See related Commentary on page viii

Intravenous Anti-IL-5 Monoclonal Antibody Reduces Eosinophils and Tenascin Deposition in Allergen-Challenged Human Atopic Skin

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Anti-IL-5 monoclonal antibody (mepolizumab) reduces baseline bronchial mucosal eosinophils and deposition of extracellular matrix proteins in the reticular basement membrane in mild asthma. Here we report the effect of anti-IL-5, in the same patients, on allergen-induced eosinophil accumulation, tenascin deposition (as a marker of repair and remodelling) and the magnitude of the late-phase allergic cutaneous reaction. Skin biopsies were performed in 24 atopic subjects at allergen- and diluent-injected sites before 6 and 48 h after, three infusions of a humanized, monoclonal antibody against IL-5 (mepolizumab) using a randomized double-blind, placebo-controlled design. Anti-IL-5 significantly inhibited eosinophil infiltration in 6 h and 48 h skin biopsies as well as the numbers of tenascin immunoreactive cells at 48 h. In contrast, anti-IL-5 had no significant effect on the size of the 6 or 48 h late-phase cutaneous allergic reaction. This study (a) suggests that eosinophils are unlikely to cause the redness, swelling, and induration characteristic of the peak (6 h) late-phase cutaneous allergic reaction and (b) shows that decreases in tenascin positive cells at 48 h correlates with reduction of eosinophils, so providing further evidence of involvement in remodelling processes associated with allergic inflammation.

Key words: anti-IL-5/eosinophil/late-phase reaction/skin/tenascin
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In addition to their role as pro-inflammatory cells in allergy, asthma eosinophils are also involved in certain repair and fibrotic processes consequent to allergic inflammation. For example, in the skin of atopic subjects, eosinophil-derived fibrogenic factors (transforming growth factor-β (TGF-β1) and interleukin (IL)-13) were temporally associated with fibroblast-associated tenascin and procollagen-I immunoreactivity, and the formation of α-smooth muscle (SM) actin + myofibroblasts, following local allergen challenge (Phipps *et al*, 2002). In addition the specific reduction of eosinophils in asthmatic airways at baseline by intravenous infusions of an anti-IL-5 monoclonal antibody decreased the deposition of the extracellular matrix (ECM) proteins, tenascin, lumican, and procollagen III, within the reticular basement membrane (Flood-Page *et al*, 2003b).

IL-5 is essential for the terminal differentiation of the committed eosinophil precursor (Sanderson, 1992). It is also involved in eosinophil migration and priming (Sehmi *et al*, 1992) and prolongs the survival of the cell in tissues (Rothenberg *et al*, 1989). More recently monoclonal antibodies against IL-5 have been prepared and administered as a single intravenous infusion to both mild atopic (Leckie *et al*, 2000), as well as chronic, severe asthmatics (Kips *et al*, 2003). These have produced no appreciable effects on either the late asthmatic reaction, airway hyperresponsiveness or other clinical outcomes including lung function. However, although anti-IL-5 almost totally ablated eosinophils in the blood and sputum (Leckie *et al*, 2000), tissue eosinophils were reduced rather than depleted (Flood-Page *et al*, 2003a), possibly as a result of downregulated IL-5Rα expression of airway eosinophils (Liu *et al*, 2002; Gregory *et al*, 2003).

In this study, we have measured eosinophils, tenascin, and the size of the late-phase reaction (LPR) in allergen-challenged skin sites before and after anti-IL-5. The cutaneous LPR, elicited in atopic subjects, is characterized by an edematous, red, and slightly indurated swelling that peaks 6–9 h after intradermal allergen challenge. The LPR is often considered as a model of allergic inflammation since it is associated with local infiltration by various cell types including eosinophils, basophils, and neutrophils. The eosinophil in particular has been considered as an important effector cell in producing the macroscopic appearance of the LPR possibly through the release of lipid mediators such as cysteinyl leukotrienes (Reshef *et al*, 1989; Zweiman *et al*, 1991; Wardlaw *et al*, 1995). Although increases in the size of the LPR accompanies increases in the numbers of eosinophils up to 6 h after allergen injection, thereafter the cell persists in tissues whereas the LPR declines fairly rapidly (Phipps *et al*, 2002). For this reason we were interested to study the effect of reduction or depletion of eosinophils, by anti-IL-5, on the magnitude of the allergen-induced LPR.

Abbreviations: APAAP, alkaline phosphatase anti-alkaline phosphatase; ECM, extracellular matrix; FEV_1, forced expiratory volume in 1 s; IL, interleukin; LPR, late-phase reaction; MBP, major basic protein; TGF-β, transforming growth factor-β

In this study, we performed skin biopsies 6 and 48 h after cutaneous allergen challenge (from the same patients described as previously by Flood-Page *et al*, 2003b) before and after the infusion of an anti-IL-5 monoclonal antibody. We chose the 6 h time point as optimal for studying the effect of eosinophil depletion on the LPR since in time-course studies up to 72 h this was shown to be the peak of the characteristic redness and swelling (Phipps *et al*, 2002). A 48 h biopsy time point was chosen to enable us to determine whether this procedure affected the later expression of a marker of repair (as previously shown for tenascin) (Phipps *et al*, 2002).

