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Pre-existing Tolerance Shapes the Outcome of Mucosal Allergen Sensitization in a Murine Model of Asthma

Timothy J. Chapman,* Jason A. Emo,* Sara A. Knowlden,† Fariba Rezaee,‡ and Steve N. Georas*

Recent published studies have highlighted the complexity of the immune response to allergens, and the various asthma phenotypes that arise as a result. Although the interplay of regulatory and effector immune cells responding to allergen would seem to dictate the nature of the asthmatic response, little is known regarding how tolerance versus reactivity to allergen occurs in the lung. The vast majority of mouse models study allergen encounter in naïve animals, and therefore exclude the possibility that previous encounters with allergen may influence future sensitization. To address this, we studied sensitization to the model allergen OVA in mice in the context of pre-existing tolerance to OVA. Allergen sensitization by either systemic administration of OVA with aluminum hydroxide or mucosal administration of OVA with low-dose LPS was suppressed in tolerized animals. However, higher doses of LPS induced a mixed Th2 and Th17 response to OVA in both naïve and tolerized mice. Of interest, tolerized mice had more pronounced Th17-type inflammation than did naïve mice receiving the same sensitization, suggesting pre-existing tolerance altered the inflammatory phenotype. These data show that a pre-existing tolerogenic immune response to allergen can affect subsequent sensitization in the lung. These findings have potential significance for understanding late-onset disease in individuals with severe asthma. The Journal of Immunology, 2013, 191: 4423–4430.
system using chicken OVA as a model allergen suggest three major conclusions. The first is that typical Th2-promoting stimuli that induce robust inflammation in naive mice are completely ineffective in the setting of previous tolerance, even when given via the mucosal route. The second is that breakdown of tolerance and initiation of allergic inflammation after administration of LPS is associated with a mixed Th2/Th17 response in mice. Third, the history of OVA exposure has an impact on the immune response following sensitization, resulting in an enhanced Th17 response. These studies indicate that pre-existing tolerance has a previously overlooked role in shaping the development of deleterious immune responses in the lung.

Materials and Methods

Mice

Male C57BL/6 mice (6–12 wk of age) were purchased from the National Cancer Institute. TCR transgenic OT-II mice (21) (a gift from Dr. David Topham, University of Rochester Medical Center) were crossed to the B6. PL (CD90.1) and B6.SJL (CD45.1) backgrounds and bred in the University of Rochester animal facility. Animal protocols were approved through the University Committee on Animal Resources and were conducted according to safety guidelines.

Allergen sensitization models

On days 0–2, naive mice were exposed daily to either PBS as control or 100 μg Grade V OVA (Sigma-Aldrich) in 30 μl intranasal inoculation (using an IL-6 bioassay in RAW macrophages, we estimated that the OVA contained 10 ng endotoxin per 100 μg OVA). For the mucosal sensitization model, mice were sensitized on days 12–14 daily by oropharyngeal administration of 100 μg OVA mixed with 100 ng LPS (E. coli strain O55:B5 from Sigma-Aldrich), 1 μg LPS, or nothing in 50 μl. For the systemic model, a mixture of 10 μg OVA and 2 mg alun (Thermo Scientific) was administered by i.p. injection on day 12. For all sensitization groups, twice-daily challenges (1 h each) were done on days 26–28 in an inhalation chamber with 1% Grade V OVA aerosolized via jet nebulizer (Salter) at 10 psi. Mice were sacrificed on day 30. Before intranasal and oropharyngeal inoculation, all mice were first sedated with Avertin (2,2,2-tribromoethanol).

CD4+ T cell adoptive transfer

Spleens were removed from OT-II mice, and single-cell suspensions were made. Following RBC lysis (eBioscience), cells were stained with Abs and sorted by FACS (BD FACSAria) based on CD3+CD4+CD25+Foxp3+ (positive). Following RBC lysis (eBioscience), cells were stained with Abs and sorted by FACS (BD FACSAria) based on CD3+CD4+CD25+Foxp3+ (positive). Sorted cells were washed and i.v. injected into C57BL/6 recipient mice, as indicated in Supplemental Fig. 1 and Fig. 6A.

