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Antagonism of Airway Tolerance by Endotoxin/Lipopolysaccharide through Promoting OX40L and Suppressing Antigen-Specific Foxp3+ T Regulatory Cells

Wei Duan, Takanori So, and Michael Croft

Respiratory exposure to allergens can lead to airway tolerance. Factors that antagonize tolerance mechanisms in the lung might result in susceptibility to diseases such as asthma. We show that inhalation of endotoxin/LPS with Ag prevented airway tolerance and abolished protection from T cell-driven asthmatic lung inflammation. Under conditions leading to tolerance, adaptive Ag-specific CD4+Foxp3+ T regulatory cells (Treg) were generated following exposure to intranasal Ag and outnumbered IL-4- and IFN-γ-producing CD4 T cells by 100:1 or greater. Inhaled LPS altered the ratio of Treg to IL-4+ or IFN-γ+ T cells by concomitantly suppressing Treg generation and promoting effector T cell generation. LPS induced OX40L expression on dendritic cells and B cells that resulted in a synergistic activity between TLR4 and OX40 signals, leading to production of IL-4, IFN-γ, and IL-6, which blocked Treg development. Furthermore, inhibiting OX40/OX40L interactions prevented LPS from suppressing tolerance, and resulted in the generation of greater numbers of adaptive Treg. Thus, cooperation between TLR4 and OX40 controls susceptibility to developing airway disease via modulating the balance between adaptive Treg and IL-4+ or IFN-γ+ T cells. Targeting OX40L then has the potential to improve the efficacy of Ag immunotherapy to promote tolerance.


A

irway exposure to harmless environmental Ags can lead to a state of tolerance, and the phenomenon of tolerance to inhaled Ag is being used in the clinic as the basis of allergen/Ag immunotherapy. The same environmental Ags that can lead to tolerance, in the case of asthatics, can also lead to an Ag-specific Th2-biased immune response, resulting in lung inflammation characterized by airway eosinophilia and mucus hypersecretion (1, 2). The precise mechanisms that regulate tolerance and those that lead to the breakdown of inhalation tolerance are then of significant interest. In particular, regulatory T cells (Treg)3 have been suggested to be critical in controlling the immune response after exposure to inhaled Ag, either suppressing existing pathogenic Th2 cells, or possibly being responsible for maintaining a tolerant state such that Th2 populations cannot be generated effectively (3, 4).

Both innate and adaptive immune systems most likely modulate these Ag-specific airway responses. TLR are key regulators of both innate and adaptive immunity. In particular, exposure to the TLR4 ligand, LPS/endotoxin, can strongly affect the intensity and type of airway disease (5). In some cases, it has been suggested that the level of LPS in the environment is related to the severity of Th2 lung inflammation (6), and experimentally suggested mechanisms that might account for this include enhancing mast cell activation or directly increasing Th2 responses (7), and promoting Th2 cell recruitment to the lung (8, 9). However, it has also been reported that LPS can suppress ongoing allergic Th2 inflammation (10, 11). Clinical studies additionally proposed that exposure to LPS in early childhood may decrease the incidence of asthma later in life (12). Adding further complexity, the amount of LPS and TLR4 signals encountered can determine whether Th1 or Th2 types of lung inflammatory responses will be generated (13). Therefore, LPS can either lead to susceptibility to asthma, exacerbate the severity of asthma, or protect from asthma, possibly dictated by the timing of exposure and the level of exposure (14).

TLR signaling might modulate adaptive immune responses through the activation and maturation of APC. Activated APC up-regulate costimulatory molecules and secrete inflammatory cytokines, priming naive T cells into effector T cells. The interactions between costimulatory ligands and their receptors are therefore likely to be critical for generating large populations of effector T cells. OX40 (CD134), a member of the TNFR superfamily, is one costimulatory receptor that is expressed by activated T cells (15). OX40 interacts with its TNF family ligand, OX40L, which is induced on professional APC such as dendritic cells, B cells, and macrophages, after their activation (16–18). OX40 signaling strongly regulates T cell division, survival, and cytokine release (19–21). Recently, we, and others, found that OX40 also can inhibit the development of adaptive Foxp3+ Treg that differentiate from naive CD4 T cell populations in response to TGF-β (22, 23). This indicates a possible dual role for OX40 in promoting effector T cells and antagonizing the induction of adaptive Treg. We previously demonstrated that OX40 plays a critically important role in driving the expansion of memory Th2 cells that mediate the effector phase of asthmatic lung inflammation, shown with studies of OX40 knockout (KO) mice and by blocking an ongoing allergic