Results

Anti-IL-5 reduces eosinophil infiltration but does not effect the size of the cutaneous LPR Figure 1 shows the skin Congo red + eosinophil counts of biopsies taken from the diluent- and allergen-injected sites in subjects receiving anti-IL-5 or placebo. At 6 h after intradermal allergen challenge there was no significant difference (pre *vs* post) in the numbers of skin eosinophils in subjects receiving placebo; however, the counts were significantly less (pre *vs* post, $p = 0.002$) in those receiving intravenous anti-IL-5 monoclonal antibody, with a between-group difference of $p = 0.0015$. This represented a median change in eosinophil numbers at 6 h of −83% for anti-IL-5 and +33% for placebo. At 48 h after intradermal allergen challenge, there was no significant difference (pre *vs* post) in the numbers of skin eosinophils in the placebo group but the counts were significantly less (pre *vs* post, $p = 0.003$) in those receiving anti-IL-5. The between-group difference at 48 h was $p = 0.0025$. The mean eosinophil counts of all the diluent-injected sites was less than 2 cells per mm^2. Similar results were obtained with anti-MBP with a between-group difference at 6 h (anti-IL-5 *vs* placebo) of $p = 0.01$ (data not shown). The number of degranulating cells was also counted (Fig 2). At 6 h after intradermal allergen challenge there was no significant difference (pre *vs* post) in the numbers of degranulating eosinophils in subjects receiving placebo; however, the counts were significantly less (pre *vs* post, $p = 0.001$) in those receiving intravenous anti-IL-5 monoclonal antibody, with a between-group difference of $p = 0.029$. At 48 h after intradermal allergen challenge, there was no significant difference (pre *vs* post) in the numbers of degranulating skin eosinophils in the placebo group but the counts were significantly less (pre *vs* post, $p = 0.0117$) in those receiving anti-IL-5.

As previously shown (Ying *et al*, 1999), there were also significant allergen-induced increases in BB1 + basophils, elastase + neutrophils and CD4 + T cells at both time points but their numbers were unaffected by infusions of anti-IL-5 (data not shown).

In contrast, administration of anti-IL-5 (or placebo) had no significant effect on the 6 h late-phase cutaneous reaction (Fig 3). The magnitude of the 6 h LPR was similar to that previously observed (Frew and Kay, 1988; Ying *et al*, 1999; Phipps *et al*, 2002). In the majority of subjects, in both the active and placebo group, the 48 h LPR had, with the exception of a few individuals, largely resolved. But in five individuals who had a small waning 48 h reaction, anti-IL-5 appeared to have a small, but NS, inhibitory effect (individual data not shown).

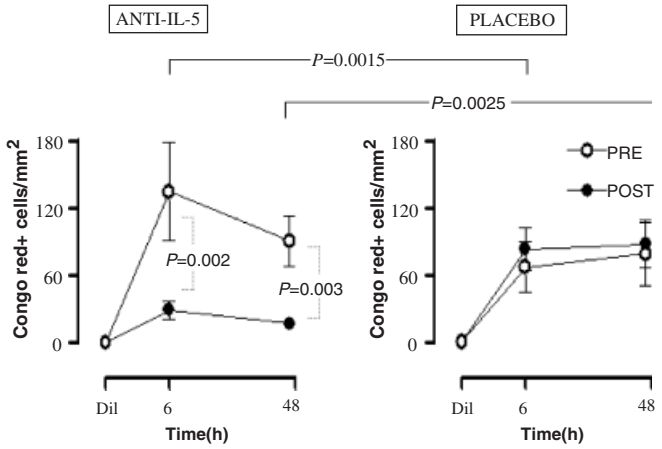

Figure 1
Effect of anti-IL-5 mAb on allergen-induced eosinophil infiltration. Eosinophil numbers are expressed as the number of Congo red + cells (mean ± SEM) per square millimeter of skin biopsy ($n = 11$–13). There was no significant between-group difference (anti-IL-5 *vs* placebo) in the allergen-induced increases in eosinophils before treatment at either 6 or 48 h ($P = 0.1858$ and 0.31, respectively). The differences between pre- and post-treatment (active or placebo) were analyzed by Wilcoxon signed-rank test. The Mann–Whitney *U* test was used for intergroup comparison. IL, interleukin; mAb, monoclonal antibody.

Anti-IL-5 deplete tenascin-positive cells in 48 h biopsies Figure 4 shows the numbers of tenascin + fibroblast-like cells from biopsies taken from diluent-and allergen-injected sites pre- and post-treatment. Tenascin + cells were predominantly located in the lower dermis and were identified morphologically as fibroblasts, appearing fusiformic in shape with elongated nuclei. There were negligible

