Fas-positive T cells regulate the resolution of airway inflammation in a murine model of asthma

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Persistent airway inflammation, mucus production, and airway hyperreactivity are the major contributors to the frequency and severity of asthma. Why lung inflammation persists in asthmatics remains unclear. It has been proposed that Fas-mediated apoptosis of inflammatory cells is a fundamental mechanism involved in the resolution of eosinophilic airway inflammation. Because infiltrating eosinophils are highly sensitive to Fas-mediated apoptosis, it has been presumed that direct ligation of Fas on eosinophils is involved. Here, we utilize adoptive transfers of T cells to demonstrate that the delayed resolution of eosinophilia in Fas-deficient mice is a downstream effect of Fas deficiency on T cells, not eosinophils. Interestingly, the mice that received Fas-deficient T cells, but not the controls, developed a persistent phase of inflammation that failed to resolve even 6 wk after the last challenge. This persistent phase correlated with decreased interferon (IFN)γ production by Fas-deficient T cells and could be reproduced with adoptive transfer of IFNγ-deficient T cells. These data demonstrate that Fas deficiency on T cells is sufficient for the development of long-term allergic airway disease in mice and implies that deregulation of death receptors such as Fas on human T cells could be an important factor in the development and/or chronic nature of asthma.

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Abbreviations used: AHR, airway hyperreactivity; B6, C57BL/6; BAL, bronchoalveolar lavage; DN, double negative; DP, double positive; FasL, Fas ligand; Rrs, respiratory system resistance; SEA, soluble egg antigen.

Asthma is a chronic disease of the airways characterized by airway inflammation, hyperreactivity, and recurrent episodes of airflow obstruction, and is often attributed to the activation of $CD4^+$ Th2 cells (1). The failure of asthmatics to resolve inflammation in their airways after exacerbations remains one of the most problematic features of the disease (1, 2). This persistent inflammation is believed to be a major contributor to the frequency and severity of asthma exacerbations and to other characteristics of asthma, including airway remodeling, smooth muscle hypertrophy, and airway hyperreactivity (AHR) (3). Inflammatory cell apoptosis is reduced in the airways and peripheral blood of asthmatics (4–6). These studies have led to the hypothesis that defects in inflammatory cell apoptosis may play a role in the inability of asthmatics to normally resolve Th2-mediated immune response.

It has been nearly two decades since the discovery that T helper lymphocytes include two subsets (Th1, Th2) with different effector functions mediated by unique patterns of cytokine production (7). Studies that have examined the relative susceptibility of Th1 and Th2 cells to Fas-mediated apoptosis have yielded conflicting results. Initially, two groups of investigators using clones of Th1 and Th2 cells found little susceptibility of the Th2 clones to Fas-induced death (8, 9). Yet another group, using cells from TCR transgenic mice, reported the equal susceptibility of Th1 and Th2 cells to Fas activation-induced cell death, whereas others found that Th2-skewed cells were resistant (10, 11). Overall, there are now several studies demonstrating that Th2 cells up-regulate c-FLIP, which protects them from Fas-mediated cell death (for review see reference 12). This up-regulation is thought to

be the result of a selective increase of PI-3K activity in Th2 cells (13, 14). Furthermore, several groups have found that Th2 cells produce less Fas ligand (FasL) than Th1 cells (15, 16), possibly as a result of differential NFAT usage in Th2 cells (16). A recent study found that ligation of CTLA-4 on Th2 cells protects them from Fas-mediated activation-induced cell death (17). Together, all of these in vitro studies suggest that Fas–FasL interactions might not play an important role in the normal resolution of Th2 inflammatory responses. Nevertheless, there are examples of in vivo Th2 responses being augmented in Fas-deficient mice (18, 19) and the question of whether Th2 responses in vivo are regulated, at least in part, by Fas is still unclear.

To address the role of Fas in the regulation of Th2-mediated inflammation, we used a previously described murine model of allergic airways disease and mice with abnormalities of the Fas signaling pathway (20). We found that Fas deficiency leads to a delayed resolution of Th2 inflammation. Furthermore, Fas deficiency, specifically on T cells, is sufficient to induce delayed resolution and can actually lead to the induction of persistent lung Th2 inflammation lasting at least 6 wk. This chronic inflammation occurs after the acute response in the absence of additional antigen challenge and includes many pathological features characteristic of human asthma, including continued inflammatory cells in the airways, dramatic mucus production, and AHR. Interestingly, although T cells from control mice that had resolved their airway and lung inflammation produced high levels of IFNγ upon antigen restimulation, Fas-deficient T cells from unresolved mice produced significantly less IFNγ. These data suggested that Fas deficiency on the T cells might lead to persistent inflammation through a defect in the development of IFNγ-producing T cells that, in turn, may fail to effectively down-regulate the Th2 response (21). In support of this hypothesis, we demonstrate that the persistent inflammation can be reproduced by the adoptive transfer of IFNγ-deficient T cells instead of Fas-deficient T cells. These data demonstrate that Fas–FasL interactions can regulate in vivo Th2 responses and suggest that immunotherapy directed at this pathway might be beneficial for chronic Th2-mediated diseases.

RESULTS

Resolution of airway inflammation is delayed in mice deficient in the Fas pathway

The role of proapoptotic surface molecules in the resolution of Th2-mediated airway inflammation is uncertain. To address whether Fas is involved in the resolution of airway inflammation, we compared the timing of resolution after *Schistosoma mansoni* sensitization and challenge in Fas-deficient (Lpr) and wild-type mice (all strains used in these experiments are on the C57BL/6 [B6] background). As reported previously, mice sensitized with *S. mansoni* eggs and challenged with SEA antigen develop a robust airway inflammation by 4 d after the challenge, consisting of 70–75% of eosinophils, 10–15% of T cells, and 10–15% macrophages (20, 22). We have previously established that eosinophilia in our model is totally dependent on both sensitization and challenge (22). In the absence of either the sensitization or the challenge, the composition of the small number of bronchoalveolar lavage (BAL) cells is predominantly macrophages and indistinguishable from BAL of mice without sensitization and challenge. To determine the length of time required to resolve the airway inflammation, the time course of airway inflammation in B6 mice was examined. Profound airway eosinophilia was still evident on day 7, but was mostly resolved by day 14 in most mice (Fig. 1 A). Although Lpr mice developed levels of airway inflammation equal to B6 at day 4 after challenge (Fig. 1 B), at day 14 after challenge the Lpr mice had significantly higher numbers of infiltrating eosinophils and T cells (Fig. 1 C). As we have reported previously for B6 mice, Lpr mice sensitized with *S. mansoni* eggs, but sham challenged with PBS, did not develop airway inflammation at either time point (Fig. 1, B and C). It should be noted that the sensitized and challenged Lpr mice had much less inflammation at day 14 than at day 4, suggesting that factors other than the Fas pathway also play a role in the clearance of airway inflammation

