

INFLAMMATION AND IMMUNOPATHOLOGY

## Key Regulators of Sensitization and Tolerance: GM-CSF, IL-10, TGF- $\beta$ and the Notch Signaling Pathway in Adjuvant-Free Experimental Models of Respiratory Allergy

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Conventional experimental models of respiratory allergy have contributed greatly to our current knowledge of the pathophysiology of allergic airway diseases; nevertheless, they are contingent upon unnatural sensitization techniques, entailing adjuvant-aided intraperitoneal (i.p) administration of antigen. Currently, there is a growing appreciation of the impact of tolerance mechanics in the pathophysiology of respiratory allergy. Thus, inasmuch as adjuvants exert a robust tolerance-modifying action, a transition from the conventional method of experimental sensitization to one that is more naturally and clinically relevant becomes important. We therefore opted to survey the literature and identify agents that could interfere with sensitization mechanics following non-adjuvant-aided airway exposure of laboratory rodents to aeroallergen. GM-CSF was found to exert robust Th<sub>2</sub>-polarizing action in this setting. Conversely, IL-10 fulfilled an important, albeit not so clear-cut, tolerance-favoring role; TGF- $\beta$  was also identified as a likely instigator of tolerogenesis. The role of Notch signaling in the sensitization *versus* tolerance dilemma appeared to be important but diverse. Collectively, these factors appeared to profoundly and diversely modulate the balance between tolerance and sensitization in naturally relevant experimental models of allergic airway disease.

**Keywords** adjuvant, aeroallergen, experimental protocol, sensitization, tolerance

### INTRODUCTION

Notwithstanding their immense contribution in yielding insights into the immunologic basis of respiratory allergy, rodent models are plagued by issues that hamper interprotocol consistency. The conventional (and unnatural) adjuvant-aided intraperitoneal (i.p) route of primary allergen administration prohibits reliable illustration of the mechanisms driving inhalational sensitization [1]. Indeed, adjuvant-aided experimental systems forcefully sensitize laboratory rodents to the employed allergen (regardless of it being innocuous or pervasive); this is attributed to the adjuvants capacity to direct the process of Th cell isotype switching, selectively favor IgE responses and create an immune-altering inflammatory background

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[2, 3]. Hence, such models consistently evoke a Th<sub>2</sub>-polarized response upon antigen re-exposure, as evidenced by the presence of hallmarks of allergy, i.e. production of Th<sub>2</sub> cytokines and IgE, blood and lung eosinophilia, goblet cell hyperplasia and bronchial hyper-reactivity. Nevertheless, such protocols – albeit prolific – are likely inappropriate for investigations on the subtle equilibrium between sensitization and tolerance in the naturally relevant setting. Therefore, experimental systems employing adjuvant-free respiratory exposure to inert allergen, would be more suitable for such studies; however, they typically fail in unambiguously eliciting an allergic response following re-exposure, presumably due to inability to achieve sensitization of rodents to said aeroallergen [4]. This precludes investigation of the pathophysiology of allergic responses [4]. Therefore, in the experimental setting, the capacity to omit the use of adjuvant – but importantly without forfeiting induction of a Th<sub>2</sub> response – has long been sought after. Furthermore, a recently proposed change in paradigm maintains that allergic sensitization could stem from a failure of tolerance mechanisms, as opposed to a sole Th<sub>1</sub>/Th<sub>2</sub> imbalance [5]. In this context, uncovering the tolerance-related immune effects of both conventional (i.p) and naturally relevant (airway) primary antigen exposure becomes important.

Within this conceptual frame, the main scope of this review is the presentation of pivotal immune-capable and tolerance-affecting agents and their effects following experimental implementation of naturally relevant sensitization techniques. Assessment of the action of these factors in the non-adjuvant-aided respiratory exposure platform may assist in dissecting the mechanisms underlying inhalation tolerance.

## LITERATURE SOURCES

Studies investigating the immune response following adjuvant-free sensitization are scarce, as opposed to those employing conventional models. Therefore, we set out to survey the literature and locate articles reporting successful sensitization and/or tolerance induction after the implementation of the former protocols. Pertinent original research studies that were published in English between 1980 and 2011 were selected through a Medline literature search. The compiled information was collated *versus* evidence stemming from conventional sensitization protocols, which were also identified and collected through our search.

Insofar as non-adjuvant-aided sensitization studies are comparably scarce, evidence in this context for numerous immunologically important players is lacking. Nevertheless, there are sufficient reports to allow discussion and hypothesis generating for three extensively researched cytokines, which have long been considered pivotal for inhalation tolerance, i.e. GM-CSF, IL-10 and TGF- $\beta$ . Furthermore, a recently identified signaling pathway, Notch, appeared to participate in protocol-dependent modulation of tolerance mechanics to a considerable extent and in a diverse fashion. The aforementioned factors will therefore be discussed in detail.

## GM-CSF

GM-CSF, a key hematopoietic cytokine, regulates the expansion of granulocyte and monocyte lineages and possesses robust adjuvant-like properties [6]. It is a powerful natural activator of macrophages and is indispensable for dendritic cell (DC) differentiation and maturation [7–9]. Furthermore, it enhances eosinophil survival and migration, upregulates macrophage expression of MHC-II molecules, facilitates expansion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and promotes bronchial accumulation of DCs; hence, it favors antigen presentation and allergen-specific T-cell activation [6, 9–11]. On the basis of these traits, it comes as no surprise that GM-CSF has been proposed to privilege an

allergic response over the homeostatic tolerogenic bias, by facilitating the development of a Th<sub>2</sub>-conducive bronchial milieu [7].