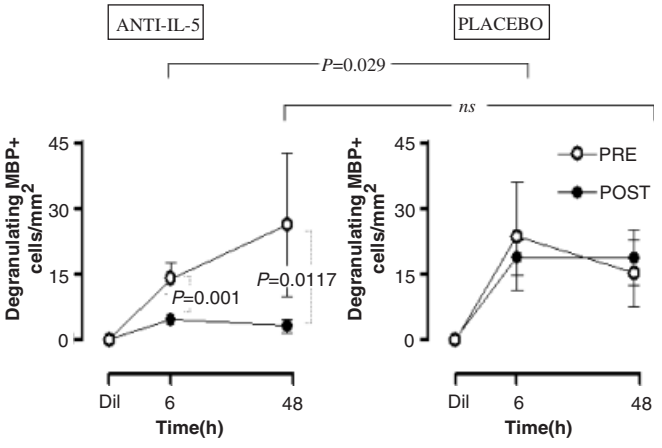

Figure 2
Effect of anti-IL-5 mAb on the numbers of MBP + degranulating eosinophils after allergen-challenge. Degranulating MBP + eosinophil numbers are expressed as the number of cells (mean ± SEM) per square millimeter of skin biopsy ($n = 11$–13). The differences between pre- and post-treatment (active or placebo) were analyzed by Wilcoxon signed-rank test. The Mann–Whitney *U* test was used for intergroup comparison. IL, interleukin; mAb, monoclonal antibody; MBP, major basic protein.

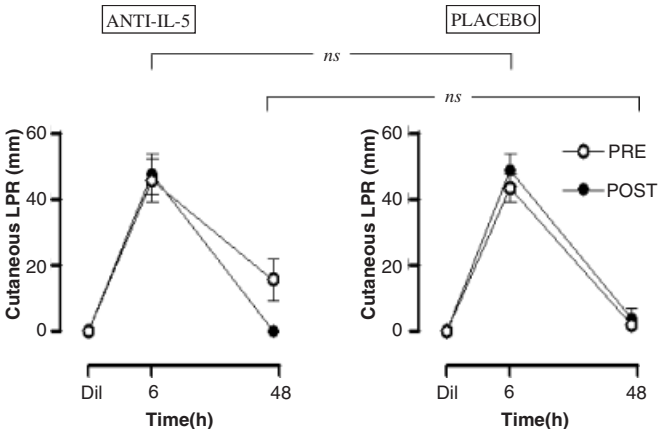

Figure 3
Effect of anti-IL-5 mAb on the size of the allergen-induced cutaneous LPR. Data represent mean ± SEM; n = 11–13. The differences between pre- and post-treatment (active or placebo) were analyzed by Wilcoxon signed-rank test. The Mann–Whitney *U* test was used for intergroup comparison. IL, interleukin; mAb, monoclonal antibody; LPR, late-phase reaction.

numbers of tenascin + cells (mean <1 cell per mm^2) at diluent-injected sites. At 6 h after intradermal allergen challenge there was no significant difference (pre-placebo *vs* post-placebo, or pre-anti-IL-5 *vs* post-anti-IL-5) in the numbers of fibroblast-like tenascin + cells. The between-group difference at 6 h was also NS. At 48 h, however, the counts were significantly less in those receiving intravenous anti-IL-5 monoclonal antibody (pre *vs* post, $p = 0.003$), but not in those receiving placebo. The between-group difference at 48 h was also significant ($p = 0.0256$). There was also a highly significant correlation between the Δ change in eosinophils and the Δ change in tenascin + cells in those receiving anti-IL-5 ($p = 0.0005$; Fig 5). Examples of Congo red + eosinophils and immunostaining for tenascin + cells, pre- and post-anti-IL-5, are shown in Fig 6.

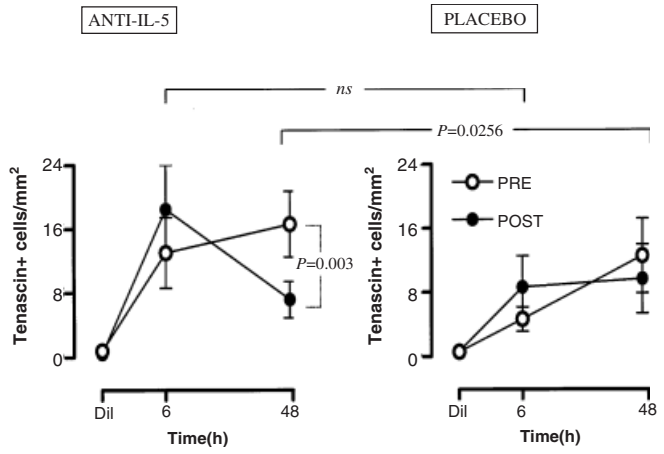

Figure 4
Effect of anti-IL-5 mAb on allergen-induced tenascin expression. The results are expressed as the number of tenascin + cells (mean ± SEM) per square millimeter of skin biopsy (n = 11–13). There were no significant between-group differences (anti-IL-5 *vs* placebo) in the allergen-induced increases in tenascin + cells before treatment at either 6 or 48 h ($P = 0.3402$ and 0.1315, respectively). The differences between pre- and post-treatment (active or placebo) were analyzed by Wilcoxon signed-rank test. The Mann–Whitney *U* test was used for intergroup comparison. IL, interleukin; mAb, monoclonal antibody.

