence is complementary to the correspondence we observed previously between the level of IL-10 in the epithelium and clinical symptoms. In both cases, high levels of IL-10 are linked to low levels of clinical symptoms. However, for the epithelium, this holds true only *before* allergen provocation; and for the endothelium, it holds true only *after* allergen provocation. The reason is for this difference and whether both regulatory mechanisms affect the same downstream targets is not entirely clear. One could speculate that epithelial expression would influence only those cells that are already in the nasal mucosa affecting the steady state. And, subsequently that endothelial IL-10 expression only affects those cells that enter the tissue during the inflammatory response. However, as it is generally accepted that the early phase response is the consequence of a direct release, mediated by IgE cross-linking, of mediators by resident mast cells (1), it is not clear in this concept how the early phase response could be influenced by endothelial IL-10. A related issue is that baseline symptoms in patients with house dust mite allergy are thought to result from constant, but low, exposure to the allergen. This would mean that even baseline symptoms are allergen-induced, but the relationship between symptoms and endothelial IL-10 levels were only evident after our experimental allergen provocation. The absence of correspondence between IL-10 levels and symptoms prior to provocation cannot be a consequence of the relatively small variation in symptoms, as these same variations are sufficient to establish a relationship with epithelial IL-10 expression.

The second point we want to make is that high levels of IL-10 seem to protect against the development of allergic symptoms. We think it is unlikely that the opposite (few allergic symptoms causing high levels of IL-10) is true as high symptom scores after provocation are not associated with a rise in endothelial IL-10 levels. In the placebo

group, endothelial IL-10 levels do not change after allergen provocation (Fig. 2A). This suggests that allergic symptoms and endothelial IL-10 levels are not regulated through a common mechanism. More specifically, the unchanged level of endothelial IL-10 after provocation in the endothelium suggests a pre-existing situation that dictates the level of symptoms an allergic individual will develop after allergen provocation.

This hypothesis raises the question of a possible mechanism. As mentioned previously (17), a mechanism in which IL-10 influences allergic symptoms via histamine is conceivable. The release of histamine by activated mast cells can be inhibited by IL-10 (23). A direct effect on cells exposed to the endothelial cells expressing IL-10 could also play a role. All inflammatory cells that play a role in the allergic cascade are known to carry the IL-10 receptor and their activity can be down-regulated by this cytokine (24). Most of these cells enter the inflamed tissue via the blood vessels, and this makes the location of the cells expressing IL-10 even more interesting. The blood vessels could function as a local checkpoint, a point where cells enter the mucosa, and where they can be adjusted or regulated upon entry, just before they start to exert their inflammatory role in the tissue.

Alternatively, some of the effects seen on the allergic symptoms could be directly related to the blood vessels. Rhinorrhoea and nasal blockage are both influenced by either increased permeability or swelling of the blood vessels (25). However, we would need to investigate how IL-10 could affect permeability or swelling.

As described by our group in a previous publication (11), local steroid treatment reduces symptoms in this provocation model. Both in the early and late phases, we saw a reduction of rhinorrhoea, nasal blockage, and sneezing compared to placebo. Moreover, treatment with steroids suppresses the relationship between IL-10 expression and allergic symptoms, while there is no difference between the percentages of IL-10-positive blood vessels after provocation in groups treated with FPANS and the placebo. Given these observations, steroid treatment does not effect symptoms via endothelial IL-10 levels, although both steroids and IL-10 may have the same downstream target.

In conclusion, we have not found, either in our own previous work or in the literature, a parameter that correlates this strongly with clinical symptoms. If we assume a causal relationship, then up to 90% of the variation in allergic symptoms can be explained by the variation in the level of expression of IL-10 in the endothelium. At present, we have only two snapshots of IL-10 expression in the endothelium, directly before provocation and 24 h after provocation. This could conceal possible transient effects of allergen provocation on endothelial IL-10 expression. Transient responses of this kind could be either a direct effect of the allergen on blood vessels, or an indirect consequence because of allergen-induced mediator release by the local nasal tissue.

