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Innate Signals from Nod2 Block Respiratory Tolerance and Program Th2 Driven Allergic Inflammation

Wei Duan, PhD, **Amit K. Mehta, PhD**, **Joao G. Magalhaes, PhD**[1], **Steven F. Ziegler, PhD**[2], **Chen Dong, PhD**[3], **Dana J. Philpott, PhD**[1], and **Michael Croft, PhD**
Division of Molecular Immunology, La Jolla Institute for Allergy and Immunology, La Jolla, California.

[1]Department of Immunology, University of Toronto, Toronto, Canada.

[2]Benaroya Research Institute, Seattle, Washington.

[3]Department of Immunology, University of Texas MD Anderson Cancer Center, Houston, Texas.

Abstract

Background—Airway tolerance is critical for protecting the lung from inflammatory disease driven by allergens. However, factors that disrupt tolerance processes and then lead to susceptibility to developing allergic asthma remain elusive.

Objective—To investigate whether recognition of bacterial microbial-associated molecular patterns in the lung may result in susceptibility to developing allergic reactions, and to understand the molecular mechanisms by which such triggers block natural tolerance.

Methods—Ligands of intracellular microbial-associated molecular pattern recognition receptors – the Nod-like receptors (NLRs), Nod1 and Nod2 - were given intranasally with antigen and their ability to modulate airway tolerance was analyzed.

Results—Intranasal Nod2 ligand rapidly induced lung expression of the innate cytokines TSLP and IL-25, and TSLP promoted expression of OX40L, a T cell costimulatory ligand, on lung CD11c+CD11b+ cells and B220+ cells. Together these three molecules blocked the generation of antigen-specific CD4+Foxp3+ adaptive Treg, and concomitantly drove IL-4-producing CD4 T cells. By altering the Treg/Th2 balance, tolerance was blocked and sensing of Nod2 ligand then resulted in subsequent susceptibility to developing eosinophil-dominated airway inflammation.

Conclusion—We show that a Nod-like receptor is a novel, previously unrecognized, pathway that adversely links innate and adaptive immunity and leads to allergic disease and asthmatic lung inflammation.

Keywords

Mouse; Nod2; asthma; TSLP; OX40L; IL-25; regulatory T cell

Corresponding author: Michael Croft, La Jolla Institute for Allergy and Immunology, 9420 Athena Circle, La Jolla, CA 92037, USA. Phone: (858) 752-6731, Fax: (858) 752 6986, mick@liai.org.

Key Messages

- Nod2 activation in the airways prevents tolerance to inhaled allergen
- Nod2 induces the innate cytokines TSLP and IL-25, and OX40L in the lung
- Nod2 activation blocks induction of CD4+Foxp3+ adaptive Treg and promotes IL-4+CD4+ T cells.

Introduction

Early immune responses to pathogens are triggered by pattern-recognition receptors (PRRs) that recognize microbial-associated molecular patterns (MAMPs). Toll-like receptors (TLR) can stimulate antigen-presenting cells to secrete inflammatory cytokines like IL-1, IFN-I, TNF, and IL-12, and to express high levels of class II and costimulatory ligands, that allow effective Th1 and Th17 responses [1]. In contrast, how Th2 responses are triggered has been less well studied, and especially whether direct or indirect recognition of microbial products would lead to susceptibility to developing allergic disease. In this regard, it has been hypothesized that the propensity to develop this Th2-driven inflammation of the lung associates with the level of microbial exposure and how clean or dirty the environment is during the formative years of development [2].

Capsule Summary

We found that bacterial peptidoglycan derivatives that target the intracellular Nod2 pattern recognition receptor are sufficient for blocking airway tolerance, via a combined action in suppressing the generation of Foxp3+ Treg and promoting Th2-effector T cells.

Logically TLR engagement that can lead to secretion of IFN-I and IL-12 should not result in allergic disease as these cytokines can direct Th1 differentiation. However, moderate TLR4 signaling can promote Th2 cells [3], and LPS can lead to susceptibility to developing asthmatic inflammation [4, 5]. Moreover, house dust mite antigen was shown to mimic a component of the TLR4 signaling complex [6]. This has led to renewed interest into how asthma and allergic disease is controlled by pattern recognition receptors. Mucosal surfaces of the respiratory tract represent a major portal of entry for airborne allergens as well as bacteria, viruses, and microbial products [7]. This suggests that the epithelium of the lung could be a critical regulatory element for controlling subsequent immune responses. In line with this, it was found that bronchial epithelial cells expressed TLR4, and signaling via radioresistant lung structural cells, that included the epithelial cells, was required for LPS, and house dust mite extract that contained LPS, to promote subsequent asthmatic lung inflammation when inhaled [5]. Thus, expression of certain pattern recognition receptors by non-hematopoietic cells might be key in controlling the susceptibility to Th2-biased disease [8].

Another class of PRR's are the intracellular Nod-like receptors (NLRs) [9]. Nod1 and Nod2 detect motifs of bacterial peptidoglycans, with Nod1 activated by mesodiaminopimelic acid (meso-DAP) and Nod2 by muramyl dipeptide (MDP). Meso-DAP is expressed by most Gram negative bacteria and MDP by most Gram positive and Gram negative bacteria, and both molecules can initiate pro-inflammatory responses inducing the release of cytokines like IL-1, TNF, and IL-6 [10]. Moreover, MDP was previously observed to be able to prevent oral tolerance and increase intraepithelial lymphocyte infiltration in the intestine mucosa [11]. Interestingly, both Nod1 and Nod2 are highly expressed in epithelial cells suggesting that, similar to TLR4, they could mediate the crosstalk between epithelial cells and other immune cells in the airway. In addition, it was recently found that Nod2 can play a critical role in sensing viral ssRNA and mediate certain anti-viral activities after infection with RSV and VSV [12]. As RSV has also been linked to susceptibility to asthma [13-15], this further suggests that Nod1 or Nod2 could be central to eliciting immunologic mechanisms that link allergen exposure in the context of bacterial or viral infection to induction or exacerbation of allergic airway disease.