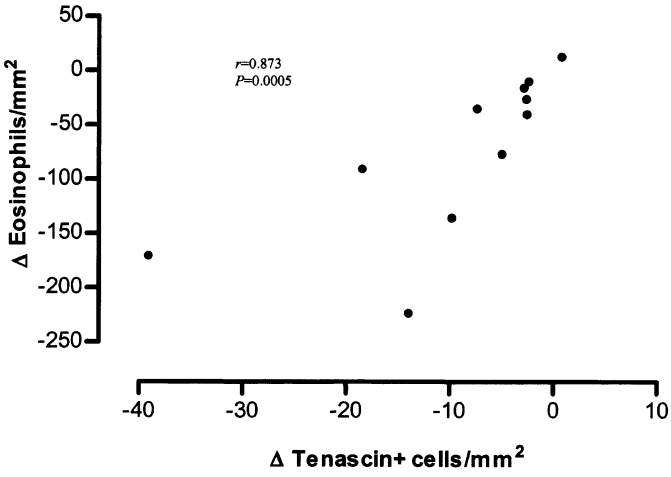

Figure 5
Correlation between the change in tissue eosinophils and change in tenascin + cells after allergen challenge. The results are expressed as the Δ change in the number of eosinophils and the Δ change in the number of tenascin + cells (mean ± SEM) per square millimeter of skin biopsy (n = 11), following active treatment at the 48 h time point. Correlations were obtained by Spearman's method with correction for tied values.

Discussion

This study shows that selective reduction of tissue eosinophils (as opposed to blood and sputum eosinophils) does not significantly affect the magnitude of the allergen-induced LPR. Although eosinophil accumulation was not completely abrogated by anti-IL-5, these data suggest, nevertheless, that eosinophils are unlikely to be essential for the development of the LPR at its peak (i.e. 6 h). It was previously shown that administration of anti-IL-5 had no effect on the late asthmatic reaction or airway hyperresponsiveness (Leckie *et al*, 2000) although in that report it was not ascertained whether the intervention actually depleted eosinophils in the relevant tissue, i.e. the bronchial mucosa. Indeed, it appears that whereas on the one hand anti-IL-5 will prevent the allergen-induced increase in eosinophils in the skin of (otherwise normal) atopics (Fig 1) (where there are virtually no eosinophils at baseline), this treatment, even when administered on several occasions, over several weeks, had only a partial effect (median depletion of 55%) on baseline eosinophils in the bronchial mucosa (Flood-Page *et al*, 2003a), which in asthmatics is already mildly inflamed and contains appreciable numbers of eosinophils even when "unprovoked" (Azzawi *et al*, 1990).

The precise mechanisms involved in the redness, swelling, and slight induration that characterizes the peak (6–9 h) late-phase skin response remains unclear. Although this study indicates that eosinophils do not appear to be essential, other cell types such as the basophil may be involved. Basophils are a rich source of mediators including histamine and cysteinyl leukotrienes (Church *et al*, 1997; Macfarlane *et al*, 2000) and respond to allergen via IgE bound to FcεR1. Furthermore, the peak of the late-phase response is usually between 6 and 24 h, at which time basophil numbers are maximal at skin sites after allergen challenge (Ying *et al*, 1999). Although IL-5 is also believed to act as a terminal basophil differentiation factor (Denburg

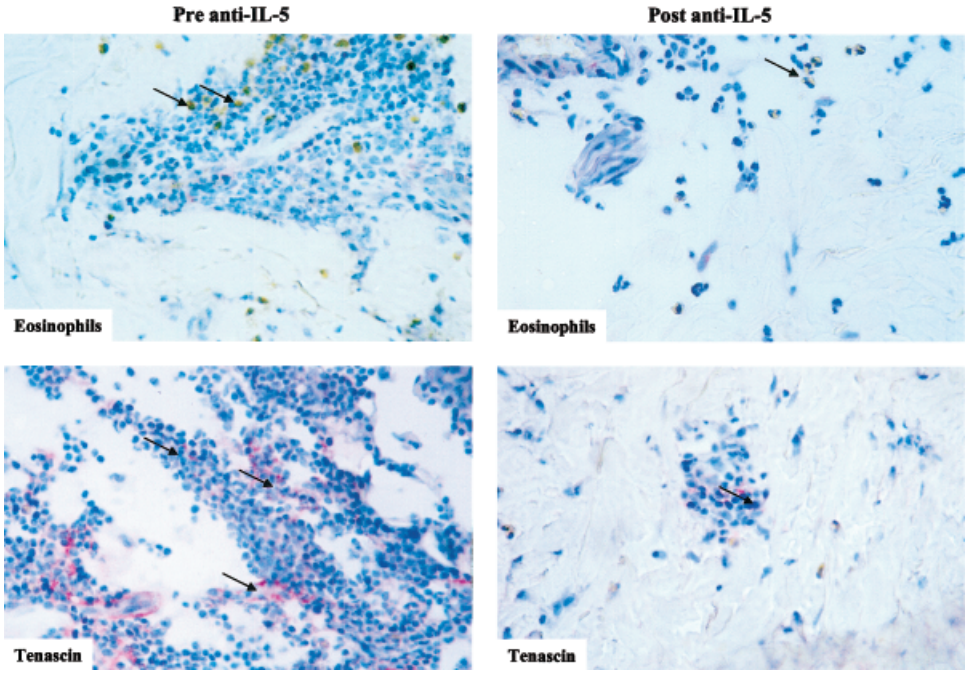

Figure 6
Effect of anti-IL-5 mAb on tissue eosinophils and tenascin + cells Examples of Congo red + eosinophils and immunostaining for tenascin + cells, pre- and post-anti-IL-5 (magnification × 200).

et al, 1991), however, anti-IL-5 did not appear to affect the numbers of infiltrating BB1 + basophils as identified either in 6 or 48 h skin biopsies (data not shown).