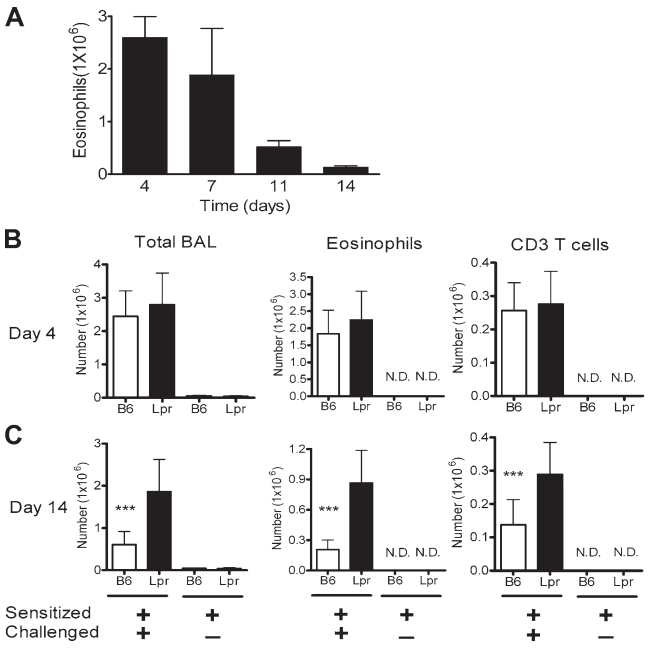

Figure 1. Resolution of airway inflammation is delayed in Fas-deficient mice. (A) Time course of BAL eosinophilia in B6 mice. Sensitized and challenged mice were killed and BAL analyzed on day 4 ($n = 11$), day 7 ($n = 4$), day 11 ($n = 7$), and day 14 ($n = 16$) after the last challenge. (B and C) Lpr and B6 mice were sensitized with inactivated *S. mansoni* eggs, challenged with SEA (10 μg/mouse), and killed on days 4 and 14 after the last challenge. Total numbers of BAL cells, $CD3^+$ T cells, and eosinophils were measured. Control mice were sensitized with inactive eggs, but challenged with only PBS (depicted as "-"). Lpr and B6 mice developed similar profound airway inflammation as measured by eosinophil and T cell numbers at day 4 after challenge, but Lpr mice had failed to resolve their airway inflammation at day 14 (Day 4: B6 $n = 7$, Lpr $n = 10$; Day 14: B6 $n = 11$, Lpr $n = 9$). ***, $P < 0.001$, N.D. indicates cell number $<10^3$. Error bars represent SEM.

in this model. However, the significant delay in resolution in the Lpr strain demonstrates that this process is, in part, regulated by Fas–FasL interactions.

Fas-positive T cells are sufficient for normal resolution of airway inflammation

We proposed two hypotheses to account for our observation that deficiency of Fas impairs resolution of airway eosinophilia. The first hypothesis was that eosinophils, which express the Fas receptor (23), are killed directly upon ligation with FasL. In this scenario, Fas-deficient B6.Lpr eosinophils would survive longer, leading to prolonged inflammation. The second hypothesis was that primarily T cells are regulated by Fas–FasL interactions. In this scenario, Th2 cells in the Lpr mice would survive longer as the result of absence of Fas expression, and these cells might indirectly prolong airway eosinophilia by increased or persistent production of eosinophil prosurvival cytokines such as IL-5.

To distinguish between these possibilities, we used an adoptive transfer model to "design" mice with differential expression of Fas on eosinophils and T cells. Lymph node T cells from either B6 mice or Lpr mice were adoptively transferred into B6.Rag$^{-/-}$ mice. Because eosinophil production occurs in the bone marrow and does not require Rag-1 expression, the resulting mice have eosinophils with the genotype of the recipient, whereas T cells have the genotype of the donor strain. Thus, the adoptively transferred mice had normal expression of Fas on their eosinophils (as well as other nonlymphoid cells), whereas expression of functional Fas on T cells depended on the donor mouse type used (B6 vs. Lpr). Mice that received B6 T cells (B6>Rag$^{-/-}$) before sensitization and challenge with *S. mansoni* had a similar course of inflammation as found previously in normal B6 (Fig. 1 A and not depicted). Mice that received Lpr T cells (Lpr>Rag$^{-/-}$) before being sensitized and challenged had similar high BAL eosinophil and CD3 T cell counts at day 4 as sensitized and challenged B6>Rag$^{-/-}$ mice (Fig. 2 A). However, strikingly, at day 14 as well as day 21, mice given Lpr T cells had significantly more airway inflammation compared with the B6 T cell transferred mice (Fig. 2, B and C).

One possible caveat of these studies is that donor eosinophils or eosinophil progenitors could be transferred with the enriched lymph node T cells. To confirm that airway eosinophils were produced from recipient progenitors, adoptive transfers were performed with CD45 congenic mice. Lymph node cells from congenic B6.CD45.1 mice were transferred into B6.Rag$^{-/-}$ (CD45.2) recipients. After the mice were sensitized and challenged, BAL was performed and the cells were analyzed for CD45 expression to determine their source. The inflammatory cells stained positively for either CD45.1 or CD45.2, but not both (Fig. 2 D, left). As expected, the CD3$^+$ T cells derive from the CD45.1$^+$ donor population (Fig. 2 D, middle). However, the eosinophil (CCR3$^+$) cells are CD45.1 negative (Fig. 2 D right), thus demonstrating that they are recipient derived. We did detect a small number of CCR3$^+$ cells that derive from the donor; however, these

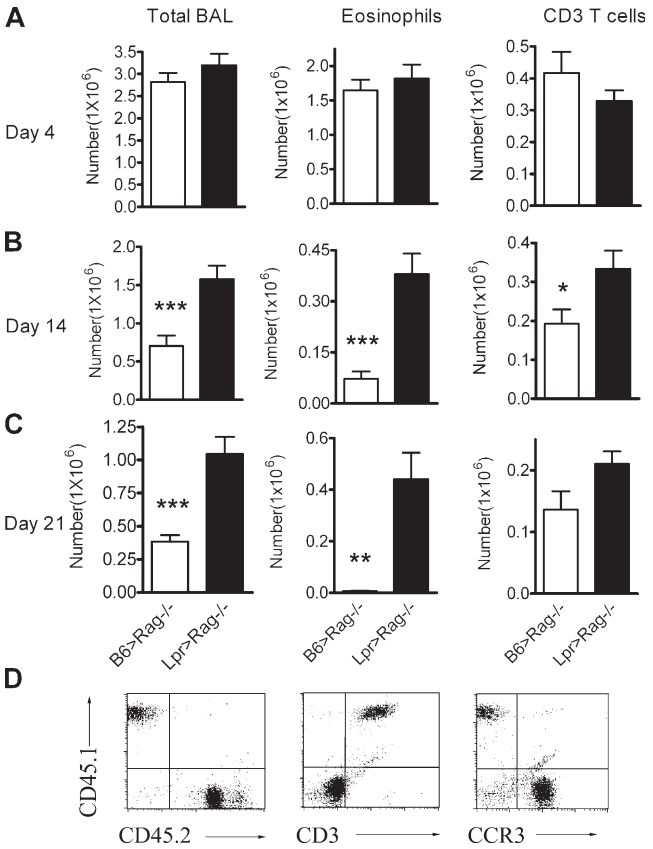

Figure 2. Fas-positive T cells are sufficient for normal resolution of airway inflammation. B6 and Lpr T cells were adoptively transferred into B6.Rag$^{-/-}$ mice 1 d before sensitization (noted as B6>Rag$^{-/-}$ and Lpr>Rag$^{-/-}$, respectively). The mice were killed on (A) day 4 (B6, $n = 15$; Lpr, $n = 11$), (B) day 14 (B6, $n = 15$; Lpr, $n = 13$), and (C) day 21 (B6, $n = 6$; Lpr, $n = 7$) after the final challenge and the BAL was analyzed. (D) B6.CD45.1 donor cells were adoptively transferred into B6.Rag$^{-/-}$ (CD45.2) recipients, sensitized, and challenged, and BAL was harvested 4 d after the final challenge. BAL cells were stained with anti-CD45.1, CD45.2, CD3, and CCR3 antibodies. The data presented in D are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars represent SEM.

cells are also CD3$^+$ and therefore represent a previously described small population of T cells that express CCR3 (24). Together, these data confirm that in our adoptive transfer model, eosinophils are predominantly, if not totally, derived from the recipients. Thus, the B6.Rag$^{-/-}$ mice adoptively transferred with Lpr donors possessed Fas-positive eosinophils and Fas-deficient lymphocytes. Together, these data validate the adoptive transfer model and demonstrate that Fas-deficient T cells are sufficient to prolong inflammation despite normal Fas expression on eosinophils.