Here, we show that airway exposure to Nod2 ligand but not Nod1 ligand prevented tolerance mechanisms from developing in the lung, suppressing the induction of antigen-specific

CD4+Foxp3+Treg cells while at the same time promoting IL-4-secreting effector CD4 T cells. Nod2 ligand resulted in selective expression of the innate cytokines TSLP and IL-25, and TSLP-dependent induction of the TNF family costimulatory molecule OX40L. Nod2 signaling subsequently resulted in full susceptibility to develop asthmatic disease, which was blocked by targeting TSLP, IL-25, or OX40L. Individually, OX40L, TSLP, and IL-25 have each been reported to promote asthmatic inflammation [16-20]. Therefore, Nod2 by engaging all of these factors fully programs the immune response to diverge from tolerance toward Th2 immunity and adverse allergic disease.

Methods

Mice

C57BL/6 and B6.PLThy1a (Thy1.1) mice were from The Jackson Laboratory.

Airway tolerance and Allergic Airway Inflammation

Airway tolerance was induced similar to already described protocols [4].

Bronchoalveolar lavage and Lung Histology

BAL was performed 24 h after the last OVA aerosol challenge. BAL fluid was examined for cytokine content by ELISA. For lung histology examination, 5-μm sections were cut and stained with H&E for examining cell infiltration. Magnification x200 was used for histologic scoring and at least 5 fields were scored to obtain the average for each mouse.

Adoptive transfer

Naïve OVA-specific CD25- CD4 cells were isolated from spleen of OT-II mice with CD4 T Cell Isolation Kits (Miltenyi Biotec, CA). 5×10^6 OT-II CD4 cells were injected i.v. into B6.PL Thy1.1 congenic mice, which were then exposed to soluble OVA or OVA mixed with Nod2 given i.n.

RT and real-time PCR

Lung tissue was homogenized with TISSUE MASTER (OMNI International, GA). Total RNA was isolated using the TRIzol reagent (Invitrogen). An aliquot of total RNA (5 μg) was reverse-transcribed to cDNA using the SuperScript III (Invitrogen). Data are presented as normalized to ribosomal protein housekeeping gene *L32*.

ELISA

Murine cytokines in BAL fluid were assayed by ELISA using paired Abs, according to the manufacturers' recommendations.

Statistical analysis

Where appropriate, data was analyzed using the Student's *t* test. Unless otherwise indicated, data represent the mean ± SEM. $P < 0.05$ was considered significant and indicated by *.

Results

Nod2 prevents induction of airway tolerance

The ability of inhaled Nod ligands to block tolerance mechanisms in the lung is highly relevant to clinical allergic disease. To test this, mice were exposed to inhaled soluble antigen (in the absence of any adjuvant), given intranasally once a day for 3 days (Fig. S1). As shown before [4, 21, 22], this results in tolerance and completely blocked the susceptibility

of these mice for developing asthmatic lung inflammation when they were subsequently immunized and challenged with antigen using a conventional protocol that promotes functional Th2 cells (Fig. 1). Significantly, we found that inhalation of the Nod2 agonist MDP with soluble antigen essentially abrogated airway tolerance. This led to full susceptibility to developing Th2-type lung inflammation, with eosinophilia (Fig. 1a), peribronchial cell infiltration in lung parenchyma (Fig. 1b), airway hyperresponsiveness (Fig. S2), and Th2 cytokines in BAL (Fig. 1c-e) that resembled the response in naïve mice that were initially exposed to PBS but not exposed to airborne antigen (Figs. 1). This effect was solely mediated by Nod2 activation as Nod2 KO mice did not respond to MDP (Fig S3). In contrast, the Nod1 agonist had little effect on preventing airway tolerance from developing, and this was regardless of the dose inhaled (Figs. 1 and not shown). The Nod2 agonist itself did not induce lung inflammation when inhaled alone in the absence of antigen (Fig. S4). Moreover, MDP only prevented tolerance when inhaled at the same time as antigen, and not before or after, suggesting that Nod2 activation induced molecules that were relevant to driving initial activation and differentiation of T cells when antigen was first presented (Fig. S5).

Nod2 prevents induction of airway tolerance through OX40L

We previously reported that the interaction of the TNFR-TNF family molecules OX40 and OX40L is crucial to the development of Th2-driven lung inflammation when mice were exposed systemically to antigen mixed with alum [16, 17]. OX40 signals can both promote naïve CD4 T cells to differentiate into the Th2 lineage [23], as well as control the survival and expansion of effector and memory Th2 cells [17]. To investigate whether Nod2 resulted in engagement of OX40, we injected a single dose of an OX40L blocking antibody at the time of exposure to inhaled Nod2 ligand mixed with antigen. Importantly, this strongly inhibited the subsequent development of airway eosinophilia (Fig 2a), bronchovascular infiltrates (Fig 2b), and Th2 cytokines in the lung (Fig 2c, 2d and 2e), to approximately baseline levels that were found in control mice that were tolerized by inhalation of soluble antigen (Figs. 2a-e). These data show that Nod2 activation results in engagement of the OX40L-OX40 pathway to initiate the Th2 response in the lung and antagonize induction of tolerance.