We were able to study the effects of anti-IL-5 on the LPR at both the 6 and 48 h time points. It was previously shown that the cutaneous LPR plateaus at 6–9 h whereas the peak of eosinophils is variable between 6 and 24 h (Phipps *et al*, 2002). Thus the 6 h time point was optimal for studying the relationship between eosinophils and the LPR, and as previously shown, tenascin + cells peaked at 48 h (Phipps *et al*, 2002). The findings presented here are in agreement with our previous observations of a dissociation between eosinophil numbers and the size of the cutaneous reaction (Phipps *et al*, 2002). Thus, whereas increases in eosinophil numbers and the size of the LPR increased in parallel at the 1, 3, 6, and 24 h time points, thereafter eosinophils persist in the tissue whereas the LPR rapidly resolves. In previous studies, we showed that tissue eosinophils persisted for up to 7 d but that the LPR was usually absent at 72 h (Ying *et al*, 1999). Although the 6 h LPR was not affected by anti-IL-5, five of the 11 individuals who had a small positive 48 h LPR before anti-IL-5 treatment showed a reduction in their LPR after treatment (Fig 3). Therefore, we cannot totally exclude a role for the eosinophil at later time points, however, classically the LPR refers to events observable between 6 and 9 h.

A previous publication showed that eosinophil granule protein deposition was prominent in the cutaneous LPR in atopics, peaking between 4 and 8 h and persisting for 48 h (Leiferman *et al*, 1990). Our study largely confirms these findings. Degranulating MBP + cells were observed in almost equal numbers at 6 and 48 h in the placebo group (pre and post) (Fig 2). After anti-IL-5 there were very few degranulating cells at either time point. Furthermore, the LPR is transient and wanes after 6–9 h (Phipps *et al*, 2002) whereas, as stated, eosinophils and eosinophil products persist for many hours and days. This makes it unlikely that degranulating eosinophil products were contributing to the 6 h LPR although in some subjects there may be a small eosinophil component to the waning 48 h response. It is also noteworthy that, although eosinophil-derived basic proteins are toxic for various cell types, including epithelial cells, there is no evidence that they cause the vasodilation and edema which characterizes the late-phase skin response.

Anti-IL-5 had a slightly more marked effect on the number of Congo red + eosinophils than MBP + cells. But basophils also contain small amounts of MBP (Ackerman *et al*, 1983).

Our second novel finding was the demonstration that reduction of the allergen-induced eosinophil response abrogated the 48 h increase in tenascin + fibroblast-like cells (Fig 4). Furthermore, there was a correlation between the decrease in the numbers of eosinophils and the decrease in the numbers of tenascin + cells (Fig 5) and so supports the view that upregulation of tenascin expression by fibroblast-like cells following intradermal allergen challenge is partly under the control of infiltrating eosinophils presumably through the release of fibrogenic factors such as TGF-β. This is in keeping with our recent study showing, in these same patients, decreased deposition of the ECM proteins, tenascin, lumican, and to a lesser extent, procollagen III, within the reticular basement membrane, together with decreased numbers of TGF-β + eosinophils in the bronchial mucosa, after anti-IL-5 treatment (Flood-Page *et al*, 2003b). Due to limitations of available tissue, we were unable to confirm that the numbers of TGF-β + eosinophils were similarly decreased in the skin. Although, in our previous study in the skin (Phipps *et al*, 2002) we had shown that allergen induces the upregulation of other markers of remodelling (i.e. α-SM actin + myofibroblasts and procollagen-I + cells), we were only able to show, in this study, an effect of anti-IL-5 on tenascin + cells. There are likely to be many other sources of TGF-β and other fibrogenic growth

factors (such as macrophages, T cells and neutrophils), however, which would not be affected by the infusions of anti-IL-5. Thus, the precise contribution of eosinophils (as compared to other cell types) to repair and remodelling processes must await further investigation.

Tenascin is a highly regulated member of the matricellular family that is expressed during development, growth, and in response to injury (Erickson and Bourdon 1989; Ruegg *et al*, 1989). In asthmatic airways the balance of the ECM proteins is altered and the deposition of tenascin, together with several other ECM proteins, is increased (Laitinen *et al*, 1997). The ECM not only forms a network of molecules that support the airways, it is also a dynamic network that has the capacity to influence cellular function. Tenascin has been demonstrated to act as a permissive substance that can prevent or allow cell migration (Treasurywala and Berens, 1998) and we have previously shown a significant upregulation of tenascin expression within fibroblast-like cells, at the vascular SM basement membrane and in and around bundles of SM in response to intradermal allergen challenge (Phipps *et al*, 2002). It is possible that tenascin, in conjunction with other proteins, facilitates cellular migration through the interstitial matrix towards sites of tissue injury and thus may play a role in remodelling.