Indeed, the tolerance-regulating role of GM-CSF is rather clear-cut. In seminal experiments entailing non-adjuvant-aided respiratory antigen exposure, Jordana's group conclusively demonstrated that, in this setting, GM-CSF exerts robust Th<sub>2</sub>-polarizing and tolerance-averting action [1, 6, 9, 12–14]. GM-CSF is produced by various cells abundant in lung tissue including epithelial and endothelial cells, fibroblasts and macrophages [1, 15]. The latter are predominant in the bronchi [10] and expand following respiratory antigen exposure in a GM-CSF enriched milieu [1]. This suggests the involvement of GM-CSF in the elicitation of a self-sustaining immune response (Figure 1). Along these lines, a GM-CSF-induced perpetuation of the allergic/inflammatory cascade is further supported on the basis of the cytokines release from Th<sub>2</sub> cells and eosinophils [7]. Indeed, these cells congregate in large numbers in allergic inflammation sites.

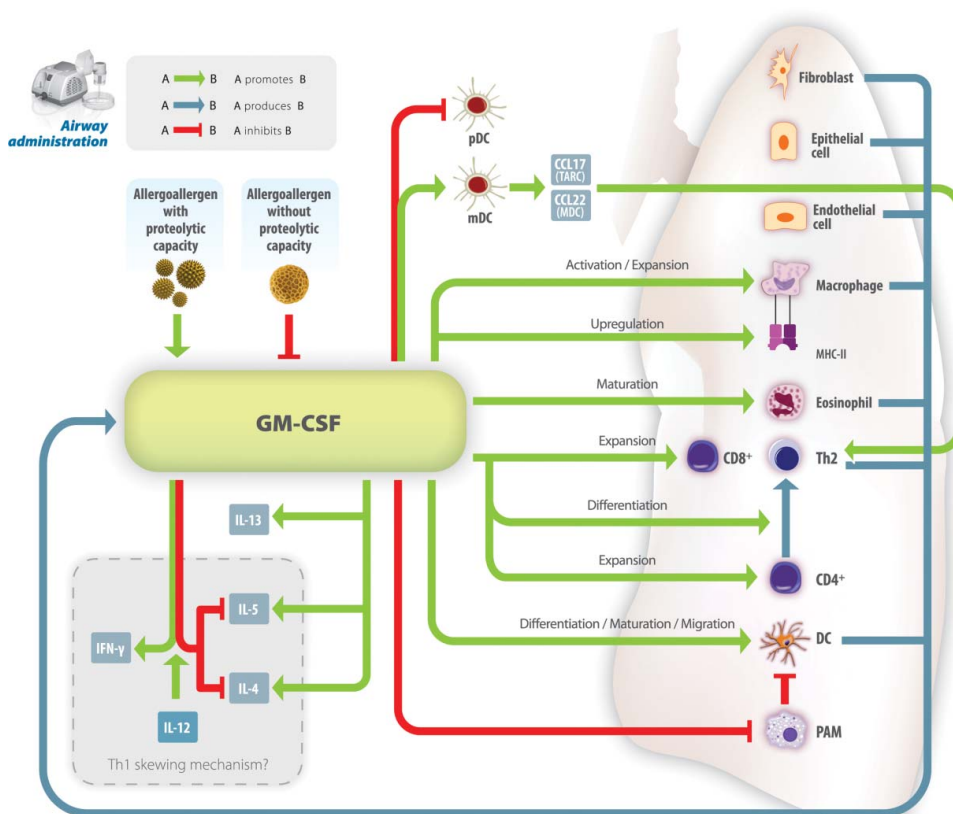


**Figure 1.** The involvement of GM-CSF in the sensitization/tolerance mechanism underlying naturally relevant exposure of laboratory rodents to antigen.

Aeroallergens with proteolytic capacity enhance GM-CSF release, as opposed to those lacking such a trait. GM-CSF upregulates Th<sub>2</sub>-favoring agents (macrophages, eosinophils, CD4<sup>+</sup> cells, dendritic cells/DCs) and downregulates those facilitating Th<sub>1</sub> polarization (pulmonary alveolar macrophages/PAM and Plasmacytoid dendritic cells/pDCs). Furthermore, it is produced by the former agents, hence fueling a self-sustaining system that perpetuates the allergic inflammatory cascade. Nevertheless, the effect of GM-CSF could be modulated by other immune-capable agents, as shown by the ability of IL-12 to bias its action toward a Th<sub>1</sub> response.

This sensitization-favoring effect of GM-CSF is likely multifaceted and driven by diverse mechanisms, which could include abrogation of the immunosuppressive activity of resident pulmonary alveolar macrophages (PAM) [15], increase in the production of IL-4, IL-5 and IL-13 [9], and/or a shift in the balance between myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) in favor of the former subset. From this perspective, van Rijt et al. [16] elegantly demonstrated that airway antigen challenge of mice sensitized in an adjuvant-free fashion lead to an increase of myeloid CD31<sup>hi</sup>Ly-6C<sup>neg</sup> DC progenitors; importantly, their co-culture with GM-CSF facilitated their differentiation into myeloid MHCII<sup>+</sup> CD11b<sup>+</sup>CD11c<sup>+</sup> DCs, which accumulated in the lungs of challenged rodents. To date, a number of mechanisms have been proposed to explain the tolerogenic inclination of plasmacytoid DCs – as opposed to myeloid DCs. These could include regulation of the function of mDCs by pDC-derived secreted or membrane proteins [17], pDC-mediated expansion of Tregs [18], pDC-induced downregulation of Th<sub>2</sub> cytokine secretion by T cells (IL-5, IL-10, IL-13), limited antigen processing and presentation efficiency, and low expression of MHC-II and co-stimulatory molecules [18]. Furthermore, pDCs antagonize the mDCs, which are known to release the Th<sub>2</sub>-selective chemokines TARC (CCL17) and MDC (CCL22). [12, 14, 17, 18]. In any event, compiling evidence supports a Th<sub>2</sub>-polarizing inclination of myeloid DCs, whose differentiation is promoted by GM-CSF [18].

