Innate signals from Nod2 block respiratory tolerance and program T_H2-driven allergic inflammation

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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 nucleotide-binding oligomerization domain (Nod)-like receptors, 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 thymic stromal lymphopoietin and IL-25, and thymic stromal lymphopoietin promoted expression of OX40 ligand, a T-cell–costimulatory ligand, on lung $CD11c^+CD11b^+$ cells and $B220^+$ cells. Together these 3 molecules blocked the generation of antigen-specific $CD4^+$forkhead box protein 3^+ adaptive regulatory T cells and concomitantly drove IL-4–producing CD4 T cells. By altering the regulatory T/T_H2–cell balance, tolerance was blocked, and sensing of Nod2 ligand 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. (J Allergy Clin Immunol 2010;126:1284-93.)

Key words: *Mouse, Nod2, asthma, TSLP, OX40L, IL-25, regulatory T cell*

Abbreviations used

BAL:	Bronchoalveolar lavage
FOXP3:	Forkhead box protein 3
H&E:	Hematoxylin and eosin
MAMP:	Microbial-associated molecular pattern
MDP:	Muramyl dipeptide
meso-DAP:	Meso-diaminopimelic acid
NLR:	Nod-like receptor
Nod:	Nucleotide-binding oligomerization domain
OVA:	Ovalbumin
OX40L:	OX40 ligand
TLR:	Toll-like receptor
Treg:	Regulatory T
TSLP:	Thymic stromal lymphopoietin

Early immune responses to pathogens are triggered by pattern recognition receptors that recognize microbial-associated molecular patterns (MAMPs). Toll-like receptors (TLRs) 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 T_H1 and T_H17 responses.[1] In contrast, how T_H2 responses are triggered has been less well studied, 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 T_H2-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]

Logically, TLR engagement that can lead to secretion of IFN-I and IL-12 should not result in allergic disease, because these cytokines can direct T_H1 differentiation. However, moderate TLR4 signaling can promote T_H2 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 are 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 radio-resistant lung structural cells, which 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 nonhematopoietic cells might be key in controlling the susceptibility to T_H2-biased disease.[8]

Another class of pattern recognition receptors is the intracellular nucleotide-binding oligomerization domain (Nod)-like receptors (NLRs).[9] Nod1 and Nod2 detect motifs of bacterial

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peptidoglycans, with Nod1 activated by meso-diaminopimelic 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 proinflammatory 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 cross-talk 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 single-stranded RNA and mediate certain antiviral activities after infection with respiratory syncytial virus and vesicular stomatitis virus.[12] Because respiratory syncytial virus 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 $CD4^+$ forkhead box protein 3 $(FOXP3)^+$ regulatory T (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 thymic stromal lymphopoietin (TSLP) and IL-25 and TSLP-dependent induction of the TNF family costimulatory molecule OX40 ligand (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 T_H2 immunity and adverse allergic disease.

METHODS

Mice

C57BL/6 and B6.PLThy1a (Thy1.1) mice were from The Jackson Laboratory (Bar Harbor, Me). See this article's Methods section in the Online Repository at www.jacionline.org for details.

Airway tolerance and allergic airway inflammation

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

Bronchoalveolar lavage and lung histology

Bronchoalveolar lavage (BAL) was performed 24 hours after the last ovalbumin (OVA) aerosol challenge. BAL fluid was examined for cytokine content by ELISA. For lung histology examination, 5-μm sections were cut and stained with hematoxylin and eosin (H&E) for examining cell infiltration. Magnification ×200 was used for histologic scoring, and at least 5 fields were scored to obtain the average for each mouse.

Adoptive transfer

Naive OVA-specific CD25− CD4 cells were isolated from spleen of OT-II mice with CD4 T Cell Isolation Kits (Miltenyi Biotec, San Diego, Calif). A total of 5×10^6 OT-II CD4 cells were injected intravenously into B6.PL Thy1.1 congenic mice, which were then exposed to soluble OVA or OVA mixed with Nod2 given intranasally.

RT-PCR and real-time PCR

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

ELISA

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

Statistical analysis

Where appropriate, data were analyzed by using the Student *t* test. Unless otherwise indicated, data represent the means ± SEMs. $P < .05$ was considered significant and is indicated by an asterisk.

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 (see this article's Fig E1 in the Online Repository at www.jacionline.org). 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 by using a conventional protocol that promotes functional T_H2 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 T_H2-type lung inflammation, with eosinophilia (Fig 1, *A*), peribronchial cell infiltration in lung parenchyma (Fig 1, *B*), airway hyperresponsiveness (see this article's Fig E2 in the Online Repository at www.jacionline.org), and T_H2 cytokines in BAL (Fig 1, *C-E*) that resembled the response in naive mice that were initially exposed to PBS but not exposed to airborne antigen (Fig 1). This effect was solely mediated by Nod2 activation because Nod2 knockout mice did not respond to MDP (see this article's Fig E3 in the Online Repository at www.jacionline.org). In contrast, the Nod1 agonist had little effect on preventing airway tolerance from developing, and this was the result regardless of the dose inhaled (Fig 1; data not shown). The Nod2 agonist itself did not induce lung inflammation when inhaled alone in the absence of antigen (see this article's Fig E4 in the Online Repository at www.jacionline.org). Moreover, MDP prevented tolerance only 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 (see this article's Fig E5 in the Online Repository at www.jacionline.org).

Nod2 prevents induction of airway tolerance through OX40L

We previously reported that the interaction of the tumor necrosis factor receptor-TNF family molecules OX40 and OX40L was crucial to the development of T_H2-driven lung inflammation when mice were exposed systemically to antigen mixed with alum.[16,17] OX40 signals can both promote naive CD4 T cells to differentiate into the T_H2 lineage[23] and control