

TGF- β , Eosinophils and IL-13 in Allergic Airway Remodeling: A Critical Appraisal with Therapeutic Considerations

Ramzi Fattouh and Manel Jordana*

Division of Respiratory Diseases and Allergy, Centre for Gene Therapeutics and Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada

Abstract: Airway remodeling is a characteristic feature of allergic asthma that is now thought to contribute to airway dysfunction and, ultimately, to clinical symptoms. A prevalent hypothesis holds that eosinophil-derived transforming growth factor- β (TGF- β) is a predominant underlying mechanism driving the development of remodeling and thus, represent promising targets for therapeutic intervention. This notion is supported by *in vivo* evidence from loss of function experiments conducted in animal models employing the surrogate allergen ovalbumin (OVA), and by indirect evidence from studies in human asthmatics. However, it is important to note that various studies in OVA systems have reported disconnects between eosinophils, TGF- β and allergic remodeling. Moreover, recent investigations in a mouse model induced by respiratory exposure to a house dust mite extract have shown that remodeling can develop independently of TGF- β . These findings challenge the above hypothesis and suggest that the mechanisms governing remodeling may be context specific. In addition to TGF- β and eosinophils, several other factors have been implicated in the development of airway remodeling. Among these, interleukin (IL)-13 may be of particular importance given its role in type-2 immunity and in the tissue repair/fibrotic response. This review will appraise the evidence pertaining to the roles of TGF- β , eosinophils and IL-13 in allergic remodeling, and will suggest that identifying robust targets for therapeutic intervention might benefit from a re-consideration of our approach to understanding remodeling.

Keywords: TGF- β , eosinophils, IL-13, allergy, airway remodeling, asthma, therapy, inflammation.

1. INTRODUCTION

It is now well recognized that allergic asthma is a chronic immune-driven inflammatory disease that is associated with the development of various structural alterations in the airway wall, collectively termed *airway remodeling* [1-3]. The pathological hallmarks of airway remodeling typically include: i) thickening of the sub-epithelial extracellular matrix (ECM) due to increased deposition of several matrix proteins such as laminin, tenascin, decorin, versican and collagen primarily types I, III and IV; ii) increased smooth muscle mass in both the central and peripheral airways resulting from smooth muscle cell hypertrophy and/or hyperplasia; iii) increased epithelial cell turnover and excessive mucous production, the latter as a result of goblet cell hypertrophy and hyperplasia; and iv) increased small blood vessel area [1-3].

At this time our understanding of airway remodeling is, beyond a thorough histological characterization, limited albeit growing rapidly. The chronic nature of the inflammatory response likely imposes unique biological stresses on the airway/lung compartment. As such, it is generally thought that airway remodeling develops in response to repetitive inflammatory insults occurring over prolonged periods of time. This notion is, in fact, supported by numerous findings including studies in animal models that have consistently demonstrated the development of a remodeling/fibrotic response in the face of chronic allergic inflammation [4-9].

Interestingly, additional investigations have revealed that remodeling develops specifically in the context of chronic type-2 but not type-1-polarized inflammation of a similar magnitude and duration [10-12]. Studies reporting evidence of airway remodeling in asthmatic children as early as three years of age have introduced some doubt as to the absolute role of inflammation in this process [13-15]. However, the lack of detailed knowledge about the immunological history of these children weakens this doubt. With respect to pathogenesis, various mediators and cell types, immune-associated and otherwise, have been directly implicated, but, which of these are fundamentally required and which are redundant is not known. In addition, the specific role and, if any, the underlying mechanism of these factors in remodeling, inflammation and immunity is not fully understood. Moreover, the extent to which any of these structural changes, once occurred, is reversible remains unclear. What is becoming increasingly clear is that at least some aspects of remodeling contribute significantly to airway dysfunction and ultimately clinical symptoms (reviewed in [3, 16]). Given that so much remains unknown it is not entirely surprising that there is a lack of effective treatments that can directly impact airway remodeling. Thus, elucidating the factors and mechanisms involved in remodeling and how they may interrelate with the immune-inflammatory component of asthma will contribute to our understanding of disease evolution and may uncover therapeutic interventions beyond anti-inflammatory treatments.

2. THE TGF- β – EOSINOPHIL PARADIGM IN ALLERGIC REMODELING

There are many molecules that have the capacity to participate either directly or indirectly in the remodeling of the

*Address correspondence to this author at the Department of Pathology and Molecular Medicine, Division of Respiratory Diseases and Allergy, McMaster University, 1200 Main Street West, Hamilton, Ontario, L8N 3Z5, Canada; Tel: (905) 525-9140, Ext. 22473; Fax: (905) 522-6750; E-mail: jordanam@mcmaster.ca

airway. They include, among others, members of the growth factor family notably, transforming growth factor (TGF)- β and vascular endothelial growth factor (VEGF); various cytokines, such as interleukin (IL)-5, IL-11, IL-13, and oncostatin M; and the enzymes matrix metalloproteinase (MMP)-9 and tryptase [17-25]. Among these molecules, TGF- β is considered to play a prominent role.

Indeed, TGF- β plays an integral role in the regulation of the ECM in the lung, and elsewhere, and is known to potentially regulate the growth and function of various resident lung cells. While most, if not all, cells can produce TGF- β , there are several studies showing that, based on immunohistochemical analysis of bronchial biopsies, eosinophils constitute the majority of the TGF- β expressing cells in the lungs of asthmatics [26-28]. Several lines of evidence have led to the, now, prominent hypothesis that eosinophil-derived TGF- β production is one of the major mechanisms that drives the development of airway remodeling in allergic airway disease. The observations in support of this hypothesis include the findings that (i) eosinophils can produce an abundance of TGF- β *in vitro* [29, 30], (ii) TGF- β expressing eosinophils are evident in the lungs of asthmatic patients [27, 28, 31-33] and in lungs of mice subjected to experimental models of allergic airway disease [34-37], (iii) TGF- β expression is positively correlated with markers of airway remodeling in asthmatics [33, 38], and (iv) TGF- β has been directly shown to mediate ovalbumin (OVA)-induced airway remodeling in experimental animal models [17]. In light of this seemingly compelling evidence, TGF- β and eosinophils have become the principal targets of therapeutic strategies aimed at ameliorating airway remodeling in asthma. In the following sections we will examine the candidacy of these factors as therapeutic targets by critically appraising the relevant literature.