Increased Th2 cytokine production is found at later time points in lung tissue of sensitized and challenged Lpr>Rag$^{-/-}$ mice

To determine whether the extended inflammation was mediated by Th2 cells, we compared cytokine levels in the lung

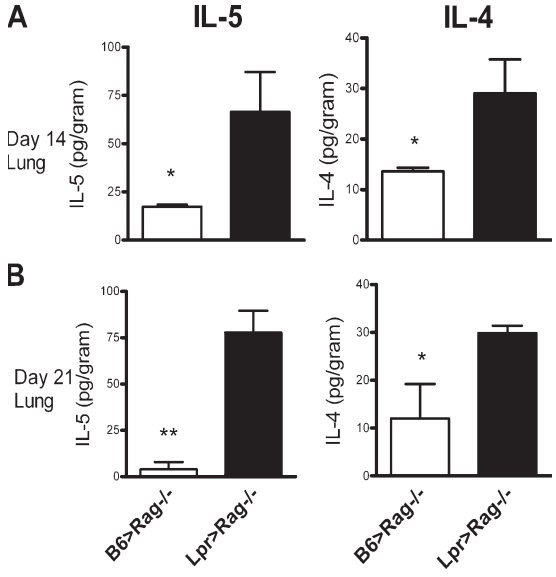

Figure 3. Increased Th2 cytokine production in the lung tissue of Lpr>Rag$^{-/-}$ mice. (A and B) Cytokine levels in lung tissue lysate at days 14 and 21 are shown. Cytokines were measured by Cytometric Bead Array as described in Materials and methods. The data are from one representative out of three experiments. Three to five mice per group per time point were analyzed. *, $P < 0.05$; **, $P < 0.01$. Error bars represent SEM.

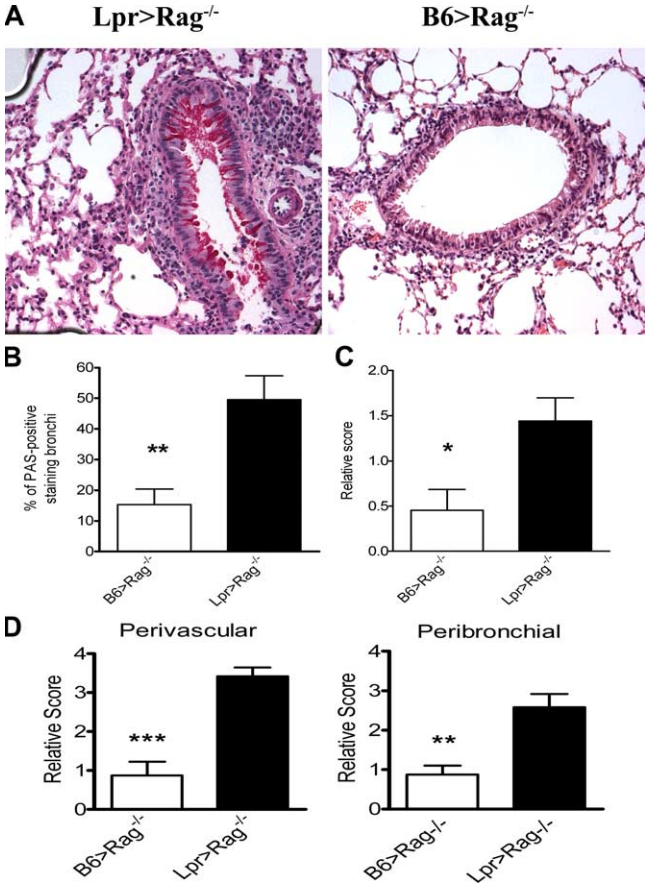

Figure 4. Increased mucus production in Lpr>Rag$^{-/-}$ mice. (A) Representative sections of lung at day 21. Lung tissues from B6>Rag$^{-/-}$ and Lpr>Rag$^{-/-}$ mice were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained PAS for analysis of mucus-containing cells. (B) Quantification of the number of positive bronchi and (C) severity of the abundance of PAS-positive mucus-containing cells. The PAS-positive bronchi scoring and the relative scores are described in detail in Materials and methods. (D) Perivascular and peribronchial inflammation were scored as described in Materials and methods. Six to seven mice per group were analyzed. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars represent SEM.

tissue between sensitized and challenged Lpr>Rag$^{-/-}$ and B6>Rag$^{-/-}$ mice. A significant difference in IL-4 and IL-5 levels between B6>Rag$^{-/-}$ and Lpr>Rag$^{-/-}$ mice was found at day 14 after the last challenge (Fig. 3 A). Furthermore, continued production of both IL-5 and IL-4 was found at day 21 from Lpr>Rag$^{-/-}$, but not B6>Rag$^{-/-}$ lung tissue (Fig. 3 B). Although a low level of IFNγ was found in lung tissue, no significant difference between these two groups was observed (unpublished data). These findings, along with the presence of eosinophils, establishes the Th2 nature of the days 14 and 21 airway and lung inflammation in the sensitized and challenged Lpr>Rag$^{-/-}$ mice.

Fas deficiency on T cells leads to increased mucus production

Increased mucus production is a common pathophysiological manifestation associated with asthma and is an important contributor to airway obstruction, which in its most severe form can lead to mortality. Goblet cell metaplasia and mucus production were determined by PAS staining of histological sections at day 21. As seen in Fig. 4 A, PAS staining in the sensitized and challenged Lpr>Rag$^{-/-}$ bronchi was noticeably intense, whereas very little PAS staining was seen in the sensitized and challenged B6>Rag$^{-/-}$ bronchi. When histological sections were scored as described in detail in Materials and methods, we found that a significantly higher percentage of bronchi showed PAS staining in the sensitized and challenged Lpr>Rag$^{-/-}$ mice compared with the sensitized and challenged B6>Rag$^{-/-}$ mice (Fig. 4 B). Furthermore, the severity of the goblet cell metaplasia was also significantly higher in the bronchi from the sensitized and challenged Lpr>Rag$^{-/-}$ mice (Fig. 4 C). Inflammation was further evaluated by scoring hematoxylin and eosin sections as described in detail in Materials and methods for the relative amounts of perivascular and peribronchial inflammation. The lungs from sensitized and challenged Lpr>Rag$^{-/-}$ mice showed more severe inflammation compared with sensitized and challenged B6>Rag$^{-/-}$ mice (Fig. 4 D). Together, these data demonstrate that both inflammation and mucus production continues through day 21 in sensitized and challenged Lpr>Rag$^{-/-}$ mice.