Sample acquisition and analysis

After sacrifice, bronchoalveolar lavage (BAL) was obtained by washing the airways twice with 750 μl PBS from a 1-ml syringe connected to a Teflon cannula. Cell counts were performed using Trypan blue exclusion, and differential counts were done as previously described (22). For cytokine measurements, BioPlex (Bio-Rad) and Milliplex (Millipore) cytokine bead arrays were performed on BAL supernatants and run on a BioPlex 200 analyzer. Lungs were processed for histological techniques, as previously described (23). Scoring of H&E-stained lung sections was done as described earlier (23). Periodic acid–Schiff (PAS) section scoring was as follows: Airways that contained >1 PAS+ cell were considered positive. Mediastinal lymph nodes (MLNs) were also removed and single-cell suspensions made. After counting, cell populations were identified by surface and intracellular FACS staining. Fluorescent Abs for CD3-FTTC and PE, CD4-PerCP/Cy5.5, CD44–Pacific Blue, CD25-PE/Cy5, CD90.1-Biotin, CD45.1–Alexa 780, RORγt-PE, and Foxp3-APC were purchased from BD, eBioscience, and BioLegend. Streptavidin–Pacific Orange was purchased from Invitrogen. For cytokine analysis, MLN cells were plated ex vivo at 2 × 10^6 cells per well in a 96-well round-bottom plate and incubated for 5 h with PMA (10 ng/ml) and A23187 ionophore (1 μM). Culture supernatants were analyzed using Bio-Plex bead array.

Measurement of airway hyperreactivity

Following sensitization and challenge, mice were sedated with Avertin and paralyzed with 0.5 mg/kg succinylcholine, and tracheostomy was performed. Airway challenges with increasing doses of methacholine and measurements via plethysmography were done as previously described (24).

Statistical analysis

Statistical analyses were performed using the Student t test. A p value < 0.05 was considered significant.

Results

Tolerized animals are protected from allergic inflammation under typical Th2-promoting conditions

To study how pre-existing tolerance influenced the subsequent immune response to allergen exposure, we used the model allergen OVA and induced inhalation tolerance by repeated intranasal inoculation with OVA in the absence of adjuvant (4, 7). Using adoptive transfer, we found that this protocol caused the expansion and differentiation of Ag-specific Foxp3+ T cells [referred to as induced regulatory T cells (iTregs) from this point on], as well as accumulation of these cells in the lung (Supplemental Fig. 1 and data not shown). At 10 d after induction of tolerance, mice were given i.p. OVA in alum as a sensitizing agent, and then were challenged with aerosolized OVA to promote Th2-type pulmonary inflammation (Fig. 1A). In control mice, robust pulmonary inflammation and eosinophilia were observed following OVA challenge. However, mice with established tolerance had little to no response (Fig. 1B and 1C). To study how pre-existing tolerance influenced the subsequent immune response to allergen exposure, we used the model allergen OVA and induced inhalation tolerance by repeated intranasal inoculation with OVA in the absence of adjuvant (4, 7). Using adoptive transfer, we found that this protocol caused the expansion and differentiation of Ag-specific Foxp3+ T cells [referred to as induced regulatory T cells (iTregs) from this point on], as well as accumulation of these cells in the lung (Supplemental Fig. 1 and data not shown). At 10 d after induction of tolerance, mice were given i.p. OVA in alum as a sensitizing agent, and then were challenged with aerosolized OVA to promote Th2-type pulmonary inflammation (Fig. 1A). In control mice, robust pulmonary inflammation and eosinophilia were observed following OVA challenge. However, mice with established tolerance had little to no response (Fig. 1B and 1C).