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3 Abbreviations used in this paper: Treg, regulatory T cell; BAL, bronchoalveolar lavage; i.n., intranasal; KO, knockout; MCC, moth cytochrome c; wt, wild type.
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response with anti-OX40L (24, 25). Whether the presence or absence of OX40 signaling also controls the induction of tolerance mechanisms has not been investigated. OX40L expression can be promoted on APC by LPS/TLR4 signals (26), raising the possibility that TLR4 and OX40 might synergize together and lead to a breakdown in tolerance at the level of Treg that could be particularly important in terms of airway exposure to environmental Ags and susceptibility to developing diseases such as asthma.

In this study, we show in a model of respiratory tolerance, mimicking allergen/Ag immunotherapy, that low-level exposure to LPS prevented tolerance mechanisms from being established, resulting in the subsequent inability to control the development of strong lung inflammatory responses. The break in tolerance mediated by LPS was dependent on OX40-OX40L interactions and involved suppressing the induction of Ag-specific CD4+ Foxp3+ Treg while concomitantly promoting IL-4- and IFN-γ-secreting effector CD4+ T cells. We found a direct synergy between TLR4 and OX40 signals in controlling the Treg balance that involved inflammatory cytokines, including IL-6. Furthermore, blocking OX40 signals restored and even enhanced the generation of adaptive Foxp3+ Treg, suppressing the action of LPS. Together, these data show that intranasal (i.n.) exposure to LPS/endotoxin leads to the interaction between OX40L and OX40, which, with other inflammatory effects of TLR4 signaling, alters the balance between Foxp3+ Treg and effector T cells, and influences susceptibility to allergic inflammatory disease. This suggests that preventing OX40-OX40L interactions might be useful to enhance the effectiveness of i.n. allergen/Ag immunotherapy and ensure the successful induction of tolerance through generating adaptive Treg.

Materials and Methods

Mice

C57BL/6 and B6.PLThy1a (Thy1.1) mice were from The Jackson Laboratory. OT-II TCR transgenic mice, bred on the BL/6 background, were used as a source of Vβ3/Vα2/Thy1.2 CD4 T cells reactive to the peptide OVA-323–339. AND TCR transgenic mice were bred on a B10.BR background, which had been precoated with 2 μg/ml anti-mouse IFN-γ or IL-4 mAb (BD Biosciences). In vitro Treg culture Ex vivo ELISPOT assay

In vitro Treg culture

Naive CD8+ T cells from wild-type (wt) or OX40−/− AND mice were plated at 3 × 105 cells/ml with 3 × 105/ml DCEK (DAP3.3C1 transfected with I-Ek) fibroblast APC, which expressed I-Ek, CD80, OX40L, and 0.5 μM MCC 88–103 peptide in the presence or absence of 1 ng/ml human rTGF-β1 (PeproTech) for 3 days. For blocking experiments, anti-IL-4 (11B11, 0.5 μg/ml), anti-IFN-γ (XMG1.2, 10 μg/ml), or anti-IL-6 (MP5-20F3, 10 μg/ml; BioLegend) was added. Membrane CD25 and intracellular Foxp3 expression on gated CD4 cells after 3 days culture was evaluated as in prior studies (22).

Results

LPS prevents airway tolerance in an OX40-dependent manner

To assess factors that might antagonize the induction of airway tolerance, we used a model in which mice were exposed to Ag given i.n. for 3 consecutive days, similar to previous published regimens that lead to tolerance (28). Inhaled Ag led to suppression of the development of airway inflammation, determined by the lack of response when mice were later immunized with Ag in alum. Low/Moderate doses of LPS, particularly notable with exposure to 1 μg, given i.n., prevented Ag-induced tolerance, as shown by subsequent immunization and challenge with the same Ag now resulting in eosinophilia in the airway (Fig. 1a), production of Th2 cytokines such as IL-4 (and IL-5 and IL-13; data not shown) in BAL (Fig. 1a), and strong lung inflammatory infiltrates (Fig. 1c).
Eosinophils in BAL; Left 24 h. LPS was given at different doses i.n. at the time of tolerance induction. Afterwards with OVA aerosol for 4 days. Inflammation was analyzed after mice were immunized with OVA in alum i.p., and then challenged 14 days after. 