Inhaled Nod2 agonist suppresses generation of antigen-specific Foxp3+ T cells and alters the Treg:IL-4+ T cell ratio through OX40L

Our, and others, prior data has shown that OX40 signaling can block expression of Foxp3 in responding naïve CD4 T cells and hence affect the number of adaptive Tregs that can form [24, 25]. We have also previously shown that the early ratio of antigen-specific Foxp3+ Treg to IL-4+ effector T cells strongly correlates with the subsequent fate of the lung [4]. Because the frequency of antigen-reactive CD4 T cells is very low, we tracked their initial response by adoptively transferring naïve Thy1.2+ CD4+CD25- T cells from OT-II TCR transgenic mice into Thy1.1+ recipients. Significantly, approximately 50% fewer antigen-specific Foxp3+ Treg were generated over 5 days in mice that inhaled the Nod2 agonist (Fig 3a). Simultaneously, IL-4-secreting CD4 T cells were analyzed by ELISPOT, the most sensitive assay for this function. Whereas very few IL-4+ CD4 T cells were found under tolerizing conditions with soluble antigen alone, Nod2 activation promoted the strong accumulation of these effector-type T cells over the same time period after Nod2 ligand exposure (Fig. 3b). Combining the two sets of data allowed a rough estimate of the ratio of induced antigen-specific Treg to that of induced early effector-like T cells. As we previously reported [4], this was biased under conditions that led to tolerance in favor of Treg, such that they outnumbered IL-4+ T cells by approximately 100:1. Treatment with Nod2 ligand strongly lowered this ratio to less than 10:1. In contrast, and correlating with the lack of activity in blocking airway tolerance, activation of Nod1 did not inhibit the generation of antigen-

specific Foxp3+ Treg and did not significantly induce IL-4-secreting cells, with the result that the ratio of these T cell subsets remained largely unchanged (Fig 3c).

Furthermore, blocking OX40L at the time of inhalation of the Nod2 ligand produced a dramatic effect in that it not only neutralized the suppressive action of Nod2 on Foxp3, but resulted in 3-4-fold more Foxp3+ adaptive Treg being generated over this initial 5 day period. Coordinately, preventing OX40L interactions inhibited the appearance of IL-4-secreting cells, such that the approximate ratio of Foxp3+ to IL-4+ T cells was even more strongly skewed in favor of the Treg (Fig 3c). This strongly contrasted with the effect of blocking OX40L in mice exposed to the Nod1 ligand, where little difference was observed and the ratio of Treg to IL-4+ T cells remained unchanged. Thus, the ability of the Nod2 agonist to block airway tolerance was completely dependent on OX40-OX40L interactions that dictated the early balance between adaptive Treg and effector T cells. We found that there was increased expression of OX40L mRNA in the lung as early as 3 hours after inhalation of the Nod2 agonist, whereas little OX40L mRNA was found in naïve mice, or mice exposed intranasally to soluble antigen, or mice exposed to antigen and the Nod1 agonist (Fig 3d). Correlating with this, OX40L was visualized at 18 hours on CD11c +CD11b+ cells (Fig. 3e) and B220+ cells (Fig. 3f) in the lungs of mice that inhaled Nod2 ligand with antigen, but was not detected on the same cells in mice that inhaled antigen alone, or antigen with Nod1 ligand (Fig. 3e and 3f).

TSLP is required for Nod2 to prevent induction of airway tolerance

Because of the rapid induction of OX40L in the lung after Nod2 stimulation, this suggested that the epithelial cells or other non-hematopoietic cells of the respiratory tract could play a role as they are the principal cells that would initially encounter inhaled Nod2 ligand. In this regard, thymic stromal lymphopoietin (TSLP) is an innate cytokine that can be produced by lung epithelial cells, fibroblasts, and smooth muscle cells [26, 27]. Transgenic mice that express TSLP in the lung, under the control of the surfactant protein C promoter, spontaneously developed asthma-like disease [18]. Furthermore, TSLP was reported to induce dendritic cells to mature into a Th2-promoting APC in vitro [27], and this activity was later found to be due to the induction of OX40L [28]. Correlating with this, intranasal administration of recombinant TSLP promoted Th2-driven lung inflammation in vivo that was blocked by inhibiting OX40L activity [29].

We therefore screened an array of inflammatory mediators in lung extracts by RT-PCR, including assessing TSLP expression. Kinetic studies showed that mRNA levels of all cytokines analyzed reached a peak at 3 hours after inhalation of Nod2 agonist (data not shown). Interestingly, both Nod1 and Nod2 agonists promoted equivalent levels of IL-6, TNF, IL-1α and IL-1β (Fig. S6), showing that the lack of activity of Nod1 was not due to defective expression in the lung, and that both agonists could be recognized in the lung compartment. In contrast, Nod2 activation promoted significantly more TSLP mRNA expression in the lung compared to Nod1 activation (Fig 4a), and this was supported by increased TSLP protein levels in BAL detected 36 hours after Nod inhalation with antigen (Fig 4b). In the absence of TSLPR in gene-deficient mice, inhaled Nod2 failed to promote eosinophilia (Fig. 4c, and Fig. S7), or any signs of broncho-vascular infiltration (Fig. 4d, and Fig. S7) and Th2 cytokines (not shown). However, TSLPR-deficient mice were also unable to mount an inflammatory response even when not tolerized (Fig. S7), showing a downstream role for TSLP as well. Because of this, we assessed early parameters that might be affected by TSLP during the induction of tolerance and active regulation. Notably, the Nod2 ligand failed to induce OX40L expression in the lungs of TSLPR-deficient mice measured at 3 hours (Fig. 4e). Furthermore, Nod2 activation promoted rather than suppressed the development of adaptive Foxp3+ Treg in the absence of TSLPR, measured over the initial 5 days of exposure to intranasal OVA, and early IL-4 producing T cells were

not found, resulting in an immune response highly skewed toward Treg and tolerance (Figs. 4f-h). This shows that Nod2 inhalation immediately promotes TSLP that is active in regulating OX40L expression and that this then controls the development of a suppressive environment. Although this link between TSLP and OX40L has been discussed for some time [30, 31], this is the first in vivo evidence that physiological induction of endogenous TSLP regulates OX40L and that this process coordinately antagonizes the induction of airway tolerance.