The late-phase skin reaction is often regarded as a model of atopic dermatitis because in both situations the histopathological picture is of an eosinophilic cell mediated hypersensitivity reaction. In atopic dermatitis, however, there is relatively little deposition of collagen and ECM proteins. Thus a single allergen challenge in otherwise healthy normal skin is unlikely to lead to marked remodelling (Ying *et al*, 1999; Phipps *et al*, 2002). Nevertheless, it is associated with type I collagen deposition and expression of the pro-fibrotic cytokines IL-11 and IL-17 (Toda *et al*, 2003) and, as such, the late-phase skin reaction could serve as a general model for events associated with the laying down of collagen and other ECM proteins, not only in skin disease but also in asthma. In the airways of asthmatics, for instance, cells of the epithelial–mesenchymal trophic unit are now recognized as active participants in the inflammatory process (Holgate *et al*, 2000). Although this remodelled phenotype is generally believed to be the result of chronic inflammation, our recent findings in the skin (Phipps *et al*, 2002) and those of Gizycki *et al* (1997) in the airways suggest that tissue remodelling is an acute/subacute process, resulting from allergen-induced interactions between eosinophils and other inflammatory cells with mesenchymal cells.

In a mouse model of atopic asthma, Blyth *et al* (2000) demonstrated a reduction in the development of subepithelial reticular basement membrane thickness by treatment with anti-IL-5 at the time of allergen challenge. In agreement with the reported effects of anti-IL-5 on subepithelial basement membrane thickening in the airways (Flood-Page *et al*, 2003b), we demonstrated that the selective depletion of eosinophils in response to intradermal challenge led to a significant reduction in the numbers of tenascin + cells at 48 h. Although in a hamster model of incisional wound healing depletion of eosinophils with anti-IL-5 accelerated the rate of wound closure by re-epithelialization (Yang *et al*, 1997), this study may simply reflect the pleiotropic activity of TGF-β on different components of the healing response. Smad-3 (a downstream signal transducer for TGF-β) heterozygous mice also demonstrate accelerated re-epithelialization compared with wild-type mice (Shipley *et al*, 1986; Ashcroft *et al*, 1999), an effect mediated by removal of the inhibitory action of TGF-β on keratinocyte chemotaxis and proliferation (Sehmi *et al*, 1992). Although together these studies suggest that eosinophils, in response to injury, signal to and activate epithelial and mesenchymal cells, it remains to be determined whether eosinophil-induced tissue remodelling is beneficial or detrimental to the host.

The observed effect of anti-IL-5 on tenascin may have been via a direct effect on fibroblasts, or other mesenchymal cells, rather than through a reduction of tissue eosinophils; however, there are no data reporting enhanced synthesis of ECM protein by IL-5 and fibroblasts do not appear to express the IL-5Rα chain (T.-T. Ou and S. Phipps, unpublished). Furthermore, there was a direct correlation between the Δ changes in eosinophils and changes in tenascin + cells in those receiving anti-IL-5 (Fig 5).

In conclusion our data suggest that eosinophils are unlikely to be essential for the swelling and induration that characterizes the late-phase skin reaction at its peak (i.e. 6–9 h) and that they may play a role in remodelling processes associated with allergic inflammation.

Materials and Methods

Volunteers for anti-IL-5 study The study was approved by the ethics committees of the Royal Brompton and Harefield NHS Trust and the London Chest Hospital, and was performed in accordance with the guidelines of the Declaration of Helsinki. All volunteers gave informed consent prior to participation in the study. 24 volunteers were recruited with a history of mild asthma, with an forced expiratory volume in 1 s (FEV_1) of $\geq$ 70% of the normal value for age and height and within an 18–55 y age range. All volunteers were atopic defined by a positive skin prick test to one or more aeroallergens (*Dermatophagoides pteronyssinus*, cat, dog, and mixed grass and tree pollen (all ALK, Horsholm, Denmark)) and were well controlled with short-acting β_2 agonist alone, had no history of worsening asthma or upper respiratory tract infection in the preceding 4 wk, and had not taken inhaled or oral corticosteroids, or other anti-inflammatory drugs or anti-histamines in the preceding 8 wk. There was documented airway hyperresponsiveness as shown by a provocative concentration causing a 20% fall in FEV_1 to histamine of $\leq$ 4.0 mg per mL. All volunteers were non-smokers for at least the preceding 6 mo with no more than a 10-pack year lifetime smoking history.

Study design and processing of specimens This was a two-center double-blind, placebo-controlled, parallel group study designed to evaluate the effects of an anti-IL-5 monoclonal antibody on baseline bronchial mucosal and bone marrow eosinophils and allergen-induced skin eosinophils. The results of studies on the effect of treatment on blood, bronchial, and bone marrow eosinophils has been reported elsewhere (Flood-Page *et al*, 2003a, b). Volunteers were randomized to receive either mepolizumab (750 mg) or placebo, administered as an intravenous infusion over 30 min. The second and third infusions of the study drug were given 4 and 8 wk after the first infusion. Skin biopsies were obtained 2 d before the first infusion of study medication and between 1 and 2 wk after the third infusion. All injections were performed with a 29-gauge needle and a 0.5 mL plastic syringe. Using this method, 30 biological units of either grass, house dust mite, cat, or dog allergen was injected intradermally into two sites