GM-CSF production from airway epithelium can be triggered by aeroallergens that possess intrinsic proteolytic activity, such as House Dust Mite extract (HDM) [9, 19, 20]. It is now well documented that exposure to such aeroallergens can efficiently cause sensitization [8]. Intriguingly, co-exposure with HDM of an allergen devoid of proteolytic activity – such as OVA – overrides tolerance induction to the latter [21]; it thus appears that protease activity impacts on the bronchial epithelium in a nonantigen specific fashion. Several mechanisms could be underlying such an effect, including cleavage of the low-affinity IgE receptor (CD23) [22] and of the IL-2R $\alpha$  subunit (CD25) [23], modulation of the IL-4/IFN- $\gamma$  equilibrium [24], triggering of pattern recognition (TLR-4) and protease-activated/c-type lectin receptors [20, 25, 26], and enhanced allergen penetration and presentation due to proteolytic degradation of the epithelium tight junctions [27]. However, emerging evidence suggests that GM-CSF released from bronchial epithelial cells *via* antigenic proteolytic activity, may in fact be a prominent component of the mechanism underlying circumvention of inhalation tolerance [21, 28, 29]. Such a key sensitization-promoting role of epithelial-derived GM-CSF has been recently shown to be dependent on TLR-4-induced release of IL-1 $\alpha$  [30]. It is therefore conceivable that the inability of OVA (or of any other inert aeroallergen) to induce naturally relevant sensitization can be partially ascribed to lack of GM-CSF-triggering capabilities. However, this supposition warrants further investigation.

To date, the evidence pointing to an immune-enhancing Th<sub>2</sub>-polarizing effect of GM-CSF in the naturally relevant setting is compelling. Nevertheless, certain contextual flexibility should not be readily dismissed. Indeed, a GM-CSF-dependent IL-12-induced Th<sub>1</sub> skew consisting of a marked downregulation of IL-4 and IL-5 and upregulation of IFN- $\gamma$  production has been reported [13]. In any event, respiratory exposure to an otherwise innocuous aeroallergen in the context of GM-CSF-inducing stimuli will likely disrupt homeostatic balance, facilitate the development of a Th<sub>2</sub>-conducive milieu and promote allergic sensitization [7]. This renders potential GM-CSF-manipulating strategies a promising future prospect in regard to asthma prevention; indeed, compiling experimental evidence builds a strong case in favor of modalities targeting this prominent immune-capable cytokine, as a means to arrest the ongoing asthma epidemic.

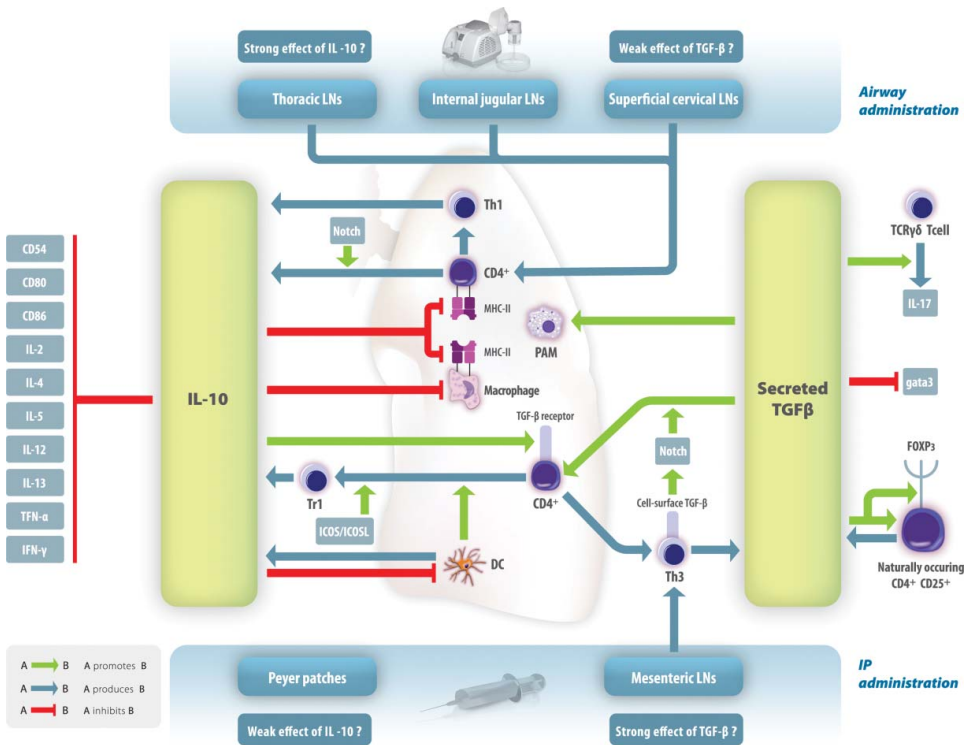


**Figure 2.** The involvement of IL-10 and TGF- $\beta$  in the sensitization/tolerance mechanism underlying naturally relevant exposure of laboratory rodents to antigen.