3. TGF- β IN ALLERGIC ASTHMA

3.1. TGF- β in Allergic Airway Remodeling

Undoubtedly, TGF- β is one of the most pleiotropic molecules and consequently one of the most difficult to dissect. Since its discovery twenty-five years ago, TGF- β has been shown to be intricately involved in basic cellular activities including proliferation and migration as well as complex processes such as embryonic development, tissue repair and immunity. In the lung, TGF- β is a major orchestrator of the ECM. It not only influences the synthesis of ECM components such as fibronectin and collagen from bronchial epithelial cells, fibroblasts, and airway smooth muscle cells but also controls the expression of matrix-degrading proteases and the levels of antiproteases such as MMPs and TIMPs (tissue inhibitor of metalloproteinases), respectively (reviewed in [39]). Moreover, *in vitro* studies have shown that TGF- β can stimulate the proliferation of mesenchymal cells, including fibroblasts and airway smooth muscle cells [40-42], trigger smooth muscle hypertrophy [43], and induce the development of myofibroblasts [44, 45]; a cell type thought to be critically involved in wound healing [46]. In addition, TGF- β can elicit the production of other profibrotic mediators such as PDGF (platelet-derived growth factor) and CTGF (connective tissue growth factor) from various cell types [40, 47, 48]. Thus, on the basis of its functional portfolio alone, TGF- β appears capable of eliciting many of the

structural alterations that have been described in the remodeled airway. Indeed, adenoviral-mediated TGF- β 1 overexpression in the lung is sufficient to induce severe pulmonary fibrosis [49].

What is the evidence that TGF- β drives allergic airway remodeling? Several studies in human asthmatics have observed a relationship between TGF- β expression and airway remodeling. Elevated levels of TGF- β mRNA and protein have been observed in bronchial biopsies from subjects with moderate-severe asthma [27, 28, 31, 33, 50, 51]. In addition, increased TGF- β levels have also been documented in the bronchoalveolar lavage (BAL) of stable atopic asthmatics versus controls [32] and TGF- β expression has been correlated with the number of fibroblasts in the remodeled airway wall in asthmatic patients but not in control subjects [38]. Notably, Vignola *et al.* observed a significant correlation between the number of TGF- β expressing epithelial or submucosal cells and basement membrane thickness (subepithelial fibrosis) in asthmatic patients compared to normal subjects [33]. Along the same lines, expression levels of activated (i.e. phosphorylated) Smad2, a TGF- β signaling molecule, was also found to be significantly associated with basement membrane thickness in asthmatics [52]. While these reports do in fact support the notion that TGF- β is involved in airway remodeling they do not demonstrate causality. Moreover, there are a number of additional studies that did not find any differences in TGF- β expression between asthmatics and controls; this issue has been meticulously reviewed by Bosse and Rola-Pleszczynski [53]. Thus, perhaps the strongest conclusion that can be made from the above observations is that TGF- β may be in some way involved in allergic asthma.

To investigate the role of TGF- β in remodeling under controlled conditions, researchers have turned to experimental animal models of chronic allergic airway disease. TGF- β expression has been consistently observed to be increased in mice following sensitization and challenge, in a variety of models [34-37]. At this time, a total of five independent studies have directly examined the role of TGF- β in airway remodeling; four in models employing the surrogate allergen OVA and one study using a model induced by exposure to a house dust mite extract (HDM). In the first of these published reports, McMillan *et al.* utilized a pan-neutralizing anti-TGF- β antibody (Ab) to block TGF- β function during the chronic exposure phase of a model that involved conventional sensitization (intraperitoneal injections of OVA + alum) followed by intermittent OVA aerosolizations [17]. Under these conditions, TGF- β blockade moderately reduced the extent of peribronchial ECM deposition and airway mucous production and dramatically decreased the number of proliferating airway smooth muscle cells compared to control groups. Using a slightly modified experimental protocol, Le *et al.* evaluated TGF- β in allergic remodeling using Smad3-deficient mice [54]. Although the Smad signaling pathway is not the only pathway by which the fibrogenic effects of TGF- β can be mediated, Smad3 has been clearly shown to play a critical role in the signaling pathways associated with TGF- β mediated wound healing and fibrosis [55]. Following repeated OVA challenge the authors observed marked decreases in airway fibrosis and total lung collagen levels, that was associated with a complete reduction in the number of peribronchial myofibroblasts in OVA-

exposed Smad3-deficient mice compared to OVA-exposed wild type (WT) mice. Moreover, a partial reduction in airway mucous production and in the thickness of the peribronchial smooth muscle layer was also noted. In agreement with these reports, Alcorn *et al.* documented a significant reduction in peribronchial collagen levels in mice subjected to a short-term OVA exposure protocol and concurrently treated with a TGF- β 1-specific neutralizing Ab [56]. Lastly, in a limited study, Leung *et al.* treated rats during OVA challenge with a TGF- β -receptor type (TGF- β R)-I kinase inhibitor (SD-208) that blocks activation of the Smad-signaling pathway [57]. Treatment with SD-208 significantly inhibited goblet cell hyperplasia and airway smooth muscle and epithelial cell proliferation. However, the impact of SD-208 treatment on the smooth muscle thickness could not be assessed for lack of a positive response in the controls. Furthermore, the effect of treatment on matrix deposition was not determined.

In contrast to the above studies, Fattouh *et al.* utilized a model of allergic airways disease induced by HDM exposure, the most clinically relevant allergen worldwide [35]. Specifically, two distinct protocols were used, (i) a continuous HDM exposure protocol whereby disease is induced *via* the respiratory mucosa by exposing mice to HDM, intranasally, several days/week for a total of five weeks and (ii) an intermittent exposure protocol that involved ten days of intranasal HDM administration, to achieve sensitization, followed by short recurrent HDM rechallenges that were separated by twelve day rest periods. Interestingly, blockade of TGF- β function, achieved using the same Ab as in the McMillan study, had absolutely no effect on the development of airway remodeling, in either the continuous or intermittent exposure systems. Indeed, HDM-exposed anti-TGF- β treated mice developed identical increases in subepithelial collagen deposition, smooth muscle thickness and airway mucous production as the HDM-exposed IgG treated controls. To confirm these results Smad3-deficient mice were also subjected to five weeks of HDM exposure and they similarly developed airway remodeling to the same extent as HDM-exposed WT littermates.

There are a number of explanations that may account for the differences in the differential requirement for TGF- β between OVA and HDM-based systems and these have been previously discussed in depth elsewhere [35]. While it is quite clear that TGF- β is required, at least in some part, for the development of allergic remodeling in OVA systems, it is equally clear that TGF- β is not critically required for remodeling to develop in an HDM system. These findings underscore the notion that the impact of TGF- β in allergic airway remodeling is contextual and, hence, may not be generally applicable. Importantly, the findings in the HDM system force reconsideration of the belief that TGF- β is, unconditionally, a central mediator of allergic remodeling, with all the attendant therapeutic implications.