IL-25 is necessary for Nod2 agonist to prevent induction of airway tolerance

Along with TSLP, we also found that IL-25 mRNA in the lung at 3 hours, and protein in BAL at 24 hours, were induced after inhalation of Nod2 ligand with antigen (Fig. 5a and b, respectively). The relative levels were higher than those in mice exposed to Nod1 ligand. IL-25, also known as IL-17E, is another innate cytokine that can be produced by bronchial epithelial cells [20], as well as other cells including eosinophils, basophils, and mast cells [32]. IL-25R is expressed on naïve T cells, and effector and memory Th2 cells, and IL-25 can enhance Th2 differentiation [19, 20, 33, 34]. Furthermore, transgenic mice expressing IL-25 in lung epithelium, under the control of the CC10 promoter, exhibited spontaneous asthma-like lung inflammation [19, 20].

IL-25 neutralizing antibody was given in a single dose at the time of exposure to Nod2 ligand. Mimicking the phenotype when OX40L was blocked, and in TSLPR-deficient mice, we found that inhibiting IL-25 essentially ablated the ability of Nod2 stimulation to overcome tolerance and lead to Th2 priming, eosinophilia, and lung inflammation (Fig. 5c and d, and not shown). In contrast to the absence of TSLPR signaling, blocking IL-25 had no effect on the early induction of OX40L in the lung at 3 hours (Fig. 5e). However, IL-25 was still required for suppression of Treg development and promotion of early IL-4-secreting T cells that were generated over the initial 5 days following inhalation of Nod2 (Figs. 5f-h). Thus, Nod2 activation in the lung can strongly influence airway tolerance and development of allergic disease. Both TSLP and IL-25 contribute to the ability of Nod2 signals to antagonize tolerance and lead to asthmatic lung inflammation, in part through promoting OX40-OX40L interactions and in part through independent activities likely directed to responding antigen-reactive naïve CD4 T cells.

Discussion

Genetic predisposition and environmental triggers have been proposed to be risk factors that lead to asthma and allergic disease [35]. By finding that bacterial peptidoglycan derivatives that target the intracellular Nod2 pattern recognition receptor were sufficient for blocking the induction of airway tolerance and eliciting Th2-driven lung inflammation, our data now uncover a new susceptibility factor for development of allergic asthma.

Many candidate genes have been proposed to associate with asthma and allergy, including genes relevant to innate immunity, epithelial biology, and mucosal adaptive immunity [35]. The intracellular pattern recognition receptors Nod1 and Nod2 have not only been linked with atopic disease [36, 37], but also with other complex diseases such as Crohn's disease [38], implying there may be a fundamental role of innate NLR in regulating mucosal immunity. In line with this, exogenous treatment with MDP was found to prevent oral tolerance and increase lymphocyte infiltration in the intestine mucosa [11]. Nod1 and Nod2 activation can result in primary antigen-specific Th2 responses when their ligands are given systemically in the peritoneal cavity [39, 40], and these data can also be viewed as evidence in support of the broad association studies. Interestingly, however, in the airway, Nod2 and Nod1 had differential activity, with only Nod2 signals blocking tolerance. Inhalation of both Nod1 and Nod2 ligands induced the pro-inflammatory cytokines IL-6, TNF, and IL-1, leading to the

conclusion that these factors alone are not sufficient to suppress the generation of Treg and initiate a productive Th2 response. Instead, we conclusively showed that Nod2 displayed these functional activities by promoting TSLP, OX40L, and IL-25 expression.

We have previously shown that TLR4-driven asthmatic lung inflammation initiated by intranasal exposure to LPS is additionally dependent on OX40-OX40L interactions [4]. Intratracheal injection of LPS, or house dust mite extract that contains LPS, was further found to promote lung expression of IL-25 and/or TSLP [5], and we have confirmed that inhalation of LPS results in equivalent induction of TSLP and IL-25 compared to inhalation of MDP (data not shown). This implies that TLR4 signals also can promote the same program as Nod2 signals, although data demonstrating the requirement for TSLP and IL-25 in breaking airway tolerance after TLR4 triggering are lacking. However, combined these data suggest it is likely that Nod2 and TLR4 signals in the lung lead to common intracellular pathways that result in TSLP and IL-25 production, and subsequently TSLP-induced OX40L expression. The source, or sources, of TSLP and IL-25 are unclear. TLR4 signals in the non-hematopoietic compartment, presumably to radioresistant lung structural cells, were shown necessary for LPS to induce Th2 cells and inflammation [5]. Therefore, in the lung, the bronchial epithelial cell is a likely candidate for producing TSLP and IL-25, although other nonhematopoietic and structural cells such as stromal cells, fibroblasts, or smooth muscle cells, are all possibilities for receiving Nod2 as well as TLR4 signals and hence being a source of these cytokines. TNF and IL-1β can promote TSLP production by human airway epithelial cells in vitro [41], and we did observe increased TNF and IL-1β expression after Nod2 was inhaled. However, Nod1 equally led to induction of these cytokines without resulting in strong TSLP expression or the induction of a Th2 response, implying that the epithelial cell might not be the only source of TSLP in the lung or more likely that other signals, perhaps direct and provided by Nod2, are required for TSLP production. IL-25 was first found to be a product of memory Th2 cells [33], but it can also be produced by airway epithelial cells, as well as several other lymphoid cells [20, 32]. Therefore, again there may not be one source of IL-25 that is promoted by Nod2.