Following non-adjuvant-aided airway exposure of lab rodents to antigen, IL-10 downregulates a variety of Th<sub>2</sub>-polarizing agents (CD54, CD80, CD86, IL-2, IL-4, IL-5, MHC-II, DCs, etc). Accordingly, TGF- $\beta$  suppresses Th<sub>2</sub> responses *via* inhibition of GATA-3, downregulation of the action of pulmonary alveolar macrophages/PAM as well as *via* promotion of the effect of naturally occurring Tregs and the expression of FOXP 3. Airway exposure could favor IL-10-driven tolerance mechanics, whereas i.p administration could privilege a TGF- $\beta$ -dependent tolerogenic response. A Th<sub>1</sub>-favoring effect of Notch and ICOS/ICOS L is also depicted.

## IL-10

IL-10, an antiinflammatory agent with robust haematopoietic suppressive capabilities is secreted, among others, by bronchial DCs – including alveolar macrophages – and CD4<sup>+</sup> T cells [31–33]. IL-10 inhibits the antigen-presenting capacity of DCs and macrophages, downregulates the expression of MHC-II and co-stimulatory molecules, and potently inhibits the secretion of T cell-regulating cytokines and chemokines [32]. In effect, IL-10 was rapidly recognized as an integral part of peripheral tolerance (Figure 2). Indeed, IL-10-secreting, Foxp3<sup>+</sup>, induced (adaptive) regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells (termed Treg1) are thought to exert a robust immune inhibitory action [34]. Adaptive immunity fulfils an important role in inhalation tolerance mechanics through the action of these cells, which secrete considerable amounts of IL-10 at a rapid pace; hence, rendering themselves pivotal for immune downregulation [32]. Conversely, the role of IL-10-producing naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> cells is much less studied and currently is a field of controversy [35]. An IL-10-mediated tolerance mechanism was uncovered in various respiratory sensitization models [36–39]. Following i.n. OVA administration, upregulation of co-stimulatory molecules, appearance of IL-10-producing suppressive T cells and

IL-10-induced downregulation of IL-4, IL-5 and IL-13 have been reported [36, 40]. Accordingly, differentiation of IL-10-producing T cells and subsequent blunting of Th<sub>2</sub> responses has been shown after i.n. administration of Derp1 [41]. The capacity of IL-10, applied at the time of primary antigen exposure to long-lastingly abrogate bronchial inflammation and inhibit IL-4 and TNF- $\alpha$  production, was also demonstrated in this setting [39]. Intriguingly, this effect was unrelated to direct modulation of the immune response *per se*, as evidenced by normal expansion of CD4<sup>+</sup> and CD8<sup>+</sup> cells.

As opposed to these reports, in other respiratory exposure studies, tolerance induction was independent of IL-10 [42–48]. It is conceivable that this disparity may partly reflect distinct immune roles of IL-10-producing naturally occurring CD4<sup>+</sup> CD25<sup>+</sup> cells *versus* inducible CD4<sup>+</sup> CD25<sup>−</sup> (treg1) cells. For instance, in an adjuvant-aided experimental system, CD4<sup>+</sup> DC25<sup>+</sup> cells were shown to exert suppressive action, which was dependent on IL-10 [49]. Intriguingly however, IL-10 needed not be secreted by CD4<sup>+</sup> DC25<sup>+</sup> Tregs themselves; they rather provided the impetus for bronchial IL-10 production by other types of cells, which could conceivably include CD4<sup>+</sup> CD25<sup>−</sup> Tregs [49]. This is in line with an accumulating body of evidence ascribing a key role in inhalation tolerance to IL-10-producing inducible Tregs. Conversely, in a naturally relevant model, CD4<sup>+</sup> CD25<sup>+</sup> tregs exerted suppressive action by interacting with pulmonary DCs (*via* regulation of CD80/CD86/MHC-II expression and promotion of IL-12 production) but – importantly – not through an effect of IL-10 [50]. It is therefore plausible that the source of IL-10 (either naturally occurring or inducible Tregs) is of relevance to the immune outcome. This could partially reconcile conflicting findings in both the adjuvant-aided and adjuvant-free platforms. Strain-dependent modulation of the immune response [50] as well as a potential role of cell-contact interactions [51, 52] could also modify the mechanism underlying inhalation tolerance and dictate a necessity (or lack thereof) for IL-10.

In any event and notwithstanding this disparity of evidence, a tolerance-favoring inclination of IL-10 is a more tangible possibility in the naturally relevant experimental setting. Indeed, such seeming discrepancies could be further reconciled by the conjecture that in diverse microenvironments, cytokines homologous to IL-10 (for instance IL-19, IL-20, IL-22, IL-24 and IL-26) may partly reciprocate for its action [53]. The effect of IL-10 could also hinge upon the distinct phase of the allergic reaction (i.e. acute *vs.* chronic), as shown in conventional sensitization protocols [53, 54]. A dependency of IL-10 action on Notch [55], ICOS-ICOSL – signaling [5], antigen nature [56] and interaction with TGF- $\beta$  [57] perplexes evaluation of its immune effect. Furthermore, the techniques employed likely affected the immune outcome. For instance, administration of recombinant IL-10 *via* diverse routes prior to i.n. antigen challenge yielded different results regarding BALF cellular accumulation [54].