3.2. TGF- β in Allergic Airway Inflammation and Immunity

There is consistent evidence, at least in experimental systems that, regardless of protocols and antigens, the levels of TGF- β are increased locally. This, on the account of immunological parsimony, raises the question of what might be the preeminent role of TGF- β in this process. The ability to

generate a robust inflammatory response is a fundamental component of host defense and, yet, it is fraught with peril. Indeed, should the inflammatory response be allowed to proceed unimpeded, the results could be devastating. Thus, it is imperative that inflammatory responses, be tightly regulated. In this regard TGF- β is one of the most powerful immunosuppressive cytokines. Indeed, TGF- β 's role as an essential regulator of immune responses is exemplified by the observation that TGF- β 1 knock out (KO) mice die shortly after birth (~4 weeks) due to a rampant, multifocal, inflammatory response [58].

Most, if not all, inflammatory cell types possess TGF- β receptors and are, thus, potentially susceptible to TGF- β mediated regulation. The ability of TGF- β to inhibit the functions of activated inflammatory cells, suggests that it may play a major part in down-regulating *established* allergic inflammation [59]; an important distinction as TGF- β likely contributes to the generation of an inflammatory response during the incipient stages [60-62]. The suppressive effects of TGF- β on eosinophils, macrophages, and lymphocytes are of direct importance to allergic airway disease. Active TGF- β inhibits eosinophil activation and differentiation and can also induce apoptosis *in vitro* [63-65]. TGF- β can also down regulate macrophage activity by interfering with their ability to respond to activating cytokines such as IFN- γ [66], increasing expression of the IL-1 receptor antagonist [67] and limiting the production of cytotoxic reactive oxygen and nitrogen intermediates [68, 69]. Furthermore, TGF- β has been shown to inhibit the proliferation of both Th1 and Th2 clones *in vitro* as well as inhibit the production of and response to the cytokines associated with each subset. Moreover, TGF- β affects B cells by impairing immunoglobulin (Ig) synthesis and secretion, interfering with the proliferation of both B cells and plasma cells and by triggering these cells to undergo apoptosis [59, 70, 71].

The implication that TGF- β may regulate the airway inflammatory response following allergen exposure is also directly supported by *in vivo* evidence from both acute and chronic experimental models. Studies conducted in transgenic mice whose T cells were rendered insensitive to TGF- β *via* over expression of either Smad7 or a dominant-negative TGF- β RII displayed increased allergic airway inflammation following acute OVA challenge in a conventional OVA model [72, 73]. Importantly, this increase was associated with enhanced airway hyperresponsiveness (AHR). Moreover, in the latter of these two reports, the increase in airway inflammation was found to be mainly due to a pronounced increase in eosinophils. Similarly, TGF- β heterozygous mice, which express approximately one-third of WT TGF- β protein levels, also developed greater airway inflammation, due exclusively to increases in eosinophils, after sensitization and challenge with OVA [74]. Notably, an increase in OVA-specific Th2-associated Igs and splenocyte cytokine production *in vitro* was also observed, suggesting that an overall enhancement in OVA-specific Th2-immunity had developed in the TGF- β heterozygous mice. Together, these studies demonstrate an important role for TGF- β in the dampening of the acute allergic inflammatory response with, perhaps, a particular emphasis on the eosinophilic component of this process. Given the genetic approaches employed, it is possible that at least some of the exaggerated responsiveness described above could be attributable to the absence

of TGF- β during the Th2 sensitization phase. However, significant increases in the levels of Th2-associated cytokines in the BAL were also reported in the Alcorn study, where TGF- β 1 function was blocked during OVA challenge but not during the sensitization period [56]. Interestingly, despite a lack of any appreciable impact on inflammation, treatment with anti-TGF- β 1 significantly enhanced AHR in terms of tissue resistance and tissue elastance [56]. In contrast to these reports, Leung *et al.* observed a decrease in the number of basement membrane eosinophils and T cells in OVA-exposed Brown Norway rats treated with SD-208 throughout challenge [57]. Surprisingly, to an extent, this treatment had no effect on AHR but, rather intriguingly, a decrease in AHR was observed when treatment was initiated mid-way through the challenge protocol. It is difficult to reconcile these findings, not only with each other but also with the results of the previous studies.

The impact of TGF- β on chronic allergic inflammation was also addressed in the study using HDM described in the previous section. In this context, neutralization of TGF- β function beginning well after sensitization was established resulted in an augmented inflammatory response characterized by an exclusive increase in eosinophilia [35]. Furthermore, this was associated with increased airway reactivity and maximal airway resistance resulting in an overall exacerbation of AHR in the continuous HDM-exposure model. Notably, treatment with anti-TGF- β also led to an increase in serum HDM-specific IgE and IgG₁, and to increased IL-5 production by splenocytes restimulated *in vitro*. Interestingly, a clear trend towards enhanced peribronchial eosinophilia was also documented in the Le study although it did not achieve statistical significance, nor was there any significant difference in AHR between OVA-exposed Smad3-deficient and WT mice [54]. Regardless, interpretations of the data pertaining to inflammation and immunity in Smad3-deficient mice may be confounded by the intrinsic immunological defects that develop in these animals. The impact of TGF- β on the regulation of chronic airways inflammation was also addressed in the McMillan study. No differences in airway inflammation were observed, nor were there any effects on the levels of IL-5 or IL-13 in lung tissue homogenates following treatment with anti-TGF- β , which was initiated long after sensitization and given over the entire course of chronic OVA-exposure [17]. The authors noted that their findings showed that it may be possible to attenuate airway remodeling without impacting inflammation. While this may indeed be the case, their findings did not necessarily rule out the possibility that TGF- β is regulating inflammation. Rather, the lack of any effect on inflammation may have been a reflection of the dose of antibody that was used. In an exact repeat of their experimental protocol we observed that mice treated with a higher dose of anti-TGF- β displayed significantly elevated airway inflammation and eosinophilia (Fig. 1). Thus, while the relevance of evaluating the role of TGF- β in the context of chronic OVA exposure is somewhat questionable as it has been clearly shown that inflammation decreases and tolerance develops with continued exposure [75, 76] we note that even in this experimental setting, TGF- β may in fact be acting as a negative regulator.

Therefore, studies in experimental models, whether acute or chronic, utilizing OVA or HDM, demonstrate consistently that, in contrast to the data on the impact on airway remodel-

ing, TGF- β acts as a negative regulator of allergic airways inflammation. This may be the preeminent role of TGF- β in allergic airway disease: to control the development of unsustainable immunopathology. Collectively, these data have profound therapeutic implications. The divergent data on the effect of TGF- β on airway remodeling in experimental systems along with the human data which is controversial in some respects and correlative in all indicates that the benefit of TGF- β blockade on the development of airway remodeling is uncertain at best. Moreover, such an intervention may, in fact, worsen lung inflammation and lung function.