on the extensor aspect of the forearms of each subject. The size of the cutaneous reaction was determined at 6 and 48 h by measuring resistance to the movement of a sharpened pencil and expressed as the mean diameter (mm) as previously described (Ying *et al*, 1999). There were no appreciable differences in the size of the late-phase skin responses between the various allergens used. An additional site was injected with a similar volume of diluent. Macroscopic responses were measured at 6 and 48 h and permanent sticky tape records of the outlines of the responses made. A 4 mm disposable biopsy punch was used to take a biopsy from the center of the reaction at 6 and 48 h after using 1% plain lignocaine for local anesthesia. The control site injected with diluent was biopsied at 6 h. In this way, each patient served as his/her control. Tissue biopsies were immediately fixed in 4% paraformaldehyde and washed in 15% PBS-buffered sucrose (Sigma, Poole, UK), embedded in OCT (optimal cutting temperature), then snap-frozen in isopentane precooled in liquid nitrogen. Cryostat sections (<8 μm) were cut from biopsies, mounted onto Superfrost Plus slides, dried overnight at 37°C, then stored with silica gel at -80°C until use (all VWR, Dagenham, UK unless stated).

Histochemistry and immunohistochemistry Eosinophil accumulation was determined by Congo red, a selective stain for eosinophils in tissue sections from the skin as described previously (Ying *et al*, 2002). Briefly, sections were washed in PBS for 5 min then incubated in 0.5% Congo red (Sigma) in ethanol/0.1 M glycine (1:1) for 5 min at room temperature. The slides were then rinsed in 70% ethanol until the background became clear, then mounted in glycergel (Dako, Cambridge, UK). The alkaline phosphatase anti-alkaline phosphatase (APAAP) technique was used to enumerate cells immunoreactive to a monoclonal antibody (mAb) against CD4, eosinophil major basic protein (MBP), neutrophil elastase, basophil BB1 (a generous gift from Dr A. Walls, University of Southampton, UK), and tenascin (Caltag-Medsystems, Towcester, UK). The APAAP technique, with source of reagents, was performed as described previously (Ying *et al*, 1999). Tissue sections were developed with fast red (Sigma) as chromogen for signal visualization (Dako). Cells were counter stained with Harris' Hematoxylin (VWR) and mounted in glycergel. Positive cells stained red after development with fast red. Substitution of the primary antibody with an irrelevant isotype-matched antibody of the same species was used as a negative control. One biopsy specimen from each time point was evaluated from each patient.

Quantitation and statistical analysis Slides were encoded and counted in a blinded fashion using an Olympus microscope (Olympus Optical Co., London, UK). The whole section was counted and the total number of single positive cells expressed as cell per square millimeter of biopsy. Data were analyzed using a statistical software package (Minitab Release 13.1, Minitab, State College, Pennsylvania). Non-parametric statistics were used throughout the study. The Wilcoxon signed-rank test was used to analyze intragroup changes in the numbers of immunoreactive-positive cells in response to allergen. The Mann–Whitney *U* test was used for intergroup comparisons. Correlation coefficients were obtained by Spearman's rank-order method. A p-value of >0.05 was accepted as non-significant (NS).

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References

Ackerman SJ, Kephart GM, Haberman TM, Greipp PR, Gleich GJ: Localization of eosinophil granule major basic protein in human basophils. J Exp Med 158:946, 1983

Ashcroft GS, Yang X, Glick AB, *et al*: Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. Nat Cell Biol 1:260–266, 1999

Azzawi M, Bradley B, Jeffery PK, *et al*: Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. Am Rev Respir Dis 142:1407–1413, 1990

Blyth DI, Wharton TF, Pedrick MS, Savage TJ, Sanjar S: Airway subepithelial fibrosis in a murine model of atopic asthma: Suppression by dexamethasone or anti-interleukin-5 antibody. Am J Respir Cell Mol Biol 23:241–246, 2000

Church MK, Bradding P, Walls AF, Okayama Y: Mast cells and basophils. In: Kay AB ed. Allergy and Allergic Diseases. Oxford: Blackwell, 1997; p 149–170

Denburg JA, Silver JE, Abrams JS: Interleukin-5 is a human basophilopoietin: Induction of histamine content and basophilic differentiation of HL-60 cells and of peripheral blood basophil–eosinophil progenitors. Blood 77:1462–1468, 1991

Erickson HP, Bourdon MA: Tenascin: An extracellular matrix protein prominent in specialized embryonic tissues and tumors. Annu Rev Cell Biol 5:71–92, 1989

Flood-Page P, Menzies-Gow A, Kay AB, Robinson DS: Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. Am J Respir Crit Care Med 167:199–204, 2003a

Flood-Page P, Menzies-Gow A, Phipps S, *et al*: Anti-IL-5 treatment (mepolizumab) reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics: Evidence for a role for eosinophils in airways remodeling. J Clin Invest 112:1029–1036, 2003b

Frew AJ, Kay AB: The relationship between infiltrating CD4 + lymphocytes, activated eosinophils, and the magnitude of the allergen-induced late phase cutaneous reaction in man. J Immunol 141:4158–4164, 1988

Gizycki MJ, Adelroth E, Rogers AV, O'Byrne PM, Jeffery PK: Myofibroblast involvement in the allergen-induced late response in mild atopic asthma. Am J Respir Cell Mol Biol 16:664–673, 1997