IL-10 is secreted by bronchial DCs and CD4<sup>+</sup> T cells [31, 32] and its target(s) are likely located in the airways [54]. Furthermore, IL-10 is probably more critical at the early stages of an immune response (antigen presentation and T cell communication) than at later stages (T effectors) [36]. It is therefore within reason to speculate that nonrespiratory sensitization (i.e. i.p. antigen administration) bypasses this line of defense. Indeed, nasal mucosa-draining lymph nodes – i.e. superficial cervical and internal jugular – are probably the predominant sites of tolerance induction following i.n. exposure [41, 43, 58]. Accordingly, early respiratory tolerance induction occurs selectively within the thoracic lymph nodes (LNs), wherein it is initially confined [48]. Thus, the above evidence collectively supports an integral role of IL-10 in airway-exposure-induced tolerance. Indeed, such unresponsiveness could predominantly be IL-10-driven, as opposed to tolerance induction in the gut, which could be more TGF- $\beta$ -dependent [36, 41].

In any case, and even though conflicting reports do exist in the i.p./adjuvant-aided platform [53, 54, 59–61], it is conceivable that a tolerance-favoring effect of IL-10 could be more potent when antigen is initially presented *via* airways, rather than systematically. This notion gains further weight in light of the well-documented dependence of the immune outcome (sensitization or tolerance) on the bronchial immunological milieu at the time of aeroallergen exposure [4, 8]. Indeed, the role of lung-based APCs in diversely driving these responses is prominent, as any condition that differentially affects their maturation, will likely promote the expansion of T cells with disparate action (regulatory *vs.* effector). For instance, respiratory exposure of immunologically naïve mice to OVA has lead to rapid accumulation of IL-10-producing pulmonary DCs within the bronchial lymph nodes; importantly, IL-10 originating from these DCs was critical for the induction of inhalation tolerance [36]. However, such a robust tolerogenic effect of DC-derived IL-10 cannot be taken for granted, as it is likely subjected to considerable modulation by changes in the bronchial microenvironment, i.e. immune perturbation brought about by viral infection as well as by exposure to airborne pollutants and ubiquitously present LPS [4]. Such a tolerance-related function of lung-based APCs could be further regulated by bronchial epithelial cells, *via* the their release of innate Th<sub>2</sub>-skewing cytokines after exposure to inhaled allergens (TSLP, IL-25, IL-33, etc.) [62, 63]

In any event, on the basis of the presented evidence, we surmise that an attempt to employ a bronchial immunosuppressive effect of IL-10, in order to contrive novel modalities for prevention of sensitization to aeroallergens, is a research goal worth pursuing. To date, production of this cytokine has been evoked through variable methods including pharmaceuticals (corticosteroids and other immunosuppressive drugs [64]) and/or infectious agents (exposure to mycobacteria [65] and heat-killed *Listeria monocytogenes* [66]). These approaches merit further investigation, as current evidence places IL-10 among the most promising candidates for a future mainstream asthma-preventive strategy.

### TGF- $\beta$

The TGF- $\beta$  superfamily consists of a multitude of regulatory proteins including five isoforms of TGF- $\beta$  (from TGF- $\beta$ 1 to TGF- $\beta$ 5) [67]. The prototype is TGF- $\beta$ 1, a pleiotropic anti-inflammatory and immunosuppressive cytokine and key regulator of immunological homeostasis [68]. TGF- $\beta$ 1 is secreted by many cells, including a subset of adaptive regulatory CD4<sup>+</sup>, Foxp3<sup>+</sup> T cells, termed Th<sub>3</sub> [69]. It impedes T-cell differentiation and function through downregulation of GATA3, T-bet and STAT4.

TGF- $\beta$ -dependent suppression of T cell responses has been reported in *in vitro* [37, 70] and *in vivo* studies [42]. Nevertheless, conflicting reports attest to the controversy surrounding TGF- $\beta$  tolerance-regulating role. For instance, Tsitoura et al. [46] demonstrated a redundancy of soluble TGF- $\beta$  for respiratory-exposure-induced tolerance. Along these lines, other investigators have put forward data suggesting that TGF- $\beta$  is superfluous to that end [56]. In an attempt to reconcile such findings, Nakamura et al. [71] provided *in vitro* data showing that CD4<sup>+</sup> CD25<sup>+</sup> T cells produce both *surface-bound* and *secreted* TGF- $\beta$ 1. Of relevance, *in vitro* systems of autoimmune diseases have furnished evidence suggesting a cell-contact-mediated tolerance mechanism (as opposed to a soluble-cytokine-dependent one) [72]. On this basis, Nakamura et al. [71] ascribed the conflicting reports on a prominent role of nonreadily detectable, non-secreted TGF- $\beta$ . Building on their premise, Ostrochova et al. [73] elegantly demonstrated that CD4<sup>+</sup> T cells, expressing cell-membrane-TGF- $\beta$ , were essential for tolerance induction in the respiratory exposure setting; conversely, soluble TGF- $\beta$  was not. Such an action could be contingent upon FOXP3 expression, which was observed only

in the TGF- $\beta$ -bearing cells; importantly, it could be downregulated by Th<sub>2</sub>-skewing agents such as alum.