One downstream target of TSLP is likely an APC such as a dendritic cell [28]. Nod2 activation resulted in OX40L expression on CD11c+ cells and B220+ cells in the lung, and this was fully dependent on TSLP. Furthermore, dendritic cells have been proposed to initiate the development of pro-allergic asthmatic reactions [42]. TSLP was reported to directly stimulate T cells [43]. However, we favor the primary action of TSLP in our studies is in promoting OX40L, as Nod2 suppression of Treg development and induction of early IL-4-secreting cells was completely dependent on host TSLPR, with neither of the latter activities being evident when wild-type (TSLPR+) transgenic T cells were responding in the knockout mice. In contrast, IL-25 did not affect OX40L expression in the lung, but it was required for determining the early balance between Foxp3+ Treg and IL-4-secreting effector T cells. Therefore, it is likely that IL-25 acts at least in part directly on the T cells providing further synergistic signals that integrate with those from OX40-OX40L interactions.

Our data lead to the question of whether Nod2 ligands play an important role in initiation or exacerbation of human asthma. In mouse models, this could be partially addressed by assessing responses to human allergens. However, there is a strong caveat to these studies in that responses to such natural allergens are generally addressed through the use of manufactured extracts, such as from *house dust mite*, *alternaria*, and *aspergillus*, which is unlikely to be the form in which the antigenic proteins from these species are encountered normally. Furthermore, these extracts might be contaminated during the extraction procedure, leading to responses dominated by the contaminant rather than a natural MAMP. For example, although house dust mite antigen has been found to possess a mimic of MD2, a component of TLR4, the response to this antigen or an extract of house dust mite has still

been shown to require LPS [6], which is presumably not from house dust mite itself. Correlating with our previous data showing that LPS can prevent airway tolerance induction in an OX40L-dependent manner [4], it has been found that lung inflammatory responses to house dust mite extract are also dependent on OX40L[44]. However, rather than the human allergen itself being unique, and possessing a Nod2 ligand, a more plausible hypothesis is that this MAMP, or an agent that would trigger Nod2 activation, would be provided during infection with bacteria such as Streptococcus pneumoniae and viruses such as Respiratory syncytial virus, which have been linked to asthma susceptibility or exacerbation [13-15, 45]. MDP is expressed in peptidoglycans of most Gram negative and Gram positive bacteria, and viral ssRNA was recently found to activate Nod2 [12]. Therefore, future studies assessing the requirement for Nod2 during lung inflammatory responses initiated by, or with a concomitant, bacterial or virus infection will be most relevant to further understanding the role of Nod2 in susceptibility to human asthma.

In summary, our data support the idea that the recognition of microbial products by innate receptors plays a critical role in regulating adaptive immunity in the airway mucosa. The hygiene hypothesis, first proposed by Strachan in 1989, posited that immune responses elicited by microbial compounds in childhood protect against the development of allergic diseases [46]. However this simple view has been challenged by evidence, including that provided here, which shows that activation of the immune system by microbial products may be a causative factor for the development of allergies and asthma. Ultimately, the exposure of humans to microbial products will be highly complex. TLR2 and TLR4 have been studied most extensively in terms of allergic disease but without any consensus regarding their activity. As mentioned above, TLR4 can certainly support Th2 cells and allergic asthma, but this is a dose dependent activity [3-5]. Similarly, TLR2 can have a Th2-biasing adjuvant activity, and can promote airway eosinophilia and hyperreactivity [47-49]. However, there are reports of TLR4 and TLR2 antagonizing asthma progression [50-53]. This is probably related to the level of signaling through these receptors, the ratio of hematopoietic to non-hematopoietic cells that are directly stimulated, and the innate inflammatory factors initially induced. Similarly, we found a dose-dependent effect of Nod2 in that a two-fold higher dose given intranasally did not break tolerance and lead to asthma susceptibility (Fig. S8). Very high doses of LPS favor IL-12 production and Th1 responses, which may not lead to TSLP and IL-25 secretion or may simply override the activity of these cytokines. Whether Nod2 stimulation will follow a similar paradigm is not clear. However, it is likely that the ultimate phenotype of the immune response, and propensity to diverge to allergic disease, will be determined by the dominant innate immunological cascade that is induced by exposure to either NLR and TLR ligands and the key to developing diseases such as asthma will lie in the concomitant or stepwise induction of TSLP, OX40L, and IL-25.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

NLRs	Nod-like receptors
PRRs	pattern-recognition receptors
MAMPs	microbial-associated molecular patterns
PAMPs	pathogen-associated molecular patterns
TLR	Toll-like receptors
MDP	muramyl dipeptide
iTreg	inducible regulatory T cell