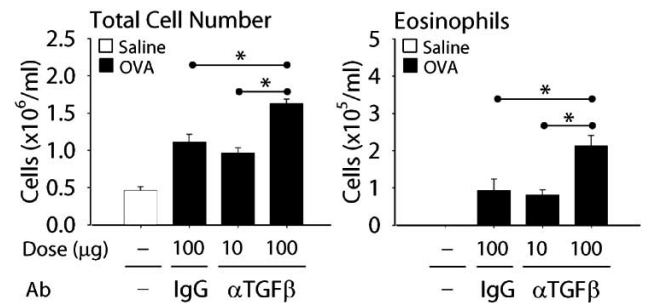


Fig. (1). Impact of TGF- β blockade on OVA-induced allergic airway inflammation. Differential cell analysis showing the number of total cells and eosinophils in the bronchoalveolar lavage. OVA mice (black bars) were sensitized by intraperitoneal injection of OVA + aluminium hydroxide on days 0 and 12, challenged with aerosolized OVA daily on days 18-23 and 3 times per week from days 26-54 and were concurrently treated with either anti-TGF- β (10 or 100 μ g/dose corresponding to 0.5 and 5 mg/kg respectively) or a control IgG (100 μ g/dose), immediately before each OVA challenge from day 26 onward by intraperitoneal injection (as described in McMillan *et al.* [17]). Saline mice (white bar) received saline instead of OVA and were not treated with either antibody. Note that the OVA mice being treated with the 10 μ g dose of anti-TGF- β (as was used in the McMillan study) display similar numbers of total cells and eosinophils as the OVA mice treated with the control antibody. In contrast, OVA-exposed mice treated with the 100 μ g dose of anti-TGF- β show significant increases in total cells and eosinophils. $n = 5-6$ /group. Data are expressed as mean $\pm$ SEM. Results were interpreted using analysis of variance with Tukey's post hoc test. Differences were considered statistically significant when P values were less than 0.05.

4. EOSINOPHILS IN ALLERGIC ASTHMA

4.1. Eosinophils in Allergic Airway Remodeling

Pulmonary eosinophilia has long been recognized as a defining feature of allergic asthma. A great deal has been learned regarding the functional capability of these cells. It is now thought that the function of eosinophils extends beyond the release of destructive effector molecules and includes leukocyte activation and survival, antigen presentation, and T-cell recruitment [77, 78]. Yet, it remains that we lack a precise understanding of the extent to which eosinophils participate in disease pathogenesis. Eosinophils are capable of producing a wide array of molecules, among which are a number of mediators that can influence the differentiation, proliferation, and function of lung structural cells [78, 79]. In this way, it is thought that eosinophils could contribute to the development of remodeling. Numerous experimental studies have investigated the role of eosinophils in allergic remodel-

ing. While the findings among these studies display greater consistency than those examining AHR (discussed below) they are not without noteworthy discrepancies. Blyth *et al.* examined the impact of anti-IL-5 treatment on the development of subepithelial fibrosis in an acute OVA model involving intraperitoneal sensitization, without adjuvant, and three intratracheal OVA challenges delivered three days apart [80]. Administration of anti-IL-5 neutralizing Abs prior to each challenge selectively ablated airway eosinophilia, and this effect was associated with a complete reduction in subepithelial reticulin thickening compared to sensitized and challenged mice treated with a control Ab. Similarly, Tanaka *et al.* showed that sensitized IL-5R α -deficient mice subjected to 'chronic' (daily challenges for 3 weeks) OVA exposure lacked airway eosinophilia, and were also protected from increases in subepithelial matrix deposition [81]. Moreover, they further demonstrated that anti-IL-5 treatment throughout the challenge period eliminated airway eosinophils and markedly inhibited increases in total lung collagen and subepithelial fibrosis in WT sensitized mice relative to controls. Kumar *et al.* also observed that anti-IL-5 treatment during the last two weeks of a six-week intermittent OVA challenge protocol (where remodeling is evident after 4 weeks) resulted in a significant, albeit partial, decrease in subepithelial fibrosis that was associated with considerable (~70%) and moderate (~35%) decreases in the number of intraepithelial eosinophils and other inflammatory cells in the trachea, respectively [82]. In yet another study, Cho *et al.* exposed IL-5-deficient mice, sensitized to OVA, to a protracted challenge protocol (2 challenges/week for 12 weeks). The authors noted a near complete reduction in the number of BAL eosinophils in the IL-5-deficient mice with no effect on the numbers of peribronchial CD4⁺ lymphocytes or F4/80⁺ macrophages or on the levels of BAL IL-13 compared to WT controls [19]. Furthermore, this was associated with the following findings: a substantial decrease in peribronchial collagen types III and V but only a moderate reduction in lung collagen overall, dramatic decreases in the thickness of the peribronchial smooth muscle layer and in the area of α -smooth muscle actin, and a modest inhibition of epithelial mucous production.

In contrast, two additional studies employing IL-5-deficient mice did not observe marked differences in airway remodeling between KO and WT animals. Indeed, Foster *et al.* reported comparable increases in the thickness of the tracheal epithelium and in the accumulation of subepithelial collagen in sensitized and chronically challenged (3 challenges/week for 6 weeks) IL-5-deficient mice compared to WT, despite significant reductions in the numbers of intraepithelial eosinophils and decreased overall inflammation in the airway wall [83]. Similarly, Leigh *et al.* noted equivalent increases in subepithelial collagen deposition between IL-5 KOs and WT mice following sensitization and chronic OVA challenge, although a decrease in mucous production and in the fold change over baseline of α -smooth muscle actin was observed in the KOs relative to WT controls [11]. The reasons for these differences are unclear. It is somewhat unlikely that the discrepancy can be explained by the differences in the experimental protocols employed as the consensus among the results of the Blyth, Tanaka, and Cho studies was strong despite protocol differences. Strain differences may be a factor, as the Cho study used IL-5 deficient mice

on a C57BL/6 background while the Foster and Leigh studies were conducted on a BALB/c background. Furthermore, it is interesting to note that the findings of the Foster IL-5 KO study also contrast with those of their subsequent report, already discussed above (Kumar *et al.*), wherein they did observe a substantial reduction in airway remodeling following treatment with an anti-IL-5 Ab; notably both studies employed the exact same experimental protocol and strain of mice.