TGF- $\beta$ -mediated tolerance in the primary respiratory exposure setting could be critically dependent on the local mucosal milieu [68]. Interestingly, both i.p and airway antigen administration primarily activate the thoracic LNs hence, perplexing discrimination of the tolerance mechanisms underlying these two methods [74]. Nevertheless, Th<sub>2</sub> effector function is likely carried out in the target organ rather than the regional LNs [75]. This highlights the importance of the differences between bronchial and i.p administration. Furthermore, i.p priming evokes Th<sub>2</sub> differentiation in Peyer's patches and mesenteric LNs [48], the latter being a main site of Th<sub>3</sub> cell production [5]. In keeping with that notion, in several i.p sensitization protocols, TGF- $\beta$  exerted robust tolerance-favoring action [60, 76], hence documenting an increased involvement in this setting. Alongside these spatial concerns, there are also temporal concerns. For instance, TGF- $\beta$  was unable to hinder cytokine production from already differentiated Th<sub>2</sub> cells [77], suggesting that it exerts its main effect at the early stages of the immune reaction. Therefore, its relative scarcity in the lung – as compared to the gut – will likely preclude a fast, and hence strong, tolerance favoring action, following respiratory antigen exposure.

Complex interactions of TGF- $\beta$  with other prominent immune-modulating agents add more layers of complexity in the evaluation of its effect. A likely TGF- $\beta$ /IL-10 synergy is suggested by an IL-10-driven augmentation of TGF- $\beta$  receptor expression [78] and a TGF- $\beta$ -induced IL-10 upregulation [79]. TGF- $\beta$ -dependent IL-17 production by TCR $\gamma\delta$  T cells [80] and a TGF- $\beta$ -induced modulation of PAM suppressive action [15] unravel further ways, whereby this cytokine could regulate tolerogenesis. Its presumed tolerance-skewing action in this setting was also shown to be critically dependent on activation of the Notch1 signaling pathway by cell-surface TGF- $\beta$  (Figure 2) [81].

To date, a considerable body of evidence supports a tolerance-instigating action of TGF- $\beta$ . A TGF- $\beta$  requirement for the *de novo* differentiation of IL-17-producing Th<sub>17</sub> cells could also fulfill a role in such mechanics [69, 82, 83]. Indeed, the Th<sub>17</sub> differentiation pathway is antagonistic to the Th<sub>2</sub> lineage [82]. IL-17 could also support mucosal barrier functions in the gut by stimulating tight junction formation [84]. Moreover, the mesenteric lymph nodes and the lamina propria of the intestine are enriched with Th<sub>17</sub> cells [82]; along these lines, IL-23, a cytokine important for Th<sub>17</sub> survival is principally produced in the intestinal ileum in mice [85]. This evidence provides an additional means whereby tolerance following i.p allergen administration could be predominantly depended on TGF- $\beta$ . From another perspective, antigen co-administration with a proinflammatory adjuvant – and thus creation of an inflammation-conducive microenvironment – could also modify the effect of DC-derived TGF- $\beta$ , further privileging differentiation of IL-17-producing CD4<sup>+</sup> T cells [86]; this has the potential to impede a robust Th<sub>2</sub> expansion. Hence, both components of a conventional experimental system (i.p administration and adjuvant utilization) may differentially impact on TGF- $\beta$ -mediated, Th<sub>17</sub>-related tolerance mechanics, as compared to a naturally relevant protocol.

On the basis of the above evidence, we propose that the effect of soluble TGF- $\beta$  could be conceivably impoverished in respiratory sensitization protocols in favor of cell-surface TGF- $\beta$  and IL-10. Indeed, IL-10-producing inducible Tregs (Treg1) are Foxp3 negative and do not require this marker to exert their action [82]; TGF- $\beta$  is however essential for the suppressive action and the expression of Foxp3 in peripheral naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> Foxp3<sup>+</sup> cells [87]. Thus, the supposition that Treg1 cells could be more important for respiratory-induced tolerance but less so for orally/i.p-induced (as articulated above), further implies a rather minor – if any – role of soluble TGF- $\beta$  in the naturally relevant setting. Conversely, the tolerance mechanics involved

in i.p administration protocols, could possibly rely on soluble TGF- $\beta$  on a greater extent. Such a protocol-dependent disparity in the magnitude of TGF- $\beta$ 's tolerogenic effect could be partially held accountable for discrepancies in pertinent findings.

## Notch

A highly conserved cell-fate-regulating pathway is mediated by a heterodimeric surface receptor termed Notch. Mammals express four Notch genes on mature lymphocytes (Notch 1–4) and two families of Notch ligands on APCs, i.e. Jagged (Jagged 1 and 2) and Delta-like (Dll) (Dll 1, 3 and 4) [88, 89]. Upon ligand-binding, Notch undergoes proteolytic cleavage resulting in the release of the intracellular domain (NICD), its nuclear translocation and subsequent activation of target genes [90].

To date, it is widely held that Notch signaling regulates TCR $\alpha\beta$  T cell and – especially – Th-cell lineage commitment [91–93]. Indeed, elicitation of a Th<sub>2</sub> response requires the pivotal transcription factor GATA-3, whose expression is regulated by Notch *in vitro* [90, 94]. Nevertheless, Notch's role across diverse experimental platforms is a field of intense controversy [93, 95, 96].