**Antagonism of airway tolerance by LPS is dependent on** 

**FIGURE 1.** 8652 LPS REGULATES ADAPTIVE Treg VIA OX40-OX40L/H9262 OVA (100 μg) in PBS given i.n. for 3 consecutive days. Nine days later, eosinophilia and IL-4 were analyzed in BAL. Results are the mean levels ± SEM from four mice per group. 

**Materials and Methods.** All results are means ± SEM from four mice per group. a. The wt or OX40 KO mice were exposed to OVA and LPS given i.n. for 3 days, and then IL-4 was analyzed in BAL. Results are the mean levels ± SEM from four mice per group. e. The mice were exposed to OVA and LPS given i.n. for 3 days, and anti-OX40L was given at the same time. Mice were subsequently sensitized and challenged as in a, and eosinophilia and IL-4 were analyzed in BAL. Results are the mean levels ± SEM from four mice per group. All results are representative of at least two experiments. In all cases, similar results compared with IL-4 were seen for IL-5 and IL-13 (data not shown).
Thy1.1 recipients. These mice were then subjected to soluble OVA given i.n. to induce airway tolerance. Flow analysis demonstrated that ~4% of OT-II CD4 cells converted into Foxp3 T cells in vivo (Fig. 2a). This is in line with other published studies (34) that have seen a similar low percentage of Foxp3 Treg being induced from naive T cells in oral tolerance models. The response appeared to be systemic in that OVA-specific Foxp3 T cells were induced in lymph nodes, spleen, and lung (data not shown). Minor conversion was also seen in the absence of Ag, as has been shown to occur in other systems (35). The OVA-specific Foxp3 T cell population enlarged in total numbers over time, being visualized within 1 day of Ag exposure, but reaching a peak by day 5, and then contracting (Fig. 2b). Similar data were obtained analyzing lymph node or lung tissue. During this period, we found no significant change (expansion or contraction) in numbers of natural Treg when analyzing the endogenous CD4 population (data not shown).

In line with tolerance being mediated by the Ag-specific Treg, we found that when mice exposed to i.n. OVA were then sensitized and challenged with MCC, an unrelated protein, no suppression was evident and MCC induced a similar level of eosinophil infiltration (and Th2 cytokines; data not shown) regardless of prior exposure to OVA (Fig. 2c). Due to the low frequency of the induced Ag-specific Foxp3 cells making their isolation impractical, we studied their function using cells generated in vitro from naive OT-II T cells driven by TGF-β. CD4 CD25 Foxp3 cells were sorted from the in vitro cultures (containing 85% Foxp3 cells; data not shown) and transferred into OVA-sensitized mice that were subsequently challenged with OVA via the Airways to induce lung inflammation. A total of 1 x 10^6 of these adaptive Treg substantially inhibited the induction of eosinophilia in the airways and Th2 cytokines in BAL (Fig. 2d, and data not shown). Further implying that CD25 Foxp3 Treg mediate i.t. tolerance, we found that transiently depleting CD25 cells with anti-CD25 shortly before exposure to i.n. OVA prevented the induction of tolerance (Fig. 2e, airway eosinophilia; Th2 cytokines; data not shown). Greater lung infiltration was subsequently observed in mice treated with anti-CD25 compared with PBS-treated nontolerized control mice, most likely indicating that natural CD25 Foxp3 Treg activity also contributes to the overall level of inflammation, even though the number of these cells does not change upon Ag exposure. Lastly, neutralization of TGF-β with Ab during the induction phase of tolerance also subsequently allowed development of eosinophilia in the airways, and elevated BAL Th2 cytokine levels (Fig. 2f, and data not shown). This coincided with a near complete block in the generation of adaptive Ag-specific Foxp3 cells as visualized 5 days after exposure to i.n. OVA (Fig. 2g). Collectively, these data strongly suggest that Foxp3 adaptive Treg, induced from naive CD4 T cells by Ag taken up in the airways and endogenously produced TGF-β, represent a primary and essential control mechanism that mediates airway tolerance.