FIGURE 1. Prevention of OVA/alum-induced allergic inflammation in tolerized mice. (A) Immunization regimen for systemic OVA/alum sensitization and challenge. aer., aerosol challenge. (B and C) Tolerized and control mice were sensitized according to the OVA/alum protocol. Total BAL cell recovery (B), differential counts (C), and absolute number of macrophages, lymphocytes, neutrophils, and eosinophils at day 30 (D) are shown. (E–G) Analysis of lung sections from OVA/alum-treated mice. (E) Representative H&E-stained lung sections from tolerized and control mice (original magnification ×10). (F) Scoring of H&E-stained lung sections for severity of inflammation; 0–4 score per lobe, maximum score of 20 per lung. (G) Analysis of PAS-stained lung sections from same mice, scored as the number of airways per lung lobe that contained >1 PAS+ epithelial cell. n = 4–8 mice per group, *p < 0.05.
no pulmonary inflammation in response to OVA challenge, with the majority of recovered BAL cells identified as macrophages (Fig. 1B–D). A > 90% reduction in both total BAL cell recovery and eosinophils comparing naive and tolerized mice was noted. This finding was also reflected in the extent of inflammation and number of mucus-secreting cells observed in lung sections from these mice taken post challenge (Fig. 1E–G). Therefore, similar to a previous report (7), we found that established inhalation tolerance prevents allergic sensitization by OVA/alum in mice.

These data raised the question of whether Th2-type OVA sensitization could be induced in tolerized mice using other sensitization strategies. To address this, we used a more physiologically relevant model of administering low-dose LPS (0.1 μg) with OVA by oropharyngeal aspiration as a means of inducing Th2-type inflammation via the respiratory mucosa (25) (Fig. 2A). We first tested the accumulation of iTregs in this model by transfer of naive OT-II cells. As expected, iTregs were induced in this model, similar to OVA/alum immunization, and were present in the draining MLNs 3 d after sensitization (708 ± 256 OT-II iTregs in tolerance group versus 334 ± 110 in no tolerance group). After Ag challenge, tolerized mice were also completely protected from the effects of sensitization with low-dose LPS plus OVA, whereas control nontolerized animals exhibited increased BAL cell recovery and eosinophilia after OVA challenge using this immunization regimen (Fig. 2). Therefore, using both systemic and mucosal models of sensitization for Th2-type allergic inflammation, we were unable to induce pulmonary inflammation in response to OVA in tolerized mice.

**Higher doses of LPS given during sensitization break established tolerance**

Published data have shown that administration of different doses of LPS can result in the generation of qualitatively distinct adaptive immune responses (25). Because low-dose LPS was insufficient to break tolerance when used as an inhaled adjuvant, we tested whether higher inhaled doses of LPS would act as an effective sensitizing agent. To test this hypothesis, mice were given a 10-fold higher dose of LPS (1 μg LPS, LPSlo) during mucosal sensitization with OVA. In contrast to the LPSlo/OVA group, LPShi/OVA sensitization induced robust pulmonary inflammation in both naive and tolerized groups after subsequent OVA challenge (Fig. 3A, 3B). Sensitization with LPS in the absence of OVA was not sufficient to induce a response after challenge (Supplemental Fig. 2). As expected, LPSlo/OVA sensitization resulted in greater overall BAL cell recovery than did LPShi/OVA (comparing Figs. 2B and 3A; p < 0.05). Of interest, increased numbers of neutrophils were also recovered compared with LPSlo, suggesting the two doses of LPS may be inducing qualitatively distinct lung inflammatory responses (comparing Figs. 2D and 3C; p < 0.05). Cytokine analysis of BAL supernatants after OVA challenge