In an attempt to circumvent the ambiguities associated with the use of IL-5 based approaches Humbles *et al.* employed Δ dbl GATA mice that are selectively devoid of the eosinophil lineage to directly investigate the role of eosinophils in allergic remodeling induced by chronic OVA exposure [84]. Eosinophil-deficient mice displayed a significant, although modest, decrease in the mean density of the submucosa, a complete reduction in total lung collagen levels and marked decreases in the total and proliferating numbers of airway smooth muscle cells following sensitization and chronic OVA challenge compared to WT controls.

Eosinophil-derived TGF- β is perhaps the most predominant mechanism by which eosinophils are thought to fuel the development of remodeling. Certainly, eosinophils can produce an abundance of TGF- β *in vitro*, and TGF- β positive eosinophils are frequently observed in the lungs of mice subjected to experimental allergy models and in patients with allergic asthma. However, there are several important considerations, beyond those previously discussed, that do not support this hypothesis. Even in a TGF- β -dependent system, such as the OVA-based model, there is a clear disconnect between eosinophils, TGF- β and remodeling. Indeed, although TGF- β expressing eosinophils can be frequently observed in the tissues so too are other cells; a statement that holds true not only for experimental OVA studies but for human investigations as well, reviewed in [53]. In the Tanaka study, a kinetic analysis of the cellular sources of TGF- β over the three-week challenge period revealed that while eosinophils, were "especially" positive for TGF- β after the first week of OVA exposures, mononuclear cells and alveolar macrophages were also expressing TGF- β at this time-point [81]. Moreover, perivascular and peribronchial mesenchymal cells as well alveolar epithelial cells became increasingly positive for TGF- β following the second and third weeks of OVA exposure. Cho *et al.* reported that eosinophils comprised less than two-thirds of the TGF- β expressing peribronchial cells following three-months of OVA challenge; F4/80⁺ macrophages made-up approximately one-third, and they also documented significant TGF- β expression by the airway epithelium [19]. With respect to the experimental model employed in both the Kumar and Foster studies, the airway epithelium was shown to be the major source of TGF- β expression [37]. In fact, there was no evidence of eosinophils expressing TGF- β 1 following chronic low-level OVA challenge. Finally, in the Humbles Δ dbl GATA study it was noted that mononuclear cells, presumably macrophages, were the main expressors of TGF- β protein during chronic OVA challenge, not eosinophils [84]. In agreement with this, the authors consistently found no differences in active TGF- β 1 expression, on either the level of protein or mRNA, between chronically challenged WT and eosinophil-deficient mice. Why TGF- β expression by eosinophils predominates in some studies and is completely ab-

sent in others is unclear. Explanations have included: limitations associated with the methodologies (e.g. immunohistochemistry, BAL) employed, subtle differences between experimental exposure protocols, and the possibility that this is a reflection of the heterogeneity of allergic responses. Ultimately, while the above studies provide evidence that eosinophils may contribute to the development of remodeling, whether eosinophil-derived TGF- β is, universally, a major underlying mechanism is tenuous at this time.

We note that all of the experimental studies examining the contribution of eosinophils to remodeling have been conducted in the context of OVA-based models, most achieving sensitization by conventional means (OVA+Alum i.p. sensitization). In our opinion, this is of particular importance given the cellular and molecular differences that are emerging between conventional OVA models and models utilizing common environmental allergens. In this regard, the notion that eosinophils are critically involved in the development of remodeling may also be contextual and perhaps limited to OVA-induced systems. To explore this issue, we have recently initiated experimentation in our laboratory and, most interestingly, our findings indicate that Δ dbl GATA mice subjected to chronic HDM exposure display similar increases in subepithelial collagen as HDM-exposed WT mice (Fig. 2). Although these studies are ongoing, they suggest that allergic remodeling, when induced by respiratory exposure to HDM, can develop independently of eosinophils.

The effect of short-term anti-IL-5 therapy (three doses separated one-month apart) on ECM deposition was examined in a small study in mild atopic asthmatics [26]. The treatment, which partially reduced bronchial eosinophil numbers, significantly decreased the total expression of tenascin and lumican relative to placebo and pre-treatment baseline levels, and decreased the expression of procollagen

III compared to placebo. However, it should be noted that the baseline expression level of tenascin was nearly ten fold higher in the treated vs placebo group and that the decrease in procollagen III was not significant compared to pre-treatment baseline measurements. Furthermore, the effects of anti-IL-5 treatment on other major remodeling-associated parameters, such as the expression of fibronectin and collagen types I and V, smooth muscle mass and overall reticular basement membrane thickness were not examined. Thus, the benefits of anti-IL-5 therapy, and presumably eosinophil depletion, on ECM deposition in humans remain to be clarified.

4.2. Eosinophils in Allergic Airway Hyperresponsiveness

It is generally assumed that eosinophils contribute to clinical symptoms and the decline in lung function, although, this issue is not without controversy. Various experimental strategies have been employed to investigate the involvement of eosinophils in AHR. We have included a table that briefly summarizes the findings of some reports that have addressed this issue. Early studies interfering with IL-5 (*via* the use of IL-5 KO or anti-IL-5 neutralizing Abs; [11, 82, 83, 85-92]) as a means of either preventing or abolishing eosinophilia yielded results that were not only conflicting (refer to Table 1, Readout: AHR) but also somewhat difficult to interpret as IL-5 is known to be involved in other relevant processes including B cell maturation and smooth muscle cell responsiveness [93, 94]. More recent studies utilizing anti-CCR3 depleting Abs and eosinophil KO strains, which result in the selective ablation of eosinophils without other apparent side-effects, have also yielded contradictory results with respect to the contribution of eosinophils to lung dysfunction (Table 1; [84, 95-97]). At least some of this controversy has been explained by the differential involvement of eosinophils and other factors in different strains of mice [98-