**LPS regulates the airway by altering the balance between Foxp3 Treg and effector cells in an OX40-dependent manner**

We then addressed whether LPS controlled the generation of adaptive Foxp3 Treg and whether OX40L played a role. Mice were exposed to a dose of LPS that prevented the induction of airway
LPS REGULATES ADAPTIVE Treg VIA OX40-OX40L

FIGURE 3. LPS suppresses induction of Ag-specific Foxp3T cells and modulates the Treg:IL-4 T cell ratio in an OX40-dependent way. Naive CD4+CD25− cells isolated from either wt or OX40 KO Thy1.2 OT-II TCR transgenic mice were transferred into wt Thy1.1 mice. Recipients were then tolerated with OVA (no LPS) or exposed to 1 μg of LPS with OVA (i.n. LPS), as described in Fig. 1. One group was given anti-OX40L (200 μg, i.p.) during the period of tolerance induction. Analyses were performed 5 days after the initial i.n. OVA instillation. a. Number of Foxp3+ OT-II CD4 cells at day 5 in lymph nodes. Data are means ± SEM from four mice per group. b and c. Total number of IL-4-secreting and IFN-γ-secreting CD4+ cells, detected by ELISPOT. Data are mean numbers ± SEM from four mice per group and represent either spontaneous cytokine secretion (medium) or induced in vitro with Ag (OVA). d and e. The ratio of Foxp3+ CD4+ cells to IL-4-secreting CD4+ cells (d) and IFN-γ-secreting CD4+ cells (e) was estimated from data in a–c. All results are representative of at least two experiments.

T cells under tolerizing conditions, we could not calculate a ratio, but it was at least 1000:1 in favor of Treg (Fig. 3e). Exposure to OVA with LPS strongly altered this balance with the ratio of Foxp3+ IL-4+ CD4 cells and Foxp3+ IFN-γ+ CD4 cells being reduced to almost 1:1 and 6:1, respectively (Fig. 3, d and e). Blocking OX40L, or analyzing the in vivo response of naive CD4 T cells derived from OX40-deficient mice, further demonstrated that OX40-OX40L interactions were essential for the action of LPS, and in their absence the balance was largely unchanged and highly in favor of Treg. Thus, airway tolerance mediated by i.n. Ag directly correlates with the early generation of adaptive Foxp3+ Treg in favor of effector-like T cells. The action of LPS in antagonizing airway tolerance suppresses the induction of Treg and allows effector T cells to develop, and this is dependent on OX40-OX40L interactions.

LPS and OX40 synergize in antagonizing development of airway tolerance

LPS might simply induce OX40L, which then promotes OX40 signaling in T cells to directly suppress Foxp3 induction as well as promote effector T cell outgrowth. Additionally, LPS might also provide other essential synergistic activities that are needed to work with OX40L to control the Treg balance. To address this, we modified the model to separate these potential activities. Agonist anti-OX40, to replace the need to induce OX40L, was given to mice exposed to i.n. OVA, but could not prevent the induction of airway tolerance regardless of dose or whether administered i.n. or i.p. (data not shown, and Fig. 4). LPS, given i.p. at any dose, also did not prevent tolerance to i.n. OVA. However, we found that a low dose of LPS given i.p. together with anti-OX400 effectively stopped the development of airway tolerance (Fig. 4). LPS and anti-OX40 treatment led to substantial eosinophilia when mice were subsequently challenged with a normal OVA sensitization
protocol (Fig. 4a), including Th2 cytokines in the BAL (Fig. 4b), and cellular infiltration in the peribronchial and perivascular areas of the lung (Fig. 4c). These data clearly show synergy between TLR4 signals and OX40 signals in blocking the induction of tolerance.