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**FIGURE 2.** Tolerized mice are protected from mucosal LPSlo/OVA induced allergic inflammation. (A) Immunization regimen for LPSlo/OVA mucosal sensitization and challenge. o.p., oropharyngeal. Total BAL cell recovery in tolerance, no tolerance, and PBS control groups (B), BAL differential cell frequency (C) and absolute differential cell number (D) were determined on day 30. (E) Scoring of inflammation from H&E stained lung sections with representative sections from each group shown (original magnification ×10). (F) Quantification of PAS+ airways from PAS-stained lung sections postchallenge. A representative “positive” and “negative” PAS-stained airway are shown for comparison (original magnification ×20). n = 5–19 mice per group. *p < 0.05.
supported this idea, and showed a reduction in IL-5 with a concomitant increase in IL-17A in LPS\textsuperscript{hi}/OVA-sensitized animals compared with LPS\textsuperscript{lo}/OVA (Fig. 3D). Of note, tolerized animals sensitized with LPS\textsuperscript{hi}/OVA had the most IL-17A recovered from BAL, suggesting the immunization history in tolerized mice influenced the subsequent effector response during the breakdown of tolerance. Despite the differences in response with LPS dose, both groups of mice developed pulmonary responses characteristic of allergic inflammation. Besides pulmonary eosinophilia, LPS/OVA-sensitized mice had increased airway resistance and prominent airway mucus production after challenge, regardless of the dose used (Fig. 3E and data not shown). Taken together, these data suggest that LPS\textsuperscript{hi}/OVA-sensitized mice induce a mixed Th2 and Th17 immune response that results in the breakdown of established tolerance and induction of allergic inflammation.

Enhanced lung inflammatory response following LPS\textsuperscript{hi}/OVA sensitization

Using LPS as an adjuvant for mucosal sensitization, we observed a dose-dependent breakdown of established inhalation tolerance to OVA. This model, which uses the same adjuvant but at different doses, enabled us to study the mechanisms of endotoxin-induced tolerance breakdown in the lung. Because the immune response in this model is initiated during the sensitization phase (see Fig. 2A), we compared inflammatory responses in tolerized animals during sensitization with LPS\textsuperscript{hi}/OVA or LPS\textsuperscript{lo}/OVA. An LPS dose-dependent increase in innate cytokines IL-1, IL-6, IL-17A, and TNF-\(\alpha\) in BAL during mucosal sensitization was noted, particularly following the first 2 d of treatment (Fig. 4). Despite the increase in innate cytokines after LPS\textsuperscript{hi}/OVA sensitization, no difference was observed in IL-10 recovery between groups (Fig. 4D). Consistent with the cytokine data, a higher dose of adjuvant yielded a more robust inflammatory response after each of the three sensitizations, as evidenced by total BAL cell recovery (Fig. 5A). Of interest, although total BAL cellularity and Foxp3\textsuperscript{+}CD4\textsuperscript{+} cells were increased in LPS\textsuperscript{hi}/OVA-sensitized mice (Fig. 5D), the recovery of CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} Treg cells was not markedly different between groups, with a significant difference observed only after the second sensitization (Fig. 5B). This result prompted us to compare the recoveries of CD4\textsuperscript{+}CD4\textsuperscript{+}Foxp3\textsuperscript{+} effector and CD4\textsuperscript{+}Foxp3\textsuperscript{+} Treg cells in BAL and MLNs during sensitization. Interestingly, the effector:Treg ratio in BAL was higher during LPS\textsuperscript{hi}/OVA sensitization compared with LPS\textsuperscript{lo}/OVA (Fig. 5F), suggesting that many more targets per suppressor cell were
present during an immunization regimen that broke tolerance. In contrast, the CD4⁺:Treg ratio in the draining MLNs was the same in both groups (Fig. 5G).