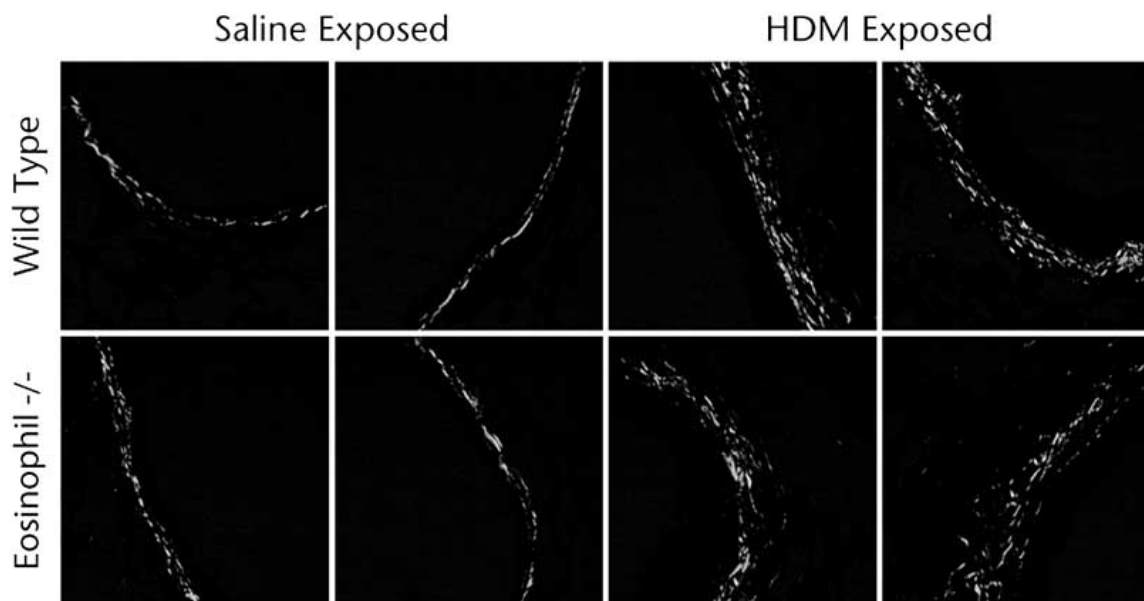


Fig. (2). Impact of eosinophils on HDM-induced subepithelial collagen deposition. Separate groups of wild type or Δ dbl GATA (eosinophil deficient) mice were exposed to saline or HDM, intranasally, 5 days per week for 5 weeks. Pictures show representative light photomicrographs of paraffin-embedded cross-sections of lung tissue obtained 72 hours after the last HDM exposure. Sections were stained with Picro Sirius red and visualized under polarized light to indicate subepithelial collagen deposition. All pictures were taken at 20x magnification. Note that the HDM-exposed eosinophil-deficient mice display increases in sub-epithelial collagen deposition equivalent to that of wild type HDM-exposed mice.

102]. Importantly, the functional consequence of eosinophils in human allergic asthma is being investigated in clinical trials although, unfortunately, the issue remains unresolved (Table 2; [103-105]).

5. REFLECTING ON ALLERGEN DIVERSITY

The differential requirement for TGF- β , and perhaps eosinophils as well, in the development of airway remodeling between OVA and HDM-based models uncovers an important, yet elemental, concept: that achieving the same output (i.e. allergic inflammation, AHR, airway remodeling) with two different inputs (OVA and HDM), does not imply the same underlying mechanisms. The reasons for this alleged mechanistic diversity may be manifold but at least two seem evident. First, the method to accomplish sensitization differs dramatically. Most OVA-based models induce sensitization by injecting OVA adsorbed to a chemical adjuvant (usually aluminium hydroxide or alum) into the peritoneum. In contrast, sensitization with HDM is attained by intranasal delivery of the extract without any additional exogenous adjuvant. The inference is that only HDM activates, and hence informs about, incipient mucosal responses. Second, from biochemical and immunological perspectives, OVA and HDM are two vastly different materials. While OVA preparations are essentially pure, HDM extracts are comprised of hundreds of protein and non-protein components, many of which (e.g. Der p1, 3, 5, 6, 9 and LPS) can engage the immune system to instigate an array of responses (reviewed in [106, 107]). We surmise that the combination of these two factors, method of sensitization and antigenic material, accredits HDM the capacity to elicit a distinct and particularly complex network of cellular responses and molecular signatures. This complexity is likely to encompass a significant degree of overlaps consistent with one of the fundamental operating principles of the immune system: a degree of redundancy to ensure survival. We must also acknowledge the diversity among common environmental aeroallergens. Indeed, HDM, roaches, fungi, and outdoor allergens such as ragweed may likely derive dissimilar immunological responses. Against this backdrop, the responses elicited by OVA-based systems may be deceiving, with implications to both our understanding of the pathogenesis of the process and the development and appraisal of therapeutics. In this regard, the list of mediators deemed "critical", in OVA-based models, to the development of allergic airway disease keeps propagating and, yet, the number of successful "single molecule based" asthma therapies remains stagnant. One wonders if this apparent paradox epitomizes the perils of the Occam's Razor ideology as applied to experimental modeling: models must be simple but not too simple.

6. THERAPEUTIC POTENTIAL OF TARGETING IL-13 IN THE TREATMENT OF AIRWAY REMODELING AND ALLERGIC AIRWAY DISEASE

Several factors, aside from TGF- β and eosinophils, have been shown to possess powerful fibrogenic activity. Of these, IL-13 may be particularly relevant in the generation of allergic airway remodeling given its critical involvement in type-2 immunity. A number of studies have investigated the role of IL-13 in AHR and remodeling in various experimental protocols of acute and chronic allergic airway disease employing a spectrum of reagents including soluble IL-13

receptors, IL-13 neutralizing Abs, IL-13-deficient animals and even vaccination against self-IL-13. Although virtually all of these studies have been conducted in conventional OVA-based models, impressively, the central findings that have emerged from these reports have been extremely consistent with one another.

Investigations in chronic OVA models have clearly demonstrated that WT mice treated with anti-IL-13 neutralizing Abs throughout challenge and IL-13-deficient mice are largely protected against increases in subepithelial collagen deposition, epithelial thickening and mucous production [11, 108, 109]. More modest effects on collagen deposition and to a lesser extent epithelial thickening and mucous production have been observed in studies that initiated IL-13 blockade at some point subsequent to the start of challenge, indicating that, once established, IL-13 blockade does little to reverse these structural changes [82, 110, 111]. In addition, functional impairment of IL-13 significantly inhibited subepithelial collagen accumulation and mucous production in an *Aspergillus fumigatus*-induced model, suggesting that the importance of IL-13 in the development of allergic remodeling may transcend OVA-based systems [22]. However, sensitization was still achieved in this model by intraperitoneal injection of allergen with adjuvant. It should be noted as well that the impact of IL-13 interference on sensitization, if any, in the above reports was not assessed. This may be of particular relevance to studies employing IL-13 KOs as these mice have displayed impaired Th2 cell development [112]. Moreover, while many of the studies interfering with IL-13 function reported substantial decreases in remodeling, and AHR for that matter, these effects were often associated with considerable decreases in eosinophilia and/or total inflammation. Hence, it becomes difficult to discriminate the direct effects of IL-13 activity on remodeling from those effects on inflammation.