**LPS and OX40 synergize in suppressing development of adaptive Treg**

Further reinforcing the conclusions from the studies with LPS given i.n., the synergistic activity of LPS and anti-OX40 when given i.p. was characterized by reduced generation of Ag-specific Foxp3⁺ Treg. Again, ~50% fewer Treg were induced over the first 5 days, although kinetic analyses demonstrated a pronounced suppression of these cells within 1–2 days after initial exposure to i.n. OVA (Fig. 5a). During this time, no change in numbers of natural Treg was observed when analyzing the endogenous CD4⁺ population (data not shown). Neither LPS nor anti-OX40 alone reduced the number of adaptive Foxp3⁺ Treg (Fig. 5b), consistent with their individual lack of effect in preventing tolerance as measured by subsequent lung inflammation (Fig. 4). Anti-OX40 promoted the early appearance of IL-4- and IFN-γ-secreting CD4 cells (spontaneous ex vivo and in vitro OVA induced), whereas LPS alone did not promote these effector-like cells (Fig. 5, c and d). Notably, only the combination of LPS and anti-OX40 resulted in an altered balance of adaptive Treg and these T cell subsets that did not significantly favor Treg, such that the approximate ratio of Foxp3⁺:T cells was less than 1:1 (Fig. 5e), and the ratio of Foxp3⁺:IFN-γ⁺ T cells dropped to ~3:1 (Fig. 5f). These data confirmed the direct correlation between the Treg balance and the resultant immune response phenotype, and demonstrated that TLR4 signals and OX40 signals synergize to antagonize the induction of i.n. tolerance by modulating this balance.
Synergy between TLR4 and OX40 is mediated through cytokines

To further understand the synergy between TLR4 and OX40, we injected anti-IL-4, anti-IFN-γ, and anti-IL-6R at the time of exposure to i.n. OVA. IL-6 was of potential interest because it is induced in APC by LPS and has been shown to in vitro to antagonize Foxp3 induction (36, 37). IL-4 and IFN-γ blockade, either alone or in combination, had no effect on the induction of adaptive Foxp3+ Treg generated to soluble OVA (Fig. 6a), in line with the observation that very few IL-4- or IFN-γ-secreting cells were found under tolerizing conditions (Figs. 3 and 5). However, the suppression generation of Foxp3+ Treg driven by LPS and anti-OX40 was prevented when these cytokotines were blocked together, implying that both Th1/Th2 lineage activities are involved in dictating the ultimate balance of adaptive Foxp3+ Treg. This is in line with a report showing that the induction of Foxp3+ Treg driven by TGF-β in vitro could be inhibited by IL-4 and IFN-γ (38). IL-6R blockade alone also prevented decreased generation of Foxp3+ Treg after exposure to LPS and anti-OX40, albeit to a lesser extent, but significantly blocking IL-6R together with IL-4 and IFN-γ resulted in much greater induction of Foxp3+ T cells (Fig. 6a), a result similar to blocking OX40-OX40L interactions when LPS was provided i.n. (Fig. 3a). This demonstrates that both innate cytokines (IL-6) and adaptive inflammatory cytokines (IL-4 and IFN-γ) triggered by OX40 and TLR4 control the generation of Ag-specific Foxp3+ Treg.

To explore this synergistic action in more detail, and to additionally understand how OX40 integrates with these control mechanisms, we cultured naïve CD4+ cells from wt or OX40 KO TCR transgenic mice with peptide-presenting fibroblast APC (Fig. 6b). These fibroblasts expressed CD80, and were transfected with OX40L, and also spontaneously produced IL-6, theoretically mimicking an in vivo activated APC that might arise upon exposure to LPS. The addition of a low dose of TGF-β into cultures with wt T cells had little effect and resulted in essentially no Foxp3+ T cells being generated (0.1%). We have previously shown that OX40-OX40L interactions suppress the induction of Foxp3 (22), but only 0.8% resulted in this system with limited TGF-β when OX40-OX40L interactions were not operative due to a deficiency in OX40. Neutralizing IL-4 or IFN-γ alone had no effect regardless of the presence or absence of OX40 signals. However, neutralizing IL-6 in combination with preventing OX40/OX40L interactions revealed strong synergy between these molecules and resulted in conversion of ~7% of the CD4 population to Foxp3+. Blocking IL-4, IFN-γ, and IL-6 together allowed a small number of Foxp3+ T cells to be generated in the presence of OX40 signals (1.3%), but removing OX40/OX40L interactions at the same time dramatically resulted in ~25% conversion (Fig. 6b). These data show that TLR4 and OX40 signals synergize to suppress the generation of Foxp3+ Treg through both direct signals from OX40 as well as the action of the innate cytokine IL-6 and the adaptive inflammatory cytokines IL-4 and IFN-γ. Their combined effect is sufficient to overcome the induction of tolerance in vivo and lead to airway inflammation.