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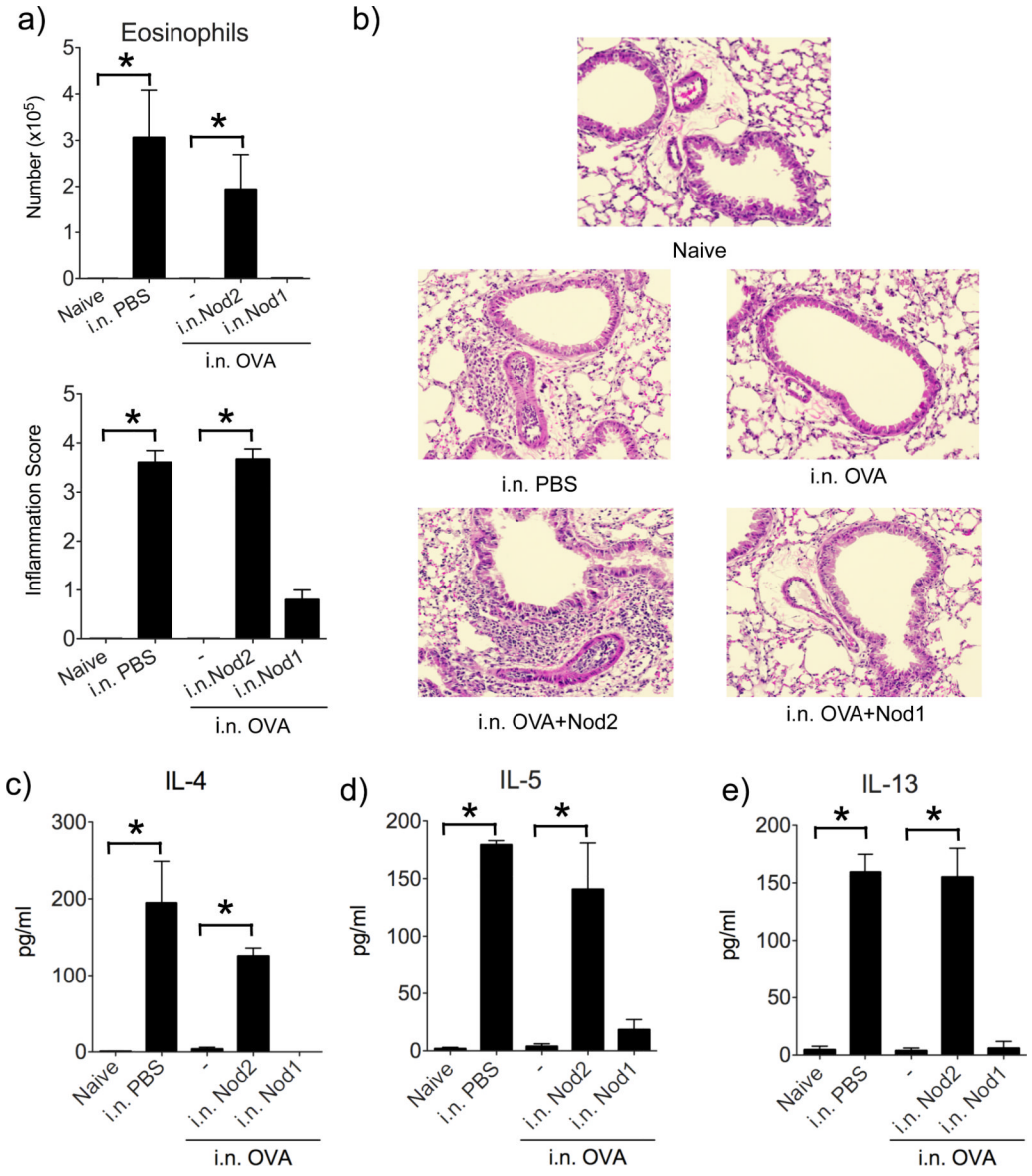

Figure 1. Inhaled Nod2 agonist prevents induction of airway tolerance

Wt B6 mice were tolerized by exposure to i.n. OVA. 9 days later, all mice were immunized with OVA/alum i.p., and then challenged 14 days afterwards with OVA aerosol for 4 days. Lung inflammation was analyzed 24h later. (a) Eosinophils in BAL. (b) H&E staining of lung sections (Right). Inflammation score (Left). (c) IL-4, (d) IL-5 and (e) IL-13 in BAL. All results are means ± SEM from 4 mice per group.