In *in vitro* models, Dll4 and Jagged1 were shown to direct Th<sub>1</sub> and Th<sub>2</sub> polarization, respectively [97, 98]. Along these lines, Dll4 upregulation privileged a Th<sub>1</sub>-conductive milieu; conversely, Jagged mediated Th<sub>2</sub> polarization [95]. Further *in vitro* evidence implicated Dll1- and Dll4-mediated signaling in Th<sub>1</sub> [98] and Th<sub>17</sub> [99] responses, conceivably through upregulation of IFN- $\gamma$  and/or downregulation of IL-4 production [95, 100, 101]. Accordingly, DC expression of Jagged, but not Dll ligands, induced Th<sub>2</sub> cell differentiation [95].

Conversely, in the *in vivo* setting, Jagged's action is disparate, insofar as the Jagged1 effect was ambiguous and Jagged2 was dispensable for Th<sub>2</sub> differentiation [88, 100, 102, 103]. In sharp contrast, the *in vivo* action of Dll4 is more straightforward and in accord with the *in vitro* studies. Indeed, Dll4-expressing Tregs downregulate IL-2 and favor Th<sub>1</sub> responses in adjuvant-aided models [100, 104].

To date, there is a scarcity of data pertaining to a potential Notch-dependent immune-modulation in naturally relevant protocols. Lamb's group [105] reported differential expression of Dll1 and Jagged1 ligands following i.n. antigen administration, as opposed to a lack of alterations following adjuvant-aided sensitization. The investigators proposed that following i.n. delivery, naive T cells receive Jagged1-dependent signals, which enabled them to differentiate into Treg cells, thus dampening pulmonary immune responses. This presumed Th<sub>1</sub>-polarizing effect of Jagged is in contrast with *in vitro* evidence from adjuvant-aided models, ascribing a Th<sub>2</sub>-favoring potential to it. Along these lines, Ray's group [81] elegantly demonstrated that inhibition of T cell activation triggered by i.n. antigen exposure was dependent on Notch1. The investigators reported elicitation of inhalation tolerance, which was likely driven by Jagged1-, Dll1- and Dll4-expressing CD4<sup>+</sup>Foxp3<sup>+</sup> cells. Although uncovering of ligand-specific action was not pursued, the robust expression of Jagged1 in the naturally relevant setting is in accord with the report by Hoyne et al. [105]. However, it again contrasts with Jagged's Th<sub>2</sub>-favoring inclination *in vitro*. Demonstrating substantial consistency, the Delta-like ligands were again correlated with a Th<sub>1</sub> skew. Finally, in a more recent report, Tan et al. [106] showed that – in a strictly non-adjuvant-aided model – i.n. sensitization with OVA significantly increased Jagged1 expression. In this setting, the authors proposed a dependency of Th<sub>1</sub>-polarization on Dll4, again congruent with its intraprotocol inclination (Figure 3).

Notch-dependent immune mechanisms are complex and still not clearly elucidated. Indeed, Notch downregulates TCR $\gamma\delta$  T cell differentiation and migration [55, 91, 96, 107], albeit conflicting results have been reported especially *in vitro* [92, 108, 109]. It is also required for the *in vitro* production of IL-10 [55] and may facilitate

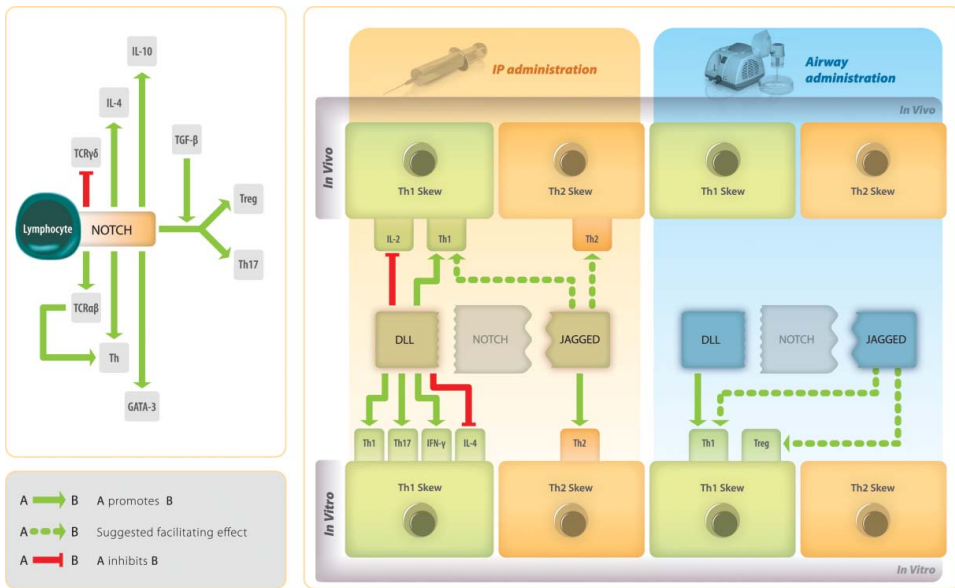


**Figure 3.** The involvement of Notch and its ligands in the sensitization/tolerance mechanism underlying conventional and naturally relevant exposure of laboratory rodents to antigen, *in vitro* and *in vivo*.

The immune effect of Notch signaling is multifaceted. Notch has been shown to upregulate both IL-4 and IL-10, as well as be necessary for GATA-3 expression. In a TGF- $\beta$  enriched milieu, Notch facilitated the differentiation of regulatory T cells (Treg). The Delta-Like Ligands (Dll) appear to consistently favor a Th<sub>1</sub> skew regardless of means of sensitization, both *in vitro* and *in vivo*. Conversely, in conventional models, Jagged ligands favor Th<sub>2</sub> polarization *in vitro*, albeit their action *in vivo* is diverse. As opposed to their Th<sub>2</sub> inclination in *in vitro* conventional models, Jagged ligands appear to privilege a Th<sub>1</sub> response in the naturally relevant setting.