In this regard, constitutive IL-13 overexpression in the lungs of naïve mice leads to the development of sub-epithelial fibrosis [21]. Interestingly, the fibrotic response that develops as a consequence of IL-13 overexpression was observed to be entirely mediated by TGF- β [113] and similar observations have been made in OVA-induced models [37, 114]. These findings suggest that IL-13 may contribute indirectly to the development of airway remodeling. On the other hand, it has been shown that IL-13 is capable of inducing differentiation of fibroblasts into myofibroblasts and stimulating lung myofibroblast proliferation *in vitro* [115, 116]. IL-13 can also activate human airway fibroblasts and can directly trigger collagen production to a similar extent as that induced by TGF- β 1, *in vitro* [117-119]. Moreover, IL-13 is a powerful stimulator of arginase expression in fibroblasts and macrophages, which further induces the production of proline, a critical component of collagen [120-123]. That IL-13 can activate reparative/fibrotic processes independently of TGF- β has been observed *in vivo*. Intraperitoneal injection of recombinant IL-13 in two-week old TGF- β 1-deficient mice led to the induction of several repair-associated genes including collagens type I and III, fibrillin, tenascin, TIMP-1 and various MMP's to levels that were equivalent to those observed in IL-13-injected WTs [124]. Of particular significance, Kaviratne *et al.* demonstrated, in a model of *Schistosoma*-induced hepatic fibrosis, that the development of the fibrotic response is TGF- β inde-

Table 1. Brief Summary of Findings from Various Studies Addressing the Impact of Eosinophils on Airway Hyperresponsiveness in Experimental Animal Models. The information provided describes the effect of treatment (i.e. Ab or gene deficiency) on the indicated readout relative to the appropriate positive control group.

		Readout:	Eosinophils		Mononuclear Cells		Th2 Immunity			AHR ^d
			BAL ^a	LG	BAL	LG	Cytokines		Ig's	
							BAL/LG	Other		
		Study Information:								
Acute Exposure Protocol	IL-5 Based Approach	Mausser <i>et al.</i> '93 [85] ^b anti-IL-5 Ab - Hartley Guinea pigs Acute OVA/Al(OH) ₃ /pertussis (i.p.)	↓ ^c (95%)	↓ (100%)	↓	N.D.	N.D.	N.D.	N.D.	↓
		Van Oosterhout <i>et al.</i> '93 [86] anti-IL-5 Ab - Dunkin-Hartley G. pigs Acute OVA (i.p.)	↓ (95%)	N.D.	N.E.	N.D.	N.D.	N.D.	N.D.	↓
		Foster <i>et al.</i> '96 [83] IL-5 KO - C57BL/6 Acute OVA/Alum (i.p.)	↓ (100%)	N.D.	↓ (lympho.)	N.D.	N.D.	N.D.	N.E.	↓
		Corry <i>et al.</i> '96 [87] anti-IL-5 Ab - BALB/c Acute OVA/Alum (s.c.)	↓ (98%)	↓ (99%)	↓	↓	N.D.	N.D.	N.D.	N.E.
		Nagai <i>et al.</i> '96 [88] anti-IL-5 Ab - BALB/c Acute OVA/Alum (i.p.)	↓ (65%)	N.D.	N.E.	N.D.	N.D.	N.D.	N.D.	N.E.
		Hessel <i>et al.</i> '97 [89] anti-IL-5 Ab - BALB/c Acute OVA (i.p.)	↓ (100%)	↓	N.E.	N.D.	N.D.	N.D.	N.D.	N.E.
		Mathur <i>et al.</i> '99 [90] anti-IL-5 Ab - C57BL/6 Acute <i>S. mansoni</i> egg Ag (i.p.)	↓ (100%)	↓ (100%)	↑ (lympho.)	N.D.	N.D.	N.D.	N.D.	N.E.
		Hamelmann <i>et al.</i> '99 [91] anti-IL-5 Ab - BALB/c Acute OVA/Al(OH) ₃ (i.p.)	↓ (95%)	↓ (95%)	N.E.	N.D.	N.D.	N.E. (LN)	N.E.	↓
		Leigh <i>et al.</i> '04 [11] IL-5 KO - BALB/c Acute OVA/Alum (i.p.)	↓ (100%)	↓ (100%)	N.D.	N.D.	N.D.	N.D.	N.D.	N.E.
	Non-IL-5 Based Approach	Justice <i>et al.</i> '02 [95] anti-CCR3 Ab - C57BL/6 Acute OVA/Al(OH) ₃ (i.p.)	↓ (100%)	↓ (100%)	N.E.	N.D.	N.E.	N.E. (spleen)	N.E.	↓
		Lee <i>et al.</i> '04 [96] PHIL (Eos KO) - C57BL/6 Acute OVA/Al(OH) ₃ (i.p.)	↓ (100%)	↓ (100%)	N.D.	N.D.	↓	N.D.	N.D.	↓
		Humbles <i>et al.</i> '04 [84] Δdbl GATA (Eos KO) - BALB/c Acute OVA/Alum (i.p.)	↓ (100%)	↓ (100%)	N.E.	N.D.	↑	N.D.	N.E.	N.E.
		Siegle <i>et al.</i> '06 [97] Δdbl GATA (Eos KO) - BALB/c Acute OVA/Alum (i.p.)	N.D.	↓ (100%)	N.D.	N.D.	N.D.	N.D.	N.D.	N.E.
Chronic Exposure Protocol	IL-5 Based Approach	Foster <i>et al.</i> '00 [92] IL-5 KO - BALB/c Chronic OVA/Alum (i.p.)	N.D.	↓ (85%)	N.D.	↓ (65%)	N.D.	N.D.	N.D.	↓
		Leigh <i>et al.</i> '04 [11] IL-5 KO - BALB/c Acute OVA/Alum (i.p.)	N.A.	N.A.	N.D.	N.D.	N.D.	N.D.	N.D.	N.E.
		Kumar <i>et al.</i> '04 [82] anti-IL-5 Ab - BALB/c Chronic OVA/Alum (i.p.)	N.D.	↓ (75%)	N.D.	↓ (40%)	N.D.	N.D.	N.D.	N.E.
	Non-IL-5	Humbles <i>et al.</i> '04 [84] Δdbl GATA (Eos KO) - BALB/c Acute OVA/Alum (i.p.)	↓ (100%)	↓ (100%)	N.E.	N.D.	↑	N.D.	N.E.	N.E.

^aAbbreviations: BAL - bronchoalveolar lavage; LG - lung; Ig's - antigen specific immunoglobulins; AHR - airway hyperresponsiveness; Ag - antigen; Ab - antibody; KO - knock out; s.c. - subcutaneous; i.p. - intraperitoneal; N.A. - not applicable; N.D. - not determined; N.E. - no effect; ↓ - decrease; ↑ - increase; lympho. - lymphocyte.