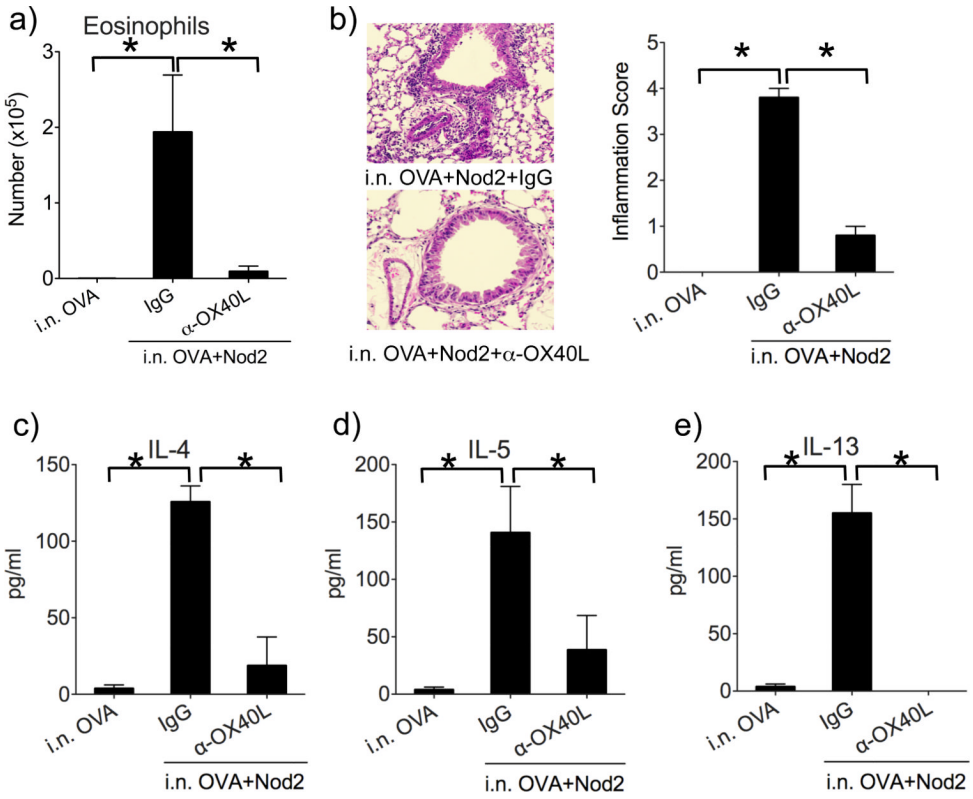

Figure 2. Inhaled Nod2 agonist prevents airway tolerance through OX40L

Wt mice were tolerized, immunized and challenged as in Fig.1. Anti-OX40L (200 μg) or control IgG was given i.p. at the time of Nod2 agonist treatment. (a) Eosinophils in BAL. (b) H&E staining of lung sections (Left). Inflammation score (Right). (c) IL-4, (d) IL-5 and (e) IL-13 in BAL. All results are means ± SEM from 4 mice per group.

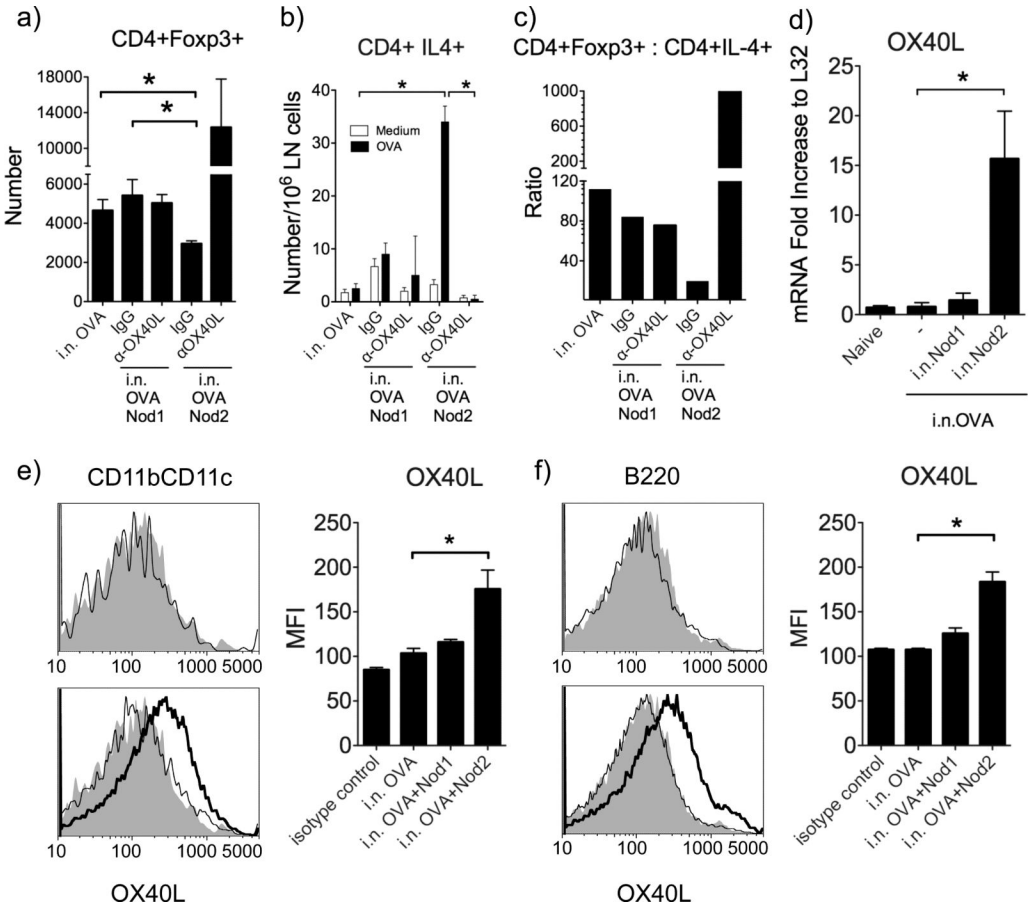

Figure 3. Inhaled Nod2 agonist suppresses generation of antigen-specific Foxp3+ T cells and alters the Treg:IL-4+ T cell ratio through OX40L

Thy1.2+ OT-II T cells were transferred into Thy1.1 mice. Recipients were then tolerized/challenged as in Fig. 1. (a) Number of Foxp3+ OT-II T cells in pooled LN. (b) Number of IL-4-secreting CD4 T cells. (c) The ratio of Foxp3+ and IL-4-secreting CD4+ cells. (d) OX40L mRNA in lung tissue. (e-f) OX40L expression on CD11c+CD11b+ cells (e) and B220+ cells (f) in lung tissue, OVA with Nod2 (bottom, thick line), OVA with Nod1 (bottom, thin line), OVA alone (top, thin line) and isotype control (gray shade). MFI of data plotted in histograms. All results are means ± SEM from 4 mice per group.