Discussion

LPS via TLR4 has been linked to airway disease, particularly asthma, and LPS/endotoxin is ubiquitously present in the environment, being readily detected in house dust (39). Whereas the exact influence of endotoxin is still being debated, and there may not be one single activity (40), exposure to LPS has been considered as a possible factor that could lead to susceptibility to developing asthma and allergic airway disease. In this study, we demonstrate that i.n. exposure to low doses of LPS at the time of inhalation of a model allergen prevented the induction of airway tolerance. Our data provide a rationale and mechanism to account for this activity, through modulating the generation of adaptive Treg. LPS antagonized the induction of Ag-specific Foxp3+ Treg and overall tolerance by allowing OX40-OX40L interactions to be active, as well
as providing essential synergistic signals through inflammatory cytokines that worked together with OX40 signals to oppose Foxp3+ Treg and allow effective development of Th2 cells. These data complement our previous analyses that found a critical role for OX40-OX40L interactions in promoting the effector and memory phases of Th2-driven lung inflammation (24, 25). We show that airway exposure to select levels of endotoxin can additionally recruit the activity of OX40 and OX40L that then opposes natural tolerance mechanisms that would protect from subsequent susceptibility to generating Th2 allergic disease such as asthma. Given that allergen/Ag immunotherapy, used clinically to promote tolerance mechanisms, might always be accompanied by exposure of individuals to environmental endotoxin, these data additionally suggest that blocking OX40-OX40L interactions may be an effective treatment to enhance the success of such a therapeutic approach.

Immunologic studies have previously found that healthy individuals possess T cells that can recognize the same peptide epitopes as allergic patients (41, 42), implying that active Ag-specific regulation of pulmonary immune responses might exist rather than simple passive ignorance of potential allergens. Growing evidence in recent years has suggested that Treg are the key players that mediate tolerance (3). Both naturally occurring thymic-derived Treg, as well as adaptive Ag-specific Treg that are thought to be generated in the periphery, have been demonstrated to suppress immune responses, with the notion that adaptive Treg might be more efficient in certain instances (34, 43, 44). Indeed, we observed that airway tolerance induced by soluble allergen is Ag specific because i.n. exposure to one Ag failed to suppress the subsequent induction of Th2 lung inflammation by another Ag. Moreover, adaptive OVA-specific CD4+CD25+ Treg, when transferred to OVA-sensitized mice, substantially suppressed lung inflammation, and this was with much higher efficiency than an equal number of polyclonal natural CD4+CD25+ Treg (data not shown). We further detected that a low number of Ag-specific Foxp3+ Treg was generated from naive CD25–Foxp3–CD4 T cells after airway exposure to soluble Ag, and depleting CD25+ T cells, or blocking TGF-β that was required for the generation of adaptive Foxp3+ T cells, prevented airway tolerance and resulted in Th2-type lung inflammation. This conclusion is in line with the finding that transfer of Ag-specific CD4+CD25+ (presumed Foxp3+) T cells isolated from TCR transgenic mice could strongly suppress the development of asthmatic lung inflammation (32), and that Ag-specific CD4+CD25+ Foxp3+ cells were induced following oral exposure to Ag in mice lacking natural thymic-derived Treg, and were sufficient to block the development of lung inflammation (33). Furthermore, increased numbers of Foxp3+ Treg have been found to be associated with resolution of lung inflammation in another model of inhalational tolerance (45), correlating with decreased numbers of Foxp3+ Treg in allergic children (46). Collectively, this supports the notion that adaptive Ag-specific CD25+Foxp3+ Treg play a dominant role in regulating the extent of airway inflammation and the induction of airway tolerance.

These studies, however, do not rule out additional roles for other Treg subsets or other types of regulation that potentially might function downstream or in conjunction with adaptive Foxp3+ Treg. Depleting CD25+ cells allowed the induction of much greater levels of lung inflammation in our studies than in nontolerized control animals, implying natural Treg also control the overall level of inflammation. IL-10-producing Treg have previously been reported to be capable of blocking lung inflammation (27, 28), and one study found that suppression of lung inflammation brought about by adoptive transfer of Ag-specific CD4+CD25+(Foxp3+) Treg was dependent on IL-10, thought to be produced by endogenous T cells, perhaps activated and/or induced by the adaptive Foxp3+ Treg (32). Studies of asthmatic patients have also suggested that the balance between IL-10-producing cells and pathogenic Th2 cells may play a role in the extent of immune responses in the airways (47). We have found that IL-10 does play a critical role at some stage in the tolerance process, in that blockade of IL-10R, similar to blockade of TGF-β, resulted in subsequent susceptibility to developing airway inflammation (our unpublished observations). However, we failed to detect IL-10 production from CD4 T cells in our model, either by flow analysis of adoptively transferred Ag-specific T cells, or by ELISPOT analysis. Thus, IL-10 may be downstream of the induction of adaptive Foxp3+ T cells, and possibly derived from non-T cells, although this needs to be investigated in more depth.