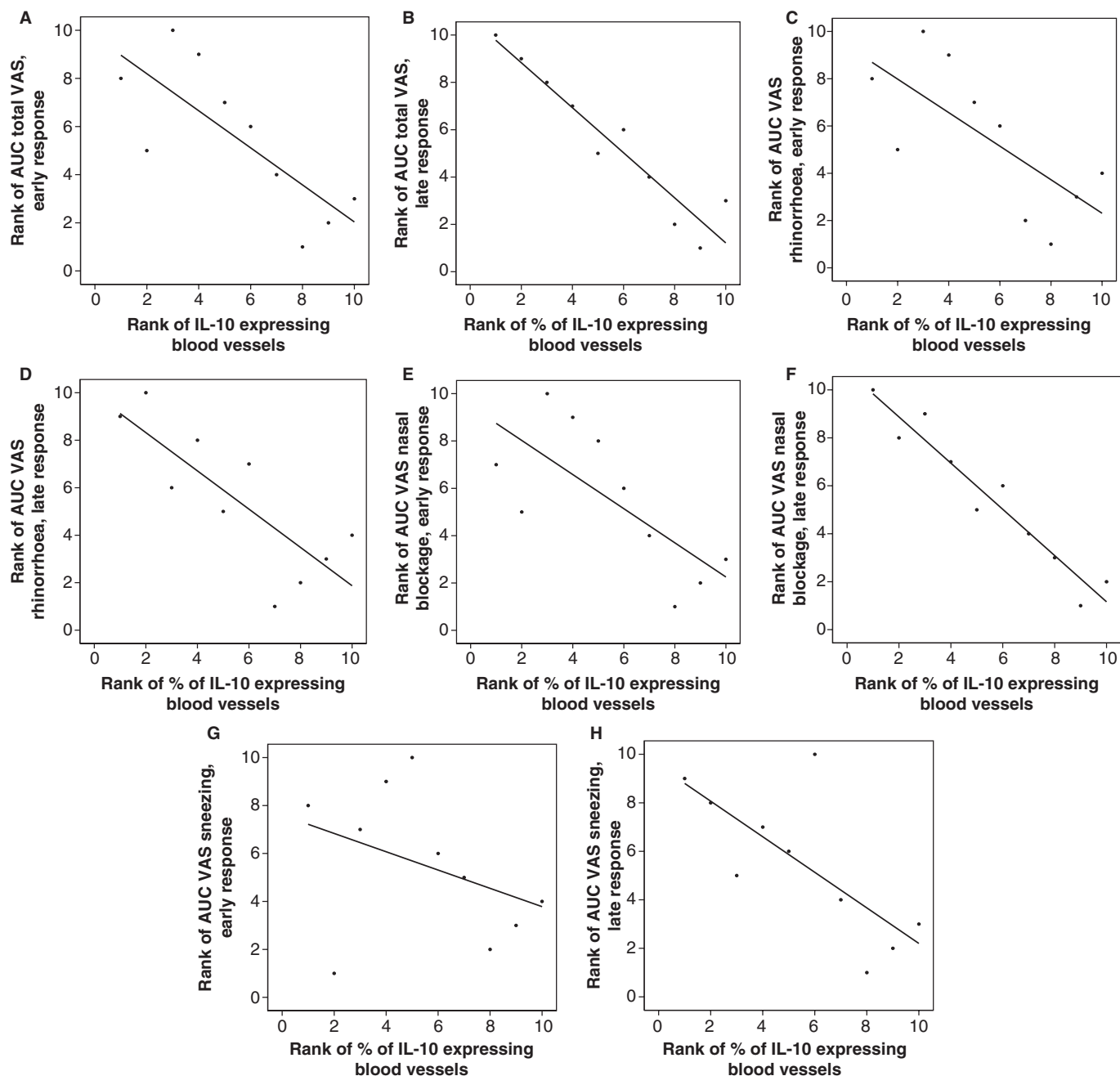


Figure 3. Endothelial IL-10 scores correlate with allergic symptoms after allergen provocation. Rank correlation plots for percentage of blood vessels expressing IL-10 with AUC of allergic symptoms after provocation for (A) total VAS early response, (B) total VAS late response, (C) VAS rhinorrhoea early response, (D) VAS rhinorrhoea late response, (E) VAS nasal blockage early response, (F) VAS nasal blockage late response, (G) VAS sneezing early response, (H) VAS sneezing late response.

Even if expression levels of IL-10 do not change within a single individual (after provocation), then it would be interesting to investigate why expression levels differ between individuals and why only a percentage of blood vessels stain positively for IL-10. Similarly, why do expression levels between healthy individuals vary? Given these observations, it seems unlikely that endothelial IL-10

expression is designed specifically for the reduction of allergic symptoms. Our data point toward a more general role of endothelial IL-10 expression in immune regulation, and this raises some interesting questions. Would individuals with lower levels of IL-10 be more prone to develop immunological disorders or would they present with more severe symptoms during a respiratory tract infection?

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