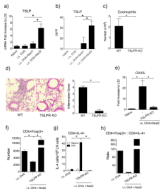

Figure 4. TSLP is required for Nod2 agonist to prevent airway tolerance

(a-b) Wt mice were tolerized/challenged as described in Fig. 1. (a) TSLP mRNA in lung tissue. (b) TSLP protein in BAL. (c-e) Wt and TLSPR knockout mice were challenged with OVA plus Nod2 agonist as in Fig. 1. (c) Eosinophils in BAL (d) H&E staining of lung sections (Left). Inflammation score (right). (e) OX40L mRNA in lung tissue. (f-h) OT-II T cells were transferred into wt or TSLPR knockout mice. Recipients were tolerized/ challenged as above. (f) Number of Foxp3+ OT-II T cells in pooled LN. (g) Number of IL-4-secreting CD4 T cells. (h) Ratio of Foxp3+ and IL-4-secreting CD4+ cells. All results are means ± SEM from 4 mice per group.

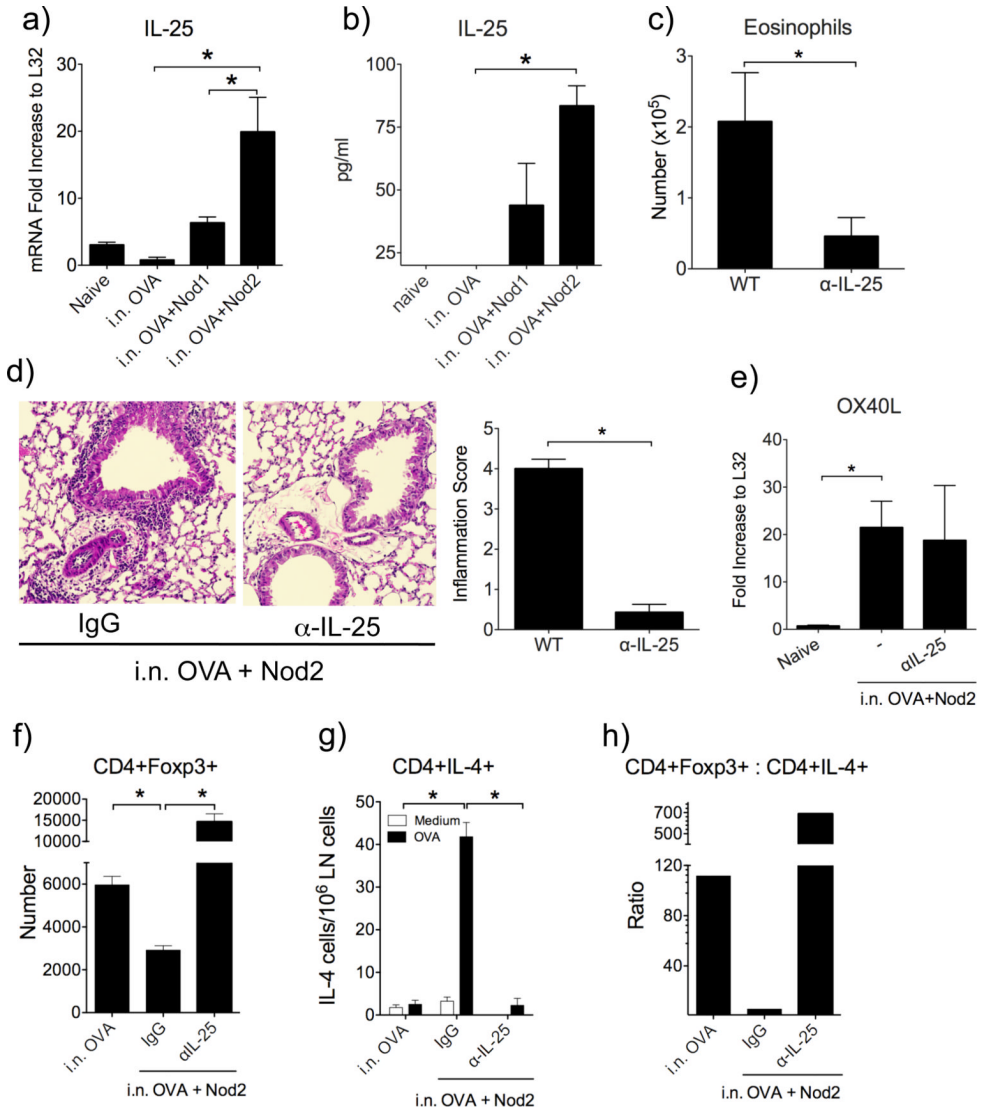

Figure 5. IL-25 is necessary for Nod2 agonist to prevent airway tolerance

(a-b) Wt mice were tolerized/challenged as described in Fig. 1. (a) IL-25 mRNA in lung tissue, and (b) IL-25 protein in BAL. (c-h) Mice were challenged with OVA plus Nod2 agonist as in Fig. 1, and treated with anti-IL-25 or control IgG. (c) Eosinophils in BAL (d) H&E staining of lung sections (Left). Inflammation score (right). (e) OX40L mRNA expression. (f-h) OT-II T cells were transferred into Thy 1.1+ mice. Recipients were tolerized/challenged as above. (f) Number of Foxp3+ OT-II T cells in pooled LN. (g) Number of IL-4-secreting CD4 T cells. (h) The ratio of Foxp3+ and IL-4-secreting CD4+ cells. All results are means ± SEM from 4 mice per group.