An interesting aspect of our study was the finding that under conditions of tolerance, the number of adaptive Foxp3+ T cells far exceeded the number of effector-like T cells, even though only a low percentage of naive T cells converted into Foxp3+ Treg. It is likely that tolerance is achieved through T cell deletion and unresponsiveness/energy, as well as active suppression from Treg, which may then further allow Treg to dominate over time. The effect of LPS was strongly emphasized by the finding that i.n. exposure altered this natural balance between adaptive Foxp3+ T cells and effector-like IL-4+ and IFN-γ+ T cells, and that this was critically dependent on promoting OX40-OX40L interactions, which were not active under neutral conditions that led to the predominance of Treg. It has been suggested that Foxp3+ Treg differentiation from naive CD4 T cells might only occur under selective conditions in the periphery (48). TGF-βR and IL-2R signals appear crucial, but the factors that antagonize their differentiative signals are not clear. We recently found from in vitro studies that OX40 signaling inhibited the generation of high numbers of Foxp3+ T cells (22), but we did not investigate whether this was a direct intracellular effect or whether additional factors cooperated with OX40. Our complementary in vivo and in vitro studies in this work highlighted that the activity of LPS was not only allowing OX40-OX40L interactions to be operative, but involved direct synergy between OX40 signals that potentiate TLR4 signals. It is well documented that LPS/TLR4 up-regulates costimulatory molecules on APC, including OX40L, but our data show that the inflammatory cytokine IL-6, also long been known to be induced by LPS, was a crucial factor in dictating the Treg balance. Other studies have also implied that IL-6 might be central to a number of inflammatory responses including respiratory disease. TLR antagonism of the suppressive effects of natural CD4+CD25+ natural Treg was described to be partly dependent on IL-6 (49), and more recently IL-6 has received much attention in that it was shown to synergize with TGF-β to promote IL-17-producing CD4 cells and at the same time suppress Foxp3 induction (36, 50). We now find that IL-6 synergizes with OX40 to further block the induction of Foxp3+ Treg, and their combined action can lead to Th2 differentiation, complementing previous studies showing OX40 or IL-6 individually can promote a bias toward Th2 responses (18, 51, 52). Our data also provide further significance to results showing that blockade of IL-6R suppressed an acute asthmatic response, and that this was linked with simultaneous expansion of Foxp3+ Treg (53). Increased IL-6 levels have additionally been reported in blood (54), BAL fluid (55), and lung tissue (56) of asthmatic patients, implying that combined targeting of IL-6 together with OX40 or OX40L might have significant benefits in terms of decreasing inflammation and at the same time allowing Foxp3+ Treg to predominate.

Lastly, we found that both IL-4 and IFN-γ acting together were also required for antagonizing the generation of Foxp3+ Treg. It
has been reported that these Th1/Th2-polarizing cytokines negatively cross-regulate the expression of Foxp3 through their corresponding transcription factors (38, 57). This lends toward the hypothesis that LPS can oppose the induction of i.n. tolerance by suppressing Foxp3 expression as well as promoting effector T cell differentiation.

In summary, we report in this study that innate immunity cooperates with adaptive immunity through TLR4 and OX40 in defining the nature of subsequent immune responses that can occur within the lung. Whether TLR4 and OX40 will synergize in suppressing Foxp3+ Treg numbers under conditions of ongoing acute or chronic lung inflammation is not known, although OX40 independently has already been shown to contribute to effector T cell survival. Future therapies using Abs that block OX40 or OX40L may have the ability to enhance the generation of Ag-specific Treg as well as suppress the development of pathogenic Th2 cells. Thus, in addition to therapeutic targeting of these molecules to reduce ongoing allergic respiratory disease (25, 58), prophylactic treatment together with conventional allergen/Ag immunotherapy might be considered to promote the induction of Treg that could protect individuals against developing lung disease.

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Disclosures

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References


