CD4+ cells are required for chronic eosinophilic lung inflammation but not airway remodeling

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Doherty TA, Soroosh P, Broide DH, Croft M. CD4+ cells are required for chronic eosinophilic lung inflammation but not airway remodeling. Am J Physiol Lung Cell Mol Physiol 296: L229–L235, 2009. First published December 5, 2008; doi:10.1152/ajplung.90543.2008.—The contribution of CD4 T cells and other CD4+ cells to lung inflammation and airway remodeling remains unclear during bouts of chronic exposure to airborne allergen. Previously, murine models have shown that CD4 T cells are required for initiation of acute inflammation and the remodeling process. However, it is unknown whether CD4 T cells or other CD4+ cells continue to be required for remodeling during ongoing allergen challenges after the development of acute eosinophilic lung inflammation. To test this, mice were sensitized and challenged with ovalbumin (OVA). After acute airway inflammation was established, a CD4 depleting antibody was administered for 4 wk during a period of chronic exposure to intranasal OVA, resulting in effective depletion of CD4+ cells from all organs, including the lung, lung-draining lymph nodes, and spleen. In these mice, levels of peribronchial inflammation, bronchoalveolar (BAL) eosinophilic, and lung CD11c+, CD8+, and Siglec-F+/CD11c- cells were significantly reduced. However, mucus metaplasia, bronchial subepithelial fibrosis, and smooth muscle mass were not affected. Additionally, depletion of CD4+ cells before the last week of chronic allergen challenges also led to significant reductions in BAL eosinophils, peribronchial inflammation, and lung CD11c+, CD8+, and Siglec-F+/CD11c- cells. These results show that CD4 T cells, and other CD4+ cells including subsets of dendritic cells, iNKT cells, and LTi cells, play a role in ongoing eosinophilic lung inflammation during periods of chronic allergen challenge, but are not required for progressive airway remodeling that develops after initial acute inflammation.

CD4 T cells; asthma; Th2

ALLERGIC ASTHMA IS CHARACTERIZED by a Th2 inflammatory response to inhaled allergens, orchestrated by CD4 T cells producing a characteristic cytokine profile responsible for eosinophilic lung inflammation (7). In asthmatics, numbers of CD4 T cells and Th2 cytokines are elevated in bronchoalveolar lavage (BAL) specimens after allergen challenge, suggesting that Th2 responses drive chronic allergic lung inflammation (34). Despite this, therapies targeting either CD4+ cells or Th2 cytokines, including IL-4 and IL-5, have not been as successful as anticipated (3, 11, 24). Although this may be due to many factors such as patient population, dose, duration, and outcome measures, it is also possible that our understanding of how CD4 T cells or other CD4+ cells contribute to chronic asthma pathogenesis, including airway remodeling, is inadequate.

Progressive decline in lung function in asthma is thought to be due to airway remodeling, characterized by structural changes including mucus metaplasia, subepithelial fibrosis, smooth muscle hypertrophy/hyperplasia, and angiogenesis (5). The relationship between inflammation and remodeling remains unclear, although some studies suggest that the two may be independent. For example, bronchial biopsies from asthmatics have shown similar levels of subepithelial fibrosis after anti-inflammatory therapy with corticosteroids (4, 8, 19). Aside from remodeling, the degree of CD4 T cell involvement in chronic allergic inflammation is unclear because other mechanisms that could support chronic inflammation may exist that are CD4 independent, including Th2 cytokine production by mast cells and eosinophils, as well as release of chemokines such as eotaxin from airway epithelium.