**Capacity of iTregs in the MLNs to suppress the effector response during breakdown of tolerance**

In contrast to the robust inflammation observed in BAL following LPShi/OVA, no significant differences were observed in the cellularity or CD4⁺:Treg ratio of the MLNs, comparing low- and high-dose LPS sensitization (Fig. 5 and data not shown). Because this lymph node is a site of initiation of pulmonary immune responses, we hypothesized that Ag-specific iTregs in the MLNs must be functionally impaired in tolerized mice to allow the development of effector T cell responses following LPS hi/OVA sensitization. To address this, we adoptively transferred OT-II TCR transgenic T cells to test the suppressive capacity of Tregs in the MLNs after sensitization. OT-II cells used for adoptive transfer were derived from transgenic mice on the congenic B6.PL (CD90.1) and B6.SJL (CD45.1) backgrounds so they could be identified by FACS analysis in the same recipient animal. In preparation for adoptive transfer, all OT-II cells were FACS sorted for CD3⁺CD4⁺CD44loCD25⁺ naive cells. This step ensured that any activation or expansion of OT-II cells observed after sensitization was the result of the in vivo immunization. Restimulation of MLN cells from immunized mice ex vivo showed increased IL-10 in tolerized groups and increased IL-4 in nontolerized groups (Fig. 6B, 6C). Further experiments to determine the cellular source of IL-10 in the MLNs suggested Tregs as IL-10 producers (data not shown). Similar to BAL measurement after OVA challenge, increased IL-17A was recovered from tolerized mice sensitized with LPShi/OVA (Fig. 6D). In the absence of established tolerance, CD45.1⁺ OT-II cells expanded following both low- and high-dose LPS/OVA sensitization, with increased expansion observed following LPShi/OVA (Fig. 7B). Surprisingly, in tolerized animals, CD45.1⁺ OT-II cell recovery from MLNs was reduced after both low- and high-dose LPS/OVA sensitization, with increased expansion observed following LPShi/OVA (Fig. 7B). Development of effector cells was also suppressed, as shown by reduced recovery of RORγt⁺CD45.1⁺ OT-II cells from MLNs (Fig. 7C). These data suggest that iTregs present in the draining lymph node function to suppress the lymph node response, even in a situation in which tolerance to a model aeroallergen was being broken.

Given the seeming contradiction between the intense inflammation and paucity of CD45.1⁺ OT-II cell expansion in MLNs during breakdown of tolerance, we further analyzed the CD90.1⁺ OT-II cells for phenotypic changes after sensitization. As with CD45.1⁺ OT-II cells, we found that RORγt⁺CD90.1⁺ OT-II cells also developed after sensitization. Interestingly, RORγt⁺CD90.1⁺
OT-II cell recovery was significantly increased in tolerized mice sensitized with LPS<sup>sh</sup>/OVA, but not following LPS<sup>lo</sup>/OVA sensitization (Fig. 7D). Because only a fraction of the CD90.1<sup>+</sup> OT-II cells became iTregs after inducing tolerance (Supplemental Fig. 1), the ROR<sub>γ</sub><sup>t</sup> cells may be developing from either Foxp3<sup>−</sup> precursor cells or Foxp3<sup>+</sup> cells. However, few CD90.1<sup>+</sup> OT-II cells expressed both Foxp3 and ROR<sub>γ</sub><sup>t</sup> after sensitization, and no difference was observed in the frequency of Foxp3 and ROR<sub>γ</sub><sup>t</sup> double positive cells in comparing LPS<sup>lo</sup>/OVA and LPS<sup>sh</sup>/OVA sensitization (data not shown). Taken together, these data suggest the stimuli resulting from LPS<sup>sh</sup>/OVA sensitization cause intense local innate inflammation that results in the conversion of distinct populations of CD4<sup>+</sup> T cells into effector cells that promote allergic inflammation.

**Discussion**

Mouse models of asthma have yielded important insights into disease pathogenesis, especially during the effector phase of the
immune response (26–30). However, less is known about the initiation of maladaptive allergen-specific immunity in the airway, in part because most studies to date have used nonphysiological routes of allergen sensitization (e.g., i.p. injection of OVA/alum). The few studies that have investigated initiation of allergen-specific immune responses in the airway have used naïve hosts, therefore not taking into account the potential impact of exposure history on the immune response. In this report, we describe a model of asthma dependent on the breakdown of established tolerance that provides new insights into the initiation of mucosal allergic immunity. Of note, the inflammatory phenotype after the breakdown of tolerance in mice showed some resemblance to that of human severe asthma, with increased inflammatory response in the lung, neutrophilia accompanying eosinophilia, increased airway hyperresponsiveness, and increased IL-17A production. We also found that a small difference in the dose of inhaled adjuvant has a powerful effect on the ability to break down tolerance and influences the subsequent memory response, both quantitatively and qualitatively. Using adoptive transfer experiments, we show that a fine balance of effector T cells and Tregs is required to maintain tolerance and that development of distinct populations of effector cells may be a hallmark of tolerance breakdown. Characterization of this model suggests it will be useful to study the mechanisms of allergic sensitization in previously tolerized animals.