Treg and Th17 differentiation in a TGF- $\beta$  enriched milieu [81, 110]. Furthermore, differences in timing could be critical. For instance, Notch action may be primarily exerted in the earliest stages of T cell development [91, 92]. Indeed, the early (in-trathymic) exon-1a is Notch-dependent; transcripts derived from it regulate GATA-3 expression [94]. Conversely, GATA-3-regulating exons, predominant at a later T cell-differentiation stage, are not dependent on Notch (i.e. exon-1b) [94]. Therefore, from a temporal perspective, the *in vitro* versus *in vivo* differences become of cardinal importance. *In vivo*-developed Th cells may already be committed to a certain cell line upon harvesting, therefore eluding Notch's *in vitro* surveillance and biasing further cell cultures toward a distinct Th phenotype [93]. In support of this contention, in the *in vivo* setting, Dll4 exerts its Th<sub>1</sub>-polarizing effect during the induction, but not the effector phase [100].

From another perspective, the strictly controlled *in vitro* setting, *ex vi termini* defies complex *in vivo* interactions of a plethora of Notch-bearing cells. It could therefore predispose to bias by virtue of its unique characteristics [102]. For instance, Dll – but not Jagged – ligands were shown to have a high *in vitro* affinity for mast cell receptors; this implies a potential Dll-driven Th<sub>1</sub> bias in this setting [111].

The presented evidence suggests that the immune microenvironment, wherein the reaction is evoked, heavily influences the effect of Notch signaling [81, 104, 112]. Therefore, it is conceivable that Notch's action could be manipulated *via* the use of adjuvants. Indeed, the Th<sub>2</sub>-adjuvant cholera toxin, stimulated Jagged2 expression in a

c-kit-dependent manner [113], whereas the Th<sub>1</sub>-adjuvant CpG oligodeoxynucleotide inhibited Jagged2 and promoted expression of Dll4 [114]. Nevertheless, although adjuvants could assist in the predominance of a specific subset, it is possible that they fail to cause a shift in its action. Indeed, the robust Th<sub>1</sub>-polarizing properties of Dll ligands transcend experimental platforms and could conceivably defy such perturbations. Dll effect is presumably related to downregulation of GATA3 and IL-2 mRNA [104] as well as IL-13 and IL-5 [112, 115]. On this basis, this potent effect could be part of a solidly structured negative feedback mechanism, assigned the task of limiting aberrant and exaggerated Th<sub>2</sub> responses [101, 104]. Conversely, Jagged1 presumed Th<sub>2</sub>-polarizing action appeared to be readily influenced by discrete protocol design; this implies an easily manipulated inductive – rather than inhibitory – Th<sub>2</sub>-skewing mechanism [90]. In any event, although the scarcity of reports does not allow for firm conclusions, Notch signaling appears to exert immune-regulating action in the naturally relevant setting, most likely in a tolerance-favoring fashion. It is therefore likely that the mechanism of tolerance induction, following exposure to antigen *via* the airways (as opposed to circumvention of tolerance in conventional experimental systems), could include a Notch-related component.

## CONCLUDING REMARKS

Conventional experimental protocols have thus far been invaluable for our investigation into the pathophysiology of respiratory allergy. Nevertheless, the importance of tolerance mechanics in the induction of allergic respiratory disease has only recently begun to be appreciated. In this context, a shift toward more naturally relevant experimental models becomes important, as it could inspire novel strategies to intercept sensitization to inert aeroallergen in the clinical field. However, the agents that perturb in this mechanism are numerous. CD8<sup>+</sup> cells and IFN- $\gamma$  are also likely involved, albeit, to date, the evidence is controversial [46, 47, 59, 60, 116, 117]. Other agents who warrant further research in this context include Thymic stromal Lymphopoietin [9], T-cell surface CTLA-4 [43, 118], the CXC chemokine Ip-10 [119], natural killer T cells [5, 34], pulmonary alveolar macrophages [11, 15, 60], the ICOS-ICOSL pathway [12, 61, 120, 121], C5aR-related signaling [122, 123], TCR $\gamma\delta$  T lymphocytes [124–127], as well as the Toll-like receptor (TLR)2, TLR4 and TLR7 signaling pathways [128–132]. In this review, we presented evidence and discussed in regard to the involvement in tolerance mechanics, of four important immune-capable agents; we also appraised their regulatory effect on protocol-dependent immune outcomes. GM-SCF clearly favored sensitization in the adjuvant-free experimental platform, whereas IL-10 appeared to be a keen instigator of tolerogenesis. A tolerance-supporting action of TGF- $\beta$  (and especially cell-surface TGF- $\beta$ ) was demonstrated in this setting, albeit it was likely less potent/important than that of IL-10. The effect of Notch-mediated signaling was ambiguous and likely diversely driven by ligand-specific differences.

## Declaration of Interest

George Guibas and Michael Makris declare no conflicts of interest. Nikolaos Papadopoulos has received payments for consultancy from ABBOT, Novartis, Menarini; for lectures from MSD, URIACH, GSK, Allergopharma, Stallergenens; and for development of educational presentations from MSD, URIACH, MEDA. The authors alone are responsible for the content and writing of the article.

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