^b[XX] - indicates citation number. ^cIndicates the effect of treatment on the given readout; value in brackets indicates the percent change relative to positive control group. ^dMethods employed to assess lung function vary among studies.

Table 2. Brief Summary of Findings from Studies Addressing the Impact of Eosinophils on Airway Hyperresponsiveness in Human Asthmatics. The information provided describes the effect of Treatment (i.e. anti-il-5 Ab) on the indicated readout relative to placebo treated and/or baseline levels.

Readout:	Eosinophils				Other Cells	FEV ₁ / Histamine PC ₂₀	Late Asthma Response ^d	Symptom Score
	Blood	Sputum	BAL ^a	Lung				
Study Information:								
Leckie <i>et al.</i> '00 [105] ^b 24 mild allergic asthmatics anti-IL-5 Ab (i.v.) [allergen challenge]	↓ ^c (90%)	↓ (90%)	N.D.	N.D.	N.D.	N.E.	N.E.	N.D.
Flood-Page <i>et al.</i> '02 [103] 24 mild asthmatics (atopic) anti-IL-5 Ab (i.v.) [baseline level]	↓ (85%)	N.D.	↓ (80%)	↓ (55%)	N.E.	N.E.	N.D.	N.D.
Kips <i>et al.</i> '03 [104] 26 severe persistent asthmatics anti-IL-5 Ab (i.v.) [baseline level]	↓ (80%)	N.E.	N.D.	N.D.	N.D.	trend ↑ (N.S.)	N.D.	N.E.

^aAbbreviations: BAL – bronchoalveolar lavage; FEV₁ – forced expiratory volume in 1 second (L/sec); Ab – antibody; i.v. – intravenous; N.D. – not determined; N.E. – no effect; N.S. – not significant; ↓ - decrease; ↑ - increase; PC₂₀ – provocative concentration of histamine causing a 20% fall in FEV₁ (mg/ml). ^b[XX] – indicates citation number. ^cIndicates the effect of treatment on the given readout; value in brackets indicates the percent change relative to placebo treated or baseline levels. ^dlate asthma response – maximum % fall in FEV₁.

pendent but IL-13 dependent [124]. Notably, fibrosis develops in this model as a consequence of a robust Th2-polarized chronic inflammatory response that is directed against the schistosome eggs. Whether the molecular regulation of fibrosis in the liver applies to airway fibrosis is uncertain. However, these findings illustrate that under certain circumstances IL-13 can, in fact, operate independently of TGF- β to drive Th2-associated reparative/fibrotic responses. It is interesting to note that both Schistosoma-induced hepatic fibrosis and HDM-induced airway remodeling develop independently of TGF- β and in response to chronic exposure to a complex Th2-inducing antigenic material, as opposed to OVA. On this basis, it is tempting to speculate that IL-13-dependent TGF- β -independent mechanisms may also underlie the development of airway remodeling induced by chronic HDM exposure. Further investigations into the role of IL-13 in HDM-induced remodeling, and in other models employing common environmental allergens, would not only be interesting from a mechanistic perspective but also from the standpoint of determining whether IL-13, unlike TGF- β and possibly eosinophils, is a critical mediator of remodeling in non-OVA-based systems.

Numerous studies have examined the impact of interfering with IL-13 function on AHR in the context of an acute allergen challenge protocol, all of which observed substantial if not complete reduction in AHR [22, 125-128]. In the case of chronic exposure protocols, studies employing IL-13-deficient mice or interfering with IL-13 activity throughout challenge also report marked reductions in AHR [11, 108, 109]. On the other hand, IL-13 neutralization initiated later during challenge has been shown to have limited to no effects [82, 110].

This outcome is relatively understandable given that AHR at chronic time points is likely attributable to the remodeling response, which does not seem to be reversible by IL-13 blockade once established. Together these reports indicate that IL-13 is involved in the development of AHR, at least in the context of the experimental regimens employed. This, coupled with the effects of IL-13 blockade on remodeling and allergic inflammation, implicate IL-13 as a multi-faceted mediator of allergic airway disease. However it would seem judicious to demonstrate the role of IL-13 in models of allergic disease employing common environmental allergens as this would strengthen its merit as a target for therapeutic intervention. As a cautionary note, based on the available evidence, we must expect that the therapeutic potential of IL-13 blocking strategies will be limited to the prevention, and not the reversal, of asthma-associated pathologies.

7. CONCLUDING PARTHIAN SHOTS

As exhibited in the preceding pages, a considerable number of studies have examined the role of TGF- β and eosinophils in allergic asthma remodeling as well as airway hyperresponsiveness. Detailed information is nearly overwhelming. Yet, an unprejudiced appraisal of the data makes it possible to discern what is 'messy' and what is not. The one issue that is clear, universally, that is regardless of the experimental system used, is that TGF- β regulates allergic airway inflammation. Indeed, there is nearly complete consensus that inhibition of TGF- β results in enhanced inflammation, mostly on the account of eosinophils. A second issue that is clear, although not universally, is that TGF- β drives the development of remodeling in OVA-based models; however, this is definitely not the case in other systems. For example, in HDM-based models

and Schistosoma-induced liver fibrosis. Somewhat unclear are the issues concerning the connection between TGF- β and eosinophils in experimental systems as well as in humans and, to a lesser extent, the role of eosinophils in OVA-induced remodeling. Some of the discrepancies are explicable; but, in other instances, they are not and, here, we suggest that there are messages in the "messiness".

Already elaborated upon is the message of context specific responses. We must appreciate that the biochemical diversity of common aeroallergens may engage distinct molecular pathways, despite the similarities in the resulting outcomes (i.e. remodeling). However, can allergen diversity also explain all the messy results? Perhaps not. For example, consider the discrepancies pertaining to the predominant cellular source of TGF- β in allergic disease. While some may be attributed to methodological differences (immunohistochemistry vs BAL, mRNA vs protein) perhaps a more enlightening assignment would be to regard the divergent results not as contradictory but as a reflection of the inherent heterogeneity of a dynamic process. This heterogeneity must be embraced as, arguably, the heterogeneity embodied by these experimental systems is but a fraction of the heterogeneity of human asthma in the real world.

What would be the benefits of accepting these predispositions? Perhaps the most conspicuous one would be that the expectation to find simple ecumenical answers about the role of a particular molecule or cell, or to the effect of a specific therapy, is candid at best and possibly misguided. Hence, it would appear that a productive endeavour would be to decode the determinants of disease heterogeneity as a way to uncover the conditions (or subsets of patients) under which a certain cell, molecule or therapy might have a distinctive impact.

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