Using this model of OVA tolerance via the respiratory route, we found that tolerized animals are resistant to systemic immunization with OVA/alum, as previously reported (7). Given the importance of innate and epithelial-derived cytokines in influencing T cell priming and polarization (17, 19), our hypothesis was that mucosal sensitization with LPS would engage the lung innate response and induce Th2 inflammation in tolerized mice. The data show that only high doses of LPS are able to promote pulmonary inflammation in previously tolerized mice. In this inflammatory setting, increased innate lung inflammation induced a Th2/Th17 response that resulted in a mixed inflammatory infiltrate. A plausible explanation based on these data is that tolerance increases the threshold of inflammation required to prime an effector response. If this is the case, low-level exposure to endotoxin will not be sufficient to induce allergic pulmonary inflammation, unlike in naive mice (25). The clinical implication is that if an individual is tolerized through allergen exposure history, allergen re-exposure in the context of robust pulmonary inflammation is required to break tolerance. This pattern may favor nonclassical clinical presentations such as neutrophilic Th17-type inflammation.

Given the strong accumulation of CD44hiCD4+ cells in BAL of high-dose LPS-sensitized mice, it is striking that recovery of Tregs was not as robust in comparison. One possibility is that different chemokine pathways regulate entry and retention of CD44hi non-Treg versus CD25+Foxp3+ Tregs (31). Alternatively, Tregs may be measured increased recovery of RORγt+CD90.1+ OT-II cells when tolerance was broken. The difference in IL-17A recovery may be related to the populations of Th17 cells generated in the presence or absence of tolerance. Recent published data have shown that Tregs can promote Th17 responses in certain conditions (34, 35). In our model, we suspect that the process of inducing tolerance via intranasal OVA generates a population of precursor cells capable of becoming Th17 cells following high-dose LPS sensitization.

Studies that attempt to sensitize naïve mice to allergens have shown that a multitude of infectious, inflammatory, and environmental stimuli are capable of acting as adjuvants for the induction of allergic inflammation. Given the rapidly increasing number of adjuvants being identified as potential risk factors for the induction of asthma, many of which are highly prevalent, it is unclear why there are not more asthmatic humans. The studies reported in this article, as well as ongoing studies in our laboratory, suggest that only a subset of adjuvants are capable of inducing allergic inflammation in tolerized mice. It is important to assess whether typical models of allergic inflammation in naïve mice are accurately reflecting allergic susceptibility in the human population. To properly identify critical risk factors for asthma, a greater understanding of allergen responsiveness in humans both before and after sensitization is needed.

Little is currently known about the relationship between allergen exposure history and the inflammatory response present in asthmatic individuals. The model used in this article suggests an association between Th17 response to allergen and breakdown of tolerance. It will be important in future studies to determine whether the findings reported in this article can be generalized to other inhaled adjuvants. If Tregs are raising the threshold of inflammation needed to develop pulmonary symptoms, strong stimuli such as viral or bacterial infection may be needed to break allergen tolerance for the eventual development of asthma. We conclude that initiation of allergic inflammation occurs through distinct mechanisms in previously tolerized versus allergen-naïve hosts. Furthermore, subtle differences in mucosal inflammation during allergen initiation can translate into qualitatively distinct effector responses. This observation may help explain the development of heterogeneous immune phenotypes in asthma despite similar environmental exposures.

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Disclosures
The authors have no financial conflicts of interests.

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