Gregory G, Kirche A, Phipps P, Gevaert P, Pridgeon P, Rankin SM, Robinson DS: Differential regulation of human eosinophil IL-3, IL-5, and GM-CSF receptor-chain expression by cytokines: IL-3, IL-5, and GM-CSF down-regulate IL-5 receptor expression with loss of IL-5 responsiveness, but up-regulate IL-3 receptor expression. J Immunol 170:5359–5366, 2003

Holgate ST, Davies DE, Lackie PM, Wilson SJ, Puddicombe SM, Lordan JL: Epithelial–mesenchymal interactions in the pathogenesis of asthma. J Allergy Clin Immunol 105:193–204, 2000

Kips JC, O'Connor BJ, Langley SJ, *et al*: The effect of SCH55700, a humanized, anti-hIL5 antbody in severe persistent asthma: A pilot study. Am J Respir Crit Care Med 167:1655–1659, 2003

Laitinen A, Altraja A, Kampe M, Linden M, Virtanen I, Laitinen LA: Tenascin is increased in airway basement membrane of asthmatics and decreased by an inhaled steroid. Am J Respir Crit Care Med 156:951–958, 1997

Leckie MJ, ten Brinke A, Khan J, *et al*: Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. Lancet 356:2144–2148, 2000

Leiferman KM, Fujisawa T, Holmes-Gray B, Gleich GJ: Extracellular deposition of eosinophil and neutrophil granule proteins in the IgE-mediated cutaneous late phase reaction. Lab Invest 62:579–589, 1990

Liu LY, Sedgwick JB, Bates ME, *et al*: Decreased expression of membrane IL-5 receptor alpha on human eosinophils: II. IL-5 down-modulates its receptor via a proteinase-mediated process. J Immunol 169:6459–6466, 2002

Macfarlane AJ, Kon OM, Smith SJ, *et al*: Basophils, eosinophils, and mast cells in atopic and nonatopic asthma and in late-phase allergic reactions in the lung and skin. J Allergy Clin Immunol 105:99–107, 2000

Phipps S, Ying S, Wangoo A, Ong YE, Levi-Schaffer F, Kay AB: The relationship between allergen-induced tissue eosinophilia and markers of repair and remodelling in human atopic skin. J Immunol 169:4604–4612, 2002

Reshef A, Kagey-Sobotka A, Adkinson NF Jr, Lichtenstein LM, Norman PS: The pattern and kinetics in human skin of erythema and mediators during the acute and late-phase response (LPR). J Allergy Clin Immunol 84:678–687, 1989

Rothenberg ME, Petersen J, Stevens RL, Silberstein DS, McKenzie DT, Austen KF, Owen WF Jr: IL-5-dependent conversion of normodense human

eosinophils to the hypodense phenotype uses 3T3 fibroblasts for enhanced viability, accelerated hypodensity, and sustained antibody-dependent cytotoxicity. J Immunol 143:2311–2316, 1989

Ruegg CR, Chiquet-Ehrismann R, Alkan SS: Tenascin, an extracellular matrix protein, exerts immunomodulatory activities. Proc Natl Acad Sci USA 86:7437–7441, 1989

Sanderson CJ: Interleukin-5, eosinophils, and disease. Blood 79:3101–3109, 1992

Sehmi R, Wardlaw AJ, Cromwell O, Kurihara K, Waltmann P, Kay AB: Interleukin-5 selectively enhances the chemotactic response of eosinophils obtained from normal but not eosinophilic subjects. Blood 79:2952–2959, 1992

Shipley GD, Pittelkow MR, Wille JJ Jr, Scott RE, Moses HL: Reversible inhibition of normal human prokeratinocyte proliferation by type beta transforming growth factor-growth inhibitor in serum-free medium. Cancer Res 46:2068–2071, 1986

Toda M, Leung DY, Molet S, *et al*: Polarized *in vivo* expression of IL-11 and IL-17 between acute and chronic skin lesions. J Allergy Clin Immunol 111:875–881, 2003

Treasurywala S, Berens ME: Migration arrest in glioma cells is dependent on the alpha integrin subunit. Glia 24:236–243, 1998

Wardlaw AJ, Moqbel R, Kay AB: Eosinophils: Biology and role in disease. Adv Immunol 60:151–266, 1995

Yang J, Torio A, Donoff RB, *et al*: Depletion of eosinophil infiltration by anti-IL-5 monoclonal antibody (TRFK-5) accelerates open skin wound epithelial closure. Am J Pathol 151:813–9, 1997

Ying S, Meng Q, Smith SJ, Larché M, Robinson DS, Kay AB: Methods for identifying human eosinophils in blood and tissue. ACI Int 14:64–71, 2002

Ying S, Robinson DS, Meng Q, *et al*: C-C chemokines in allergen-induced late-phase cutaneous responses in atopic subjects: Association of eotaxin with early 6-h eosinophils, and of eotaxin-2 and monocyte chemoattractant protein-4 with the later 24-h tissue eosinophilia, and relationship to basophils and other C-C chemokines (monocyte chemoattractant protein-3 and RANTES). J Immunol 163:3976–3984, 1999

Zweiman B, Atkins PC, Von Allmen C, Gleich GJ: Release of eosinophil granule proteins during IgE-mediated allergic skin reactions. J Allergy Clin Immunol 87:984–992, 1991