Fas deficiency on T cells leads to AHR

AHR is a hallmark of asthma and is often associated with increased airway inflammation (25, 26). To investigate if the persistent airway inflammation in Lpr>Rag$^{-/-}$ mice also leads to AHR, we measured respiratory system resistance

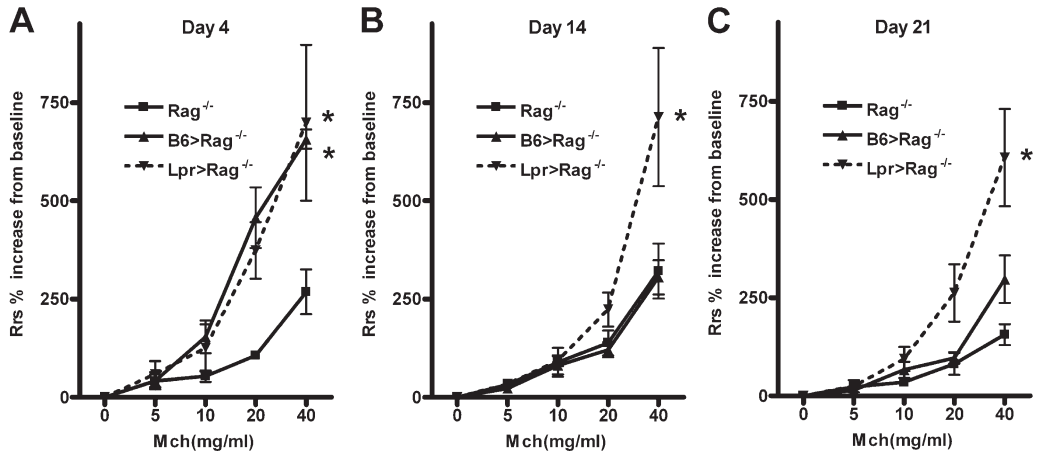

Figure 5. Fas deficiency on T cells leads to AHR. Respiratory system resistance (Rrs) to methacholine was measured at days 4 (A), 14 (B), and 21 (C) after the last challenge. Control $Rag^{-/-}$ mice, without T cell adoptive transfer, but with sensitization and challenge are shown. Analysis of variance was used to analyzed the differences in airway response to methacholine between $B6>Rag^{-/-}$ and $Lpr>Rag^{-/-}$ mice. Three to eight mice per group per time point were analyzed. *, $P < 0.05$. Error bars represent SEM.

(Rrs) changes in response to methacholine aerosol at days 4, 14, and 21 after last challenge. $Rag^{-/-}$ mice that did not receive any T cells, but were sensitized and challenged were used as controls at days 4 and 14. As previously demonstrated by others, in the absence of T cells, the $Rag^{-/-}$ mice fail to develop any eosinophilic airway or lung inflammation or AHR in our model (reference 27 and unpublished data). In both sensitized and challenged $Lpr>Rag^{-/-}$ and $B6>Rag^{-/-}$ mice, Rrs responses at day 4 to inhaled methacholine were greater than in control sensitized and challenged $Rag^{-/-}$ mice, but there was no significant difference in airway responsiveness between $Lpr>Rag^{-/-}$ and $B6>Rag^{-/-}$ mice at this time (Fig. 5 A). In marked contrast, at days 14 and 21, $Lpr>Rag^{-/-}$ mice remained hyperresponsive to methacholine, whereas sensitized and challenged $B6>Rag^{-/-}$ mice returned to normal responsiveness by day 14 (Fig. 5, B and C). Hence, after the induction of Th2-mediated airway inflammation, Fas deficiency on T cells leads to prolonged AHR.

Fas deficiency on T cells leads to persistent airway inflammation

The partial resolution of airway inflammation and cholinergic hyperresponsiveness in sensitized and challenged $Lpr>Rag^{-/-}$ mice at day 21 implied that complete resolution might just be delayed, rather than fully defective. To determine when complete resolution, similar to sensitized and challenged $B6>Rag^{-/-}$ mice occurs, we tested several later time points. To our surprise, the BAL eosinophil counts in the sensitized and challenged $Lpr>Rag^{-/-}$ mice at day 28, and even 2 wk later at day 42, were still significantly higher than the sensitized and challenged $B6>Rag^{-/-}$ mice (Fig. 6 A). BAL T cell counts remained higher in sensitized and challenged $Lpr>Rag^{-/-}$ mice at day 28, but matched the $B6>Rag^{-/-}$ mice at day 42 (Fig. 6 A). Strikingly, histological evaluation of the lungs from sensitized and challenged $Lpr>Rag^{-/-}$ mice at these later time points revealed dramatically severe inflammation and mucus production compared with sensitized and challenged $B6>Rag^{-/-}$ mice (Fig. 6 B). Even at day 42, $Lpr>Rag^{-/-}$ mice remained highly inflamed, whereas $B6>Rag^{-/-}$ mice had mild or no inflammation (Fig. 6 B, panels 2 and 5, and C). Goblet cell metaplasia and mucus production were also scored on PAS-stained sections at these time points. $Lpr>Rag^{-/-}$ mice still had dramatically intense positive PAS staining bronchi at day 42 (Fig. 6 B, panels 3 and 6, and D). Furthermore, the percentage of PAS positive bronchi and the severity of the goblet cell metaplasia were also significantly higher in the sensitized and challenged $Lpr>Rag^{-/-}$ mice compared with the sensitized and challenged $B6>Rag^{-/-}$ mice at days 28 and 42 (Fig. 6 D). Finally, increased airway reactivity was found in $Lpr>Rag^{-/-}$ mice compared with sensitized and challenged $B6>Rag^{-/-}$ at day 42 (Fig. 6 E), although both groups of mice had reduced reactivity compared with earlier time points (Fig. 5). Together, these data demonstrate that the sensitized and challenged $Lpr>Rag^{-/-}$ mice develop a chronic inflammatory response that includes many of the major features of human asthma.

Recent studies have suggested that Fas-deficient T cells may cause lung inflammation independent of antigen sensitization and challenge (28). To control for this possibility, $Rag^{-/-}$ mice were adoptively transferred with either B6 or Lpr T cells, but were not sensitized or challenged. These control mice were killed 8 wk later (which is the time equivalent of day 42 after the last challenge in our sensitized and challenged mice) and evaluated histologically. No airway inflammation or mucus production was found in any of these control mice (Fig. 6 B, panels 1 and 4). Thus, this control experiment demonstrates that the inflammation found in the sensitized and challenged $Lpr>Rag^{-/-}$ mice at day 42 is not the result of the mere presence of Fas-deficient T cells, but to an antigen-driven effect of the Lpr T cells.

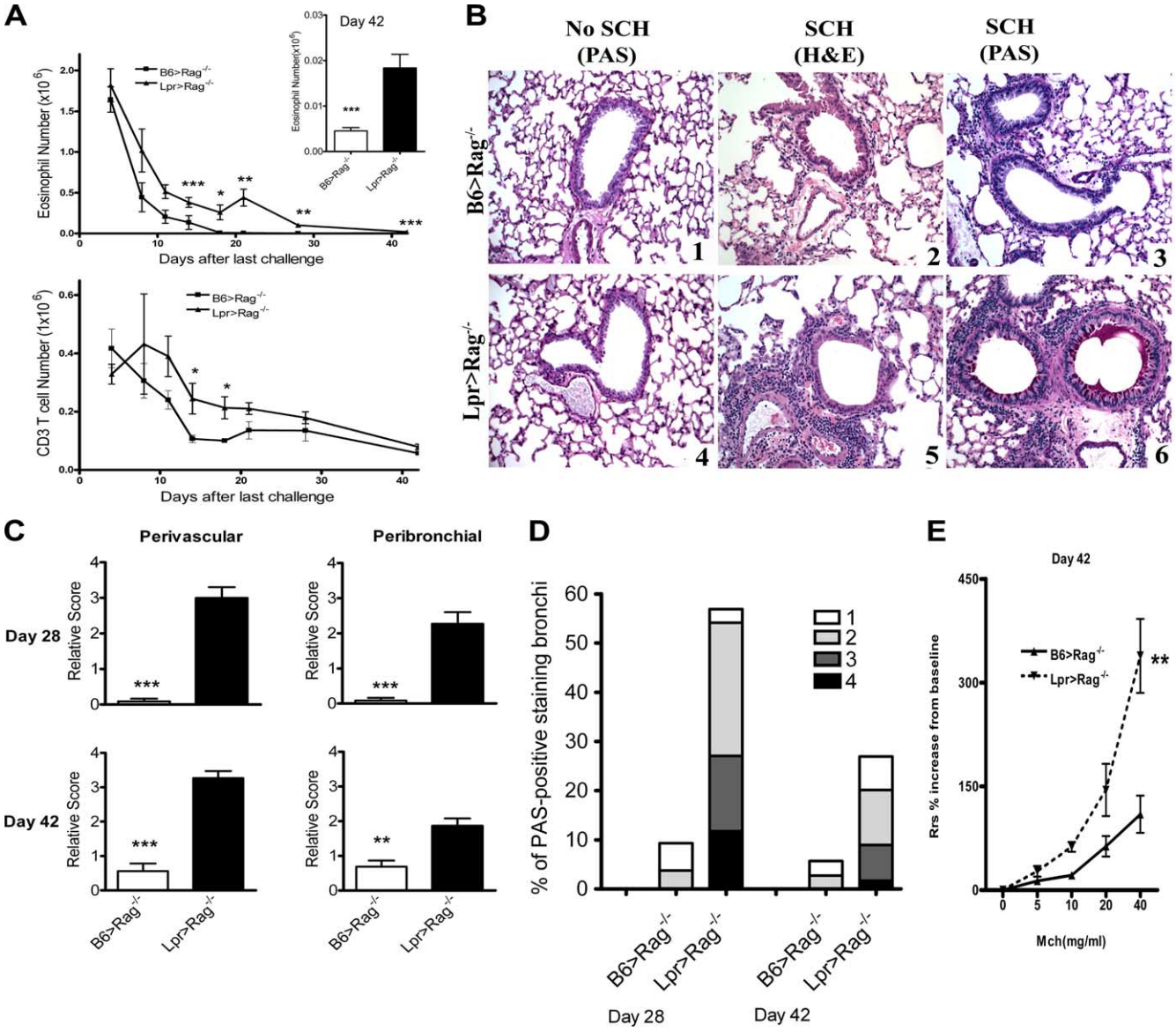

Figure 6. Fas deficiency on T cells leads to persistent airway inflammation and AHR. (A) BAL T cell and eosinophil counts were determined for days 8, 11, 28, and 42. The data from Fig. 2 for days 4, 14, and 21 are also included to provide a more complete picture of the entire time course that was tested. (B) Representative sections from B6>Rag$^{-/-}$ (panels 1–3) and Lpr>Rag$^{-/-}$ (panels 4–6) mice are shown. In panels 2–3 and 5–6, the mice were sensitized and challenged (SCH) as described in Materials and methods and killed 42 d after the last challenge (which was 57 d after the adoptive transfer of the T cells). In panels 1 and 4, the mice were adoptively transferred, but left without sensitization and challenging for 57 d to control for a nonspecific effect of the adoptive transfer of Lpr T cells on lung histology. The sections were stained with PAS or hematoxylin and eosin as noted. (C) Perivascular and peribronchial inflammation and (D) goblet cell metaplasia was scored as described in Materials and methods. (E) Respiratory system resistance (Rrs) to methacholine was measured at day 42 after last challenge. In all sections, four to five mice per group per time point were analyzed. Data in A–D are representative of two separate experiments. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. Error bars represent SEM.

Persistent airway inflammation in Lpr>Rag$^{-/-}$ mice is not the result of lymphoproliferation of CD4$^-$CD8$^-$ Lpr T cells in the chronic phase

Lpr mice have an inherited disorder of the immune system that causes massive lymphoproliferation at even early stages of life; for instance, it has been found that the pathogenic CD4$^-$CD8$^-$ CD3 T cells develop over time in Lpr strains (29). To determine if it is possible that Lpr T cells have developed other characteristics as a "side effect" of defective Fas that are causing the persistent airway inflammation in our model, we examined the proportion of CD4$^+$ and CD8$^+$ T cells and the phenotype of T cells from B6 and Lpr mice before adoptive transfer. The proportions of CD4$^+$ and CD8$^+$ cells are normal between B6 and Lpr mice as shown in Fig. 7 A, and we also didn't find any enhanced numbers of double negative (DN) and double positive (DP) Lpr T cells at mice

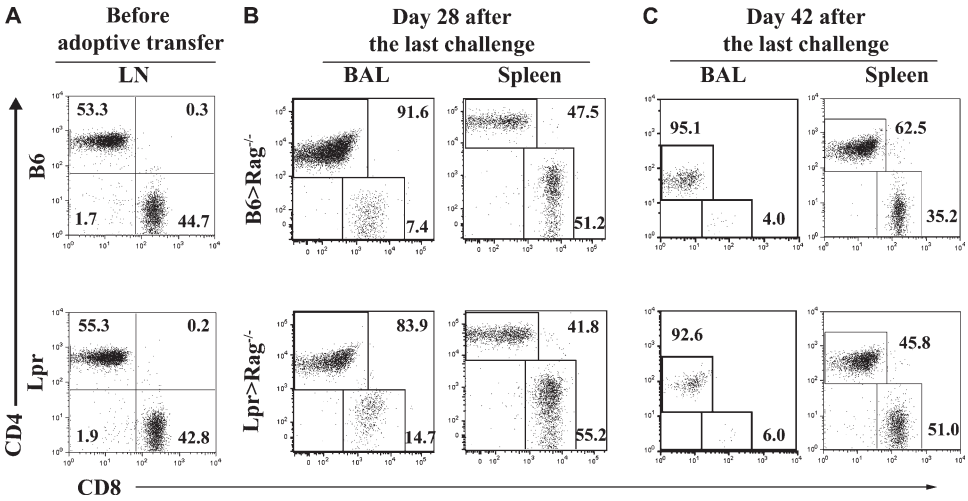

Figure 7. Persistent airway inflammation in Lpr>Rag$^{-/-}$ mice is not the result of lymphoproliferation of the pathological CD4$^-$CD8$^-$ Lpr T cells. (A) The percentage of CD4$^+$ and CD8$^+$ T cells and the phenotype of T cells from B6 and Lpr mice were examined after T cell isolation from B6 and Lpr mice before adoptive transfer. CD4 and CD8 expression was analyzed from the gated CD3 T lymphocytes. (B and C) The percentage and phenotype of CD4$^+$ and CD8$^+$ T cells from the BAL and spleens at days 28 and 42 after the last challenge were examined in B6>Rag$^{-/-}$ and Lpr>Rag$^{-/-}$ mice.

aged 5–7 wk old. This age of mice is used for T cell isolation from LN for adoptive transfer. More importantly, we also examined the phenotype of T cells from the BAL, spleen, lungs, and LN at days 28 and 42 in B6>Rag$^{-/-}$ and Lpr>Rag$^{-/-}$ mice after the last challenge. It is notable that the sum of the CD4$^+$ and CD8$^+$ T cells is >97% of total CD3$^+$ T cells in both groups and that very few CD3$^+$CD4$^-$CD8$^-$ (DN) and CD3$^+$CD4$^+$CD8$^+$(DP) T cells were observed in either the BAL, spleen (Fig. 7, B and C), lungs, and LN (not depicted) in Lpr>Rag$^{-/-}$ mice. Our data strongly suggest that persistent airway inflammation in Lpr>Rag$^{-/-}$ mice is not the result of the development of DN and DP pathological T cells populations.

Failure to resolve airway inflammation is correlated with decreased IFNγ production by Fas-deficient T cells

To determine the nature of the T cell response at day 42, BAL and lung T cells were restimulated with SEA overnight on ELISPOT plates for IFNγ and IL-5 (Fig. 8 A). IL-5–producing cells were not detected from the BAL of either B6>Rag$^{-/-}$ or Lpr>Rag$^{-/-}$ mice (unpublished data) and were equivalent in the lungs from the two groups of mice (Fig. 8 A). Interestingly, the number of antigen-responsive IFNγ-producing cells was significantly greater in the B6>Rag$^{-/-}$ BAL and lung cell cultures compared with the Lpr>Rag$^{-/-}$ cultures (Fig. 8 A). To further investigate the source of IFNγ in the B6>Rag$^{-/-}$ mice, we repeated the experiment, and on day 28, performed intracellular staining for IFNγ. As seen at day 42, day 28 lung T cells from B6>Rag$^{-/-}$ mice produced more IFNγ$^+$ cells than Lpr>Rag$^{-/-}$ mice and, interestingly, the IFNγ-producing cells in the lungs were mostly CD4$^+$ T cells (Fig. 8 B). Thus, although neither B6>Rag$^{-/-}$ nor Lpr>Rag$^{-/-}$ mice produced a significant IFNγ response at the peak of inflammation (Fig. 3 and not depicted), over time the B6>Rag$^{-/-}$, but not the Lpr>Rag$^{-/-}$ mice, develop antigen-specific IFNγ-producing CD4 T cells.

Similar to Fas-deficient T cells, IFNγ-deficient T cells can induce persistent airway inflammation

We hypothesized that the development of IFNγ$^+$ CD4$^+$ T cells may be a key mechanism involved the timely resolution in the sensitized and challenged B6>Rag$^{-/-}$ mice and that the failure to develop these IFNγ$^+$ CD4$^+$ T cells may explain the persistent inflammation found in sensitized and challenged Lpr>Rag$^{-/-}$ mice. To test this hypothesis, IFNγ$^{-/-}$ T cells were transferred to Rag$^{-/-}$ mice (IFNγ$^{-/-}$>Rag$^{-/-}$), and sensitized and challenged as described in previous paragraphs. We found that there is a similar level of airway inflammation at day 4 between IFNγ$^{-/-}$>Rag$^{-/-}$ and B6>Rag$^{-/-}$ mice. Interestingly, similar to the Lpr>Rag$^{-/-}$ mice, IFNγ$^{-/-}$>Rag$^{-/-}$ mice have a delayed resolution of eosinophilia in the airway at days 14 and 28 (Fig. 9 A). At day 28 after the last challenge, we also observed significantly higher levels of lung, peribronchial, and perivascular inflammation in IFNγ$^{-/-}$>Rag$^{-/-}$ mice than in B6>Rag mice (Fig. 9 B). These data demonstrated that IFNγ production by T cells is involved in the resolution of airway inflammation in the B6>Rag$^{-/-}$ mice and suggested that the failure of Lpr T cells to produce IFNγ in the Lpr>Rag$^{-/-}$ mice may play an important role in their inability to resolve Th2-mediated inflammation.

DISCUSSION

Why asthmatics fail to resolve inflammation in their airways remains one of the unsolved problems in asthma (1, 2). Persistence of T cells and eosinophils in bronchial biopsies was found in persistent asthma and even in intermittent asthma (4).

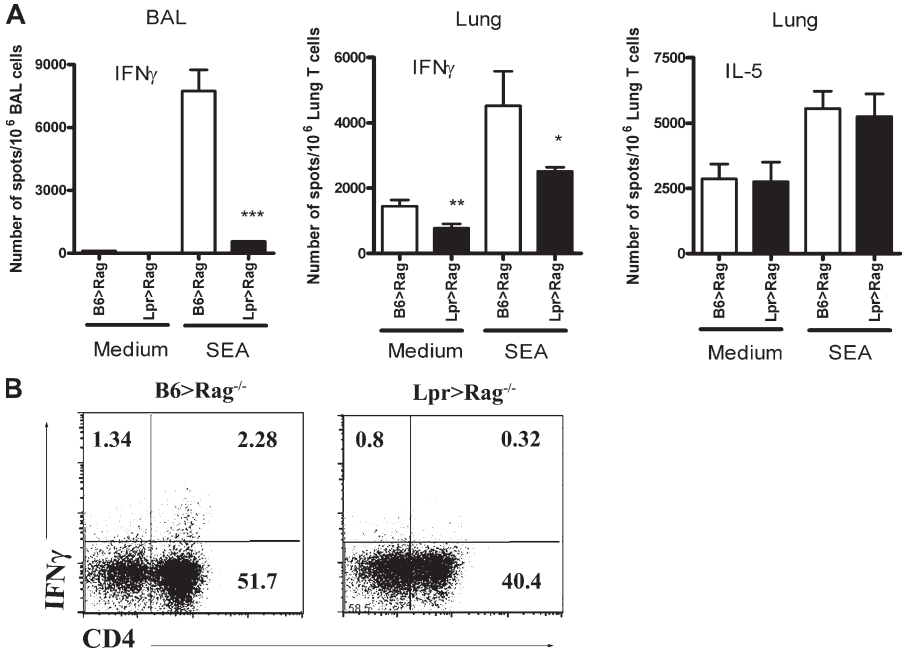

Figure 8. Failure to resolve airway inflammation is correlated with decreased IFNγ production by Fas-deficient T cells. (A) BAL cells and lung T cells were restimulated with SEA for 24 h on ELISPOT plates specific to measure IFNγ and IL-5. Data are expressed as positive points per well. Three or six wells per group were averaged and means ± SEM are shown. Higher numbers of IFNγ-producing cells were measured in cultures of day 42 B6>Rag$^{-/-}$ BAL and lung cells stimulated with exogenous SEA antigen (5 μg/ml; left and middle). Similar numbers of IL-5–producing cells were measured in cultures of day 42 Lpr>Rag$^{-/-}$ and B6>Rag$^{-/-}$ lung T cells with or without exogenous SEA antigen (right). (B) Lung T cells were restimulated with PMA and ionomycin for 4 h. The cells were stained with anti-IFNγ antibody of intracellular cytokine staining. Lung T cells from Lpr>Rag mice had sevenfold less IFNγ$^+$ T cells than those from B6>Rag$^{-/-}$ mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

These findings led us to question how Th2 inflammatory responses normally resolve in murine models of asthma. The objective of this investigation was to determine the potential role of the death receptor, Fas (CD95), in antigen-induced Th2-mediated airway inflammation in mice. We find that Fas$^+$ T cells play a pivotal role in the resolution of airway inflammation, mucus production, and AHR. These findings in mice may shed light on potential mechanisms of asthma pathogenesis.

Several studies have suggested that dysregulated apoptosis of eosinophils may be involved in human asthma. Eosinophils express the Fas receptor and undergo apoptosis in vitro and in vivo when bound by FasL or an agonizing antibody (30–32). However, eosinophils from asthmatic lungs undergo less apoptosis than normal controls and express more Bcl-2, and treatment of human asthma with corticosteroids leads to an increase in the number of apoptotic eosinophils (33–35). In fact, asthma severity has been directly correlated to reduced eosinophil apoptosis in induced-sputum samples (35). Yet, how eosinophil apoptosis is regulated in asthma remains undefined.

In our study, we demonstrated that Fas-deficient Th2 cells were sufficient to drive the extended course of airway inflammation in sensitized and challenged Lpr mice. These data suggest that the fate of eosinophils in the lungs and airways is largely dependent on T cells. Our results do not discount the effect of eosinophil apoptosis mediated by Fas or other mechanisms. Furthermore, because we have not examined eosinophil apoptosis directly in our model, we cannot exclude the possibilities that the lack of resolution is the result of lack of clearance of eosinophils or to enhanced or persistent recruitment of cells. Nevertheless, our findings suggest that resolution of airway eosinophilia inflammation is largely downstream of Fas expression on T cells. In human asthma, there were several reports that T cells also undergo less apoptosis. One report found that mitogen-stimulated peripheral blood T cells from asthmatic subjects failed to undergo the same degree of Fas-mediated apoptosis as T cells from the normal control subjects, thereby providing direct evidence for a defect in programmed cell death in the pathogenesis of asthma (5). Additionally, defective expression of Fas messenger RNA and Fas receptor was found on pulmonary T cells from patients with asthma (36). De Rose et al. reported that the defect in IFNγ production involved in the allergic immune response may be responsible for a decrease in apoptosis of allergen-activated T lymphocytes in the airways of atopic asthmatic patients (37). Thus, our results in this new model have direct correlations with the findings of these studies in human asthma. In addition to dysfunctional Fas pathway on T cells, markedly reduced Fas ligand mRNA and protein in the airway epithelium during allergic airway inflammation induced by OVA in mice may be also involved in the pathogenesis of certain inflammatory conditions of the airway (38).

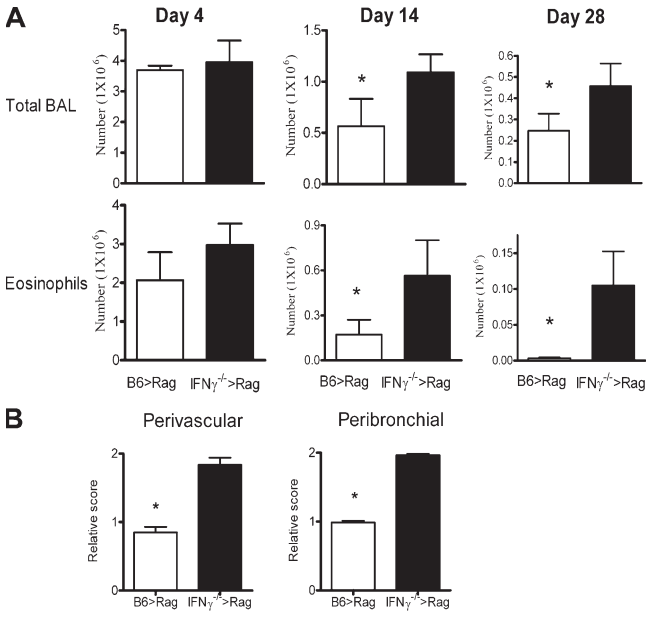

Figure 9. IFNγ-deficient T cells can induce persistent airway inflammation similar to Lpr T cells. (A) B6 and IFN$\gamma^{-/-}$ T cells were adoptively transferred into Rag$^{-/-}$ mice 1 d before sensitization. BAL was analyzed on days 4, 14, and 28 after the last challenge. IFN$\gamma^{-/-}$>Rag and B6>Rag$^{-/-}$ mice produced similar levels of airway inflammation at day 4, but at days 14 and 28, higher levels of eosinophils were found in the IFN$\gamma^{-/-}$>Rag$^{-/-}$ mice compared with B6>Rag$^{-/-}$ mice. (B) Perivascular and peribronchial inflammation was scored as described in Materials and methods. At days 14 (not depicted) and 28 (B), significantly higher levels of lung peribronchial and perivascular inflammation was observed in IFN$\gamma^{-/-}$>Rag$^{-/-}$ mice than in B6>Rag$^{-/-}$ mice. Three to four mice per group were analyzed. *, $P < 0.05$. Error bars represent SEM.

Persistent Th2 inflammation has been one of the most difficult aspects of authentic asthma to model in animals. Many models for persistent inflammation require continued antigen exposure for long periods of time. When antigen exposure is ended, airway remodeling and AHR can remain for 3–4 wk; however, inflammation resolves quickly after the last antigen challenge (39–41). Recently, there has been the development of other models that use either mite allergens (42) or conventional OVA allergen with new technology for aerosol delivery (40). However, all of these models require 20–40 nebulizations after one to two immunizations. Mice with a targeted deletion of the T-bet gene spontaneously demonstrated multiple physiological and inflammatory features characteristic of asthma (43). Transgenic mice that overexpress IL-5 (44), IL-9 (45), IL-11 (46), and IL-13 (47) showed AHR, eosinophilic inflammatory response, and collagen deposition in the airways, indicating that chronic exposure to Th2 cytokines could also induce airway remodeling. Airway inflammation in these animal models occurs spontaneously without any antigen exposure. Thus, current methods for inducing chronic airway inflammation fall into two categories: those models in which the mice are repeatedly challenged for weeks or even months, and models of genetically manipulated mice that develop a spontaneous Th2-type airway inflammation in the absence of allergen exposure.

In this study, we now present data on a third method of inducing persistent airway inflammation. This novel model is not spontaneous, but allergen-induced, yet only requires two challenges after a single sensitization. Although it has been proposed that the persistent inflammation found in asymptomatic asthmatics is the result of chronic low level exposure to allergens, it is just as likely that the persistent inflammation in asymptomatic asthmatics is the result of defects in resolution of airway inflammation. Thus, the Fas-deficient T cell mice represent an altogether new model of persistent airway inflammation that does not require continuous challenges to induce this key feature of asthma.

Recently, Rajewsky et al. published their findings on ablation of Fas specifically in the T cell compartment (28). They reported that between 8 and 12 mo, these mice develop severe pulmonary fibrosis and accumulation of inflammatory cells. Although T cell–specific Fas$^{-/-}$ mice and our adoptively transferred Lpr>Rag$^{-/-}$ mice are both Fas deficient on only T cells, the timing and type of lung pathology differs in the two models. Nevertheless, to test whether our findings could be explained by spontaneous lung disease, we adoptively transferred B6 and Lpr T cells into Rag$^{-/-}$ mice but did not sensitize and challenge these animals. After 8 wk, no evidence of inflammation could be found (Fig. 6 B). Thus, it is highly unlikely that persistence of Th2-type inflammation in our model is related to the pulmonary fibrosis and inflammation that develops at a much later stage of life in T cell specific Fas$^{-/-}$ mice.

Our study is supported by previous work from Gelfand et al., who demonstrated that AHR is increased in OVA-sensitized and challenged Fas-deficient mice compared with wild type at day 4 after the last challenge and that blockade of inflammation with anti–IL-5 treatment attenuates the response (18). Interestingly, unlike our findings, these investigators did not find a delay in the resolution of inflammation in the Lpr mice. More importantly, in our adoptive transfer model, we now demonstrate that Fas deficiency specifically on T cells is sufficient to prolong inflammation despite normal Fas expression on eosinophils.

To investigate the possible mechanisms involved in the development of persistent airway inflammation in sensitized and challenged Lpr>Rag$^{-/-}$ mice, T cell function was studied. We found that lung T cells from B6>Rag$^{-/-}$ mice produced more IFNγ^+ cells than lung T cells from Lpr>Rag$^{-/-}$ mice and, interestingly, that the IFNγ-producing cells in the lungs were mostly CD4$^+$ T cells. To test the hypothesis that development of IFNγ^+ CD4$^+$ T cells may be a key mechanism involving timely resolution, IFN$\gamma^{-/-}$ T cells were transferred to Rag$^{-/-}$ mice. We found that IFN$\gamma^{-/-}$>Rag$^{-/-}$ mice have a delayed resolution of eosinophilia and lung peribronchial perivascular inflammation (Fig. 9). These data suggest that the failure of Lpr T cells to produce IFNγ in the Lpr>Rag$^{-/-}$ mice may play an important role in their inability to resolve their Th2-mediated inflammation. These

results were also consistent with previous reports (21, 37, 48, 49) in which Th1 T cells inhibited Th2-induced eosinophilia and mucus production. Why effective antigen-specific Th1 cells fail to develop in the Lpr>Rag$^{-/-}$ mice remains unclear. In fact, several reports suggest that Fas deficiency leads to greater Th1 responses (for review see reference 12). Thus, further studies will be required to resolve these apparent contradictory studies.

There has been much controversy over the role of Th1 cells in asthma (for review see reference 1). Although some studies have suggested a protective role for Th1 cytokines in allergy and asthma (50, 51), other investigators have suggested that Th1 cells enhance pulmonary inflammatory responses and AHR (52–54). In our model, the data clearly demonstrate that Th2 cells play a stimulating role in the early phase of the airway inflammation, but that Th1 cells play an inhibiting role in the chronic phase of airway inflammation. Nevertheless, the development of our novel animal model of asthma provides a new opportunity to study the mechanisms involved in chronic Th2 inflammation as well as the pathological outcomes of this long-term inflammation. Further investigations in the role of Fas in regulating Th2 immunity may translate into better treatments for asthma and other Th2-mediated diseases.

MATERIALS AND METHODS

Animals. B6 mice were purchased from either The Division of Cancer Treatment at the National Cancer Institute or from The Jackson Laboratory. B6.MRL-Tnfrsf6^{lpr} (Lpr), B6.129S7-Ifng^{tm1Ts}/J (IFN$\gamma^{-/-}$), B6.129S7-Rag1^{tm1Mom} (B6.Rag$^{-/-}$), and B6.SJL-Ptprc^{a}Pep3^{b}/BoyJ (B6.CD45.1) mice were purchased from The Jackson Laboratory and bred and housed in a specific pathogen-free barrier facility maintained by the University of Chicago Animal Resources Center. The studies reported here conform to the principles outlined by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

Antibodies and flow cytometry. Anti–mouse CD3 (clone 17A2), CD4 (clone L3T4), CD8 (clone 53–6.7), CD45.1 (clone A20), and CD45.2 (clone 104) antibodies were obtained from BD Biosciences. Anti–mouse CCR3 antibody (clone 83101.111) was obtained from R&D Systems. Anti-IFNγ–PE was obtained from eBioscience. For intracellular staining, the cells were fixed with 4% paraformaldehyde for 10 min after surface staining and washed 2× in permeabilization buffer (0.03% saponin/PBS/0.25% gelatin). Staining antibodies, diluted to the predetermined concentration in permeabilization buffer, were added to the samples, and the samples were incubated for 30 min at 4°C. The samples were washed and analyzed on a FACS LSR-II (Becton Dickinson).

***S. mansoni* sensitization and challenge.** S. mansoni sensitization and challenge to induce murine allergic airway disease was described previously (20). In brief, S. mansoni eggs were harvested and SEA was produced as described previously (55). At day −14, mice were immunized by i.p. injection of 5,000 inactivated S. mansoni eggs, which induced a natural Th2 response in the absence of active infection. A days −7 and 0, the mice were challenged with 10 μg of SEA by intranasal and intratracheal aspiration, respectively. The mice were studied between 4 and 42 d after the last challenge. To ensure that they had not yet developed lymphoproliferative disease, Lpr and IFN$\gamma^{-/-}$ mice were used between 5–7 wk of age.

BAL analysis. BAL was performed by delivering 0.8 ml of cold PBS into the cannulated trachea and gently aspirating the fluid. The lavage was repeated a total of four times to recover a total volume of 2.5–3 ml. The lavage was centrifuged and supernatant was stored at −20°C for cytokine analysis. The percentage of cell types found within BAL fluid was determined by FACS analysis with cell type–specific markers.

Th1/Th2 cytokine determination in lung tissue. The lung tissue was weighed, homogenized in 500 μl 1× PBS containing protease inhibitors, and collected by centrifugation at 3,000 revolutions/min for 15 min. Cytokines in the lung tissue were detected using a murine Th1/Th2 Cytometric Bead Array kit (BD Biosciences) according to manufacturer's instructions, except that the beads were incubated in 10-fold more volume of lung lysate (500 μl) than recommended. The detection limits were 5 pg/ml for IL-4, 5 pg/ml for IL-5, and 2.5 pg/ml for IFNγ.

ELISPOT. ELISA spot plates (Cellular Technology Limited) were coated with rat anti–mouse IL-5 and IFNγ antibodies (2 μg/ml) (BD Biosciences). Plates were blocked with PBS/0.1% BSA and washed with PBS. BAL and lung cells were cultured on the plates for 16 h at 37°C in the presence or absence of SEA (5 μg/ml). The ELISPOT plates were scanned by ImmunoSpot Series 2 Analyzer (Cellular Technology Limited).

Adoptive transfer. B6 and Lpr T cells were harvested from lymph nodes from donor mice and enriched by nonadherence to a nylon wool column. 10^7 cells were adoptively transferred into each recipient intravenously. The purity by flow cytometry was between 90 and 95% CD3$^+$ T cells.

Histology. Lungs were removed from mice after BAL and fixed by immersion into 4% paraformaldehyde. Lobes were sectioned sagitally, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin for analysis. An inflammation score was assigned in a blinded fashion by a pathologist. The score of peribronchiolar and perivascular inflammation was determined as follows: 0, normal; 1, few cells; 2, a ring of inflammatory cells one cell layer deep; 3, a ring of inflammatory cells two to four cells deep; and 4, a ring of inflammatory cells of more than four cells deep. Additional sections were stained with periodic acid schiff (PAS) for analysis of mucus-containing cells. Scoring was performed by examining at least 20 consecutive fields. Numerical scores for the abundance of PAS-positive goblet cells (56) in each airway were determined as follows: 0, <5% goblet cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75%, with 0 being negative and 1–4 being positive for PAS-staining bronchi.

Airway responsiveness. Methacholine challenge experiments were performed at 4, 14, 21, and 42 d after the last antigen challenge. Rrs was measured through a computer-controlled small animal ventilator (Flexivent; SCIREQ) as described previously (57). Each mouse was challenged with increasing doses of methacholine aerosol (0, 5, 10, 20, and 40 mg/ml in saline) for 12 s. After each challenge, Rrs was recorded during tidal breathing every 10 s for 2 min. Maximum values of Rrs were taken and expressed in terms of percentage change from baseline after saline aerosol.

Statistical analysis. Graph generation and statistical analysis were performed by using Prism software (version 4.00; GraphPad). Differences between groups for lung histology parameters, total BAL cells, BAL eosinophils, BAL T cells, and cytokine content in BAL fluid and lung tissues were determined by using an unpaired Student's two-tailed *t* test. For analyses of the data on days 28 and 42 in Figs. 6 and 9, the more conservative (less powerful), Mann-Whitney nonparametric comparison was used. Error bars represent SEM. Two-way analysis of variance was used to analyze the differences in airway response to methacholine among groups of mice. Statistical significance was claimed whenever *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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