Murine models of acute Th2-driven airway inflammation have shown that CD4+ cells are required for eosinophilic lung inflammation and airway hyperresponsiveness, and adoptively transferred Th2 CD4 T cells can induce these features (15, 16). In addition, models using repetitive antigen challenge to induce airway remodeling have shown that if CD4+ cells are depleted at the time of the initial acute airway challenge, there is reduced inflammation and remodeling (12, 23). Because iNKT cells (up to 90%), lymphoid tissue inducer cells (LTi) (up to 50%), and a subset of dendritic cells (up to 50%) can express CD4, these data might also indicate roles for these cells as well as conventional CD4 T cells for promoting the inflammatory process (1, 22, 30). Furthermore, adoptively transferred CD4 T cells from ovalbumin (OVA)-sensitized rats have been reported to induce increases in airway smooth muscle mass when recipients were repetitively challenged with OVA via the airways (33). Thus, CD4 T cells, and perhaps other CD4+ cells, appear to be required for both initiation of airway inflammation and induction of remodeling. Conversely, CD4 depletion 3 wk after the termination of chronic antigen challenge was found, not surprisingly, to have no effect on features of airway remodeling (28). However, to our knowledge, there are no studies addressing the contribution of CD4+ cells to continued airway inflammation and the progressive remodeling that occurs during ongoing chronic allergen challenge, but after the establishment of acute airway inflammation. Using a CD4 depletion strategy at two different time points during such a chronic allergen challenge, we investigated the effect on airway inflammation and remodeling. Interestingly, we found that although mice depleted of CD4+ cells did display significantly reduced signs of lung inflammation, they still underwent the process of lung remodeling.
MATERIALS AND METHODS

Mouse model of OVA-induced airway inflammation and remodeling. Eight- to ten-week-old C57/BL6 mice (Jackson Laboratories) were given intraperitoneal (IP) injections on days 0 and 12 with 50 μg of OVA (Sigma) adsorbed to 0.5 mg of Alum (Pierce). Intranasal challenges of 20 μg of OVA in 20 μl of PBS were given on days 24, 26, and 28. Some mice were killed on day 29 to confirm induction of acute lung inflammation. Further intranasal challenges using the same OVA dose were then performed two times per week for 4 wk to allow progressive airway remodeling (Fig. 1A). Control groups of mice received IP injections of alum without antigen and intranasal challenges with PBS without antigen. Mice were killed 2 days after the last OVA challenge, and BAL fluid, lungs, lung-draining lymph nodes, and spleens were obtained. BAL was performed by intratracheal insertion of catheter and lavaging with 0.8–0.9 ml of 2% filtered BSA (Sigma). The right hilum was tied off and lung was isolated for cellular analysis by FACS. The left lung was instilled with 0.4 ml of 4% paraformaldehyde (PFA) and placed in PFA overnight for one experimental protocol (Fig. 1A). In another protocol, 750 μg of GK 1.5 or control rat IgG (Millipore) was administered IP on days 30, 35, 43, and 50 for one experimental protocol (Fig. 1A). In another protocol, 750 μg of GK 1.5 or control rat IgG were given IP once, 3 days before the last two intranasal challenges (see Fig. 3A). In all experiments, verification of CD4 depletion by FACS was performed in lungs, lung-draining lymph nodes, and spleen. For CD4 depletion before the last two intranasal challenges, peripheral blood was taken via capillary tube eye bleed and processed for FACS analysis.

Airway inflammation analysis. BAL cells were obtained by centrifugation and resuspended in 250 μl of Hanks’ balanced salt solution (Gibco). Total cell counts were performed using a hemacytometer (Hauser-Scientific). Differential cell counts of cytopsins were performed after Hema 3 (Fisher) staining, a modified Wright-Giemsa stain, by counting 200 leukocytes at ×400. Paraformaldehyde-fixed lung sections were stained with hematoxylin and eosin (H&E). Slides were blinded, peribronchial regions, six to eight per mouse, were evaluated at ×200, and inflammatory infiltrates around airways were graded for severity (0, normal; 1, <3 cell diameter thick; 2, 3–10 cells thick; 3, >10 cells thick) and extent (0, normal; 1, <10% of sample; 2, 10–25%; 3, >25%). Scores were calculated by multiplying severity by extent (max 9).

Airway remodeling analysis. Paraformaldehyde-fixed lung sections were stained with Masson’s Trichrome, periodic acid-Schiff (PAS), and anti-α-smooth muscle antibody (Sigma). Mucus metaplasia was determined by counting the number of PAS+ bronchial epithelial cells. The area of peribronchial fibrosis on trichrome-stained sections was evaluated using an image analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD) (9). Smooth muscle area was evaluated in the same manner. All slides were blinded until completion. Results are expressed as the area of staining per micrometer length of basement membrane of bronchioles. At least six bronchi were counted in each slide.

Flow cytometry. Lungs were digested in 2 mg/ml collagenase (Roche) for 30 min, and lung, spleen, and lymph node cells were purified using a 70-μm cell strainer (BD Falcon). Red blood cell lysis was used for spleen and peripheral blood (Sigma). Cells were blocked for 10 min and then stained for 30 min with various combinations of PerCP-conjugated anti-CD4, CD8, PE-conjugated Siglec-F, FITC-conjugated CD11c, and anti-CD3 (BD Biosciences). After being washed, cells were fixed with 2% PFA and analyzed with a FACSCalibur flow cytometer (BD Biosciences). Further analyses were performed with Flow Jo software (Tree Star).

Statistical analysis. Statistical analysis was performed using GraphPad Prism Software (San Diego, CA). The Mann-Whitney test was used where indicated.

RESULTS

CD4 depletion in the lung and lung-draining lymph nodes. Mice were first sensitized and then challenged on days 24–28 with intranasal OVA on three alternating days and killed 1 day...
after the last challenge. The hallmark of the OVA-induced allergic airway model is eosinophilic lung inflammation, and we observed high numbers of BAL leukocytes containing 75–80% eosinophils (Fig. 1B). Marked peribronchial cellular infiltration and epithelial mucus production were present as well. Thus, before CD4 depletion, we confirmed the presence of acute eosinophilic lung inflammation and mucus production. Similar to previous analyses, at this time, we found little/no evidence of airway subepithelial fibrosis or smooth muscle changes (data not shown).

Anti-CD4 antibody was initially given 2 days after the last acute intranasal challenge (day 30) and then once a week during the remaining weeks of chronic antigen challenge (Fig. 1A). After the 8-wk protocol, lung, lung-draining lymph node, and spleen were processed to confirm efficient depletion of CD4+ cells. Lung-draining lymph node and splenic cell populations in control mice had 19–24% CD3+CD4+ cells and less than 2% CD3-CD4+ cells (Fig. 1C). The group receiving anti-CD4 had a near complete absence of CD4 cells with less than 2% of the total CD4+ cells remaining. In the lung, the percent of CD3+ cells was reduced by one-third after anti-CD4 administration (data not shown), with an approximate 98% depletion of CD4+ lung cells (Fig. 1D). Thus, anti-CD4 treatment given after the establishment of acute airway inflammation was effective in depleting the majority of CD4+ cells from the animals.

CD4 depletion during chronic airway antigen challenge reduces lung inflammation. Mice undergoing the 8-wk experimental protocol depicted in Fig. 1A were killed 2 days after the last allergen challenge. Mice that received the CD4-depleting antibody for 4 wk had on average 30–40% reduced levels of lung tissue were 70% lower than in control mice (Fig. 2), further experiments were performed to evaluate if this reflected only an early role, or if continued involvement of CD4 cells was required at later times when mice had already been exposed to chronic allergen for several weeks. Anti-CD4 or control antibody was therefore administered for 1 wk, 3 days before the last two OVA challenges (Fig. 3). Lung cell suspensions made at the time of death also confirmed CD4 depletion (Fig. 3C).

In mice receiving anti-CD4, total lung CD8+(CD3+) T cells were moderately reduced suggesting a subpopulation of CD8 T cells present in the chronically inflamed lung is dependent on CD4+ cells. Interestingly, 80% fewer total lung CD11c+ cells were found, which would partially result from direct depletion of CD4+ dendritic cells, but also indicated that a majority of infiltrating dendritic cells were dependent on the presence of CD4+ T cells or other CD4+ cells. Overall, these results show that CD4+ cells play a substantial role in contributing to the inflammatory infiltrate that is found with chronic allergen challenge, and they are required after the establishment of initial acute inflammation. However, CD4+ cells are not absolutely necessary as significant eosinophilia was found even when they were absent.

CD4 depletion after several weeks of chronic airway challenge reduces eosinophilic inflammation. Given the requirement for CD4+ cells to maintain strong lung inflammation during 4 wk of allergen challenge (Fig. 2), further experiments were performed to evaluate if this reflected only an early role, or if continued involvement of CD4 cells was required at later times when mice had already been exposed to chronic allergen for several weeks. Anti-CD4 or control antibody was therefore administered for 1 wk, 3 days before the last two OVA challenges on wk 8, as shown in Fig. 3A. Analysis of peripheral blood, taken 2 days after anti-CD4 was given, revealed successful depletion of CD4+ cells before the remaining intranasal challenges (Fig. 3B). Lung cell suspensions made at the time of death also confirmed CD4 depletion (Fig. 3C).

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Fig. 2. CD4 depletion reduces lung inflammation during chronic allergen challenge. A: mice undergoing the protocol in Fig. 1A were killed 2 days after the last intranasal antigen challenge. BAL leukocytes (left) and eosinophils (right) were counted. B: H&E-stained lung sections (left) were scored for levels of peribronchial inflammation (top right), and total lung cells were counted (bottom right). C: pooled lung cells were evaluated by FACS for CD3+CD4+, CD3+CD8+, CD11c+, and Siglec-F+CD11c- cells. Results are from 8 mice per OVA group and 4 mice in the Alum/PBS control group. Pooled lung cells are from 4 mice/group. The Mann-Whitney test was used for P values.
were modestly reduced (Fig. 3C). The number of BAL leukocytes was taken 1 day before the second to last intranasal challenge and analyzed by FACS for CD4+ cells. Results in B are 1 representative of 4 mice/group, and pooled lungs from 4 mice/group were analyzed in C; 1 of 2 independent experiments.

Significantly, mice receiving anti-CD4 showed an ~50% reduction in total CD11c+ cells and CD8 T cells that accumulated in the lung (Fig. 3C). The number of BAL leukocytes were modestly reduced (P = 0.0070) after 1 wk of CD4 depletion (Fig. 4A). However, these mice had ~50% fewer BAL eosinophils (P = 0.0019), total lung cells, and peribronchial inflammation as measured by histological scoring (<0.001) (Fig. 4, A–C). Assessment of lung eosinophils by analysis of Siglec-F+CD11c- cells also revealed an ~60% reduction compared with mice receiving the control antibody. Thus, CD4+ cells continue to play a strong role in controlling the extent of lung inflammation during periods of exposure to airborne antigen, even weeks after the onset of chronic exposure. However, again, they are not absolutely necessary as eosinophilia and influx of some dendritic cells and CD8 T cells can occur in their absence.

CD4 depletion during chronic allergen challenge does not reduce airway remodeling. Last, we assessed airway remodeling. Previous studies have shown that remodeling occurs progressively over 4 wk of chronic exposure to antigen following the initial acute lung inflammatory response (6). After repetitive antigen challenge, airway remodeling features were present in control mice that included strong increases in peribronchial subepithelial fibrosis, smooth muscle area, and the number of mucus-producing epithelial cells (Fig. 5). However, most significantly, the extent of fibrosis and smooth muscle mass was not any different between mice receiving the CD4-depleting antibody and the control antibody (P = 0.8076). There was a slight trend toward a reduction in PAS-positive epithelial cells, representing mucus metaplasia, but this did not reach statistical significance (P = 0.1195). Therefore, although CD4+ cells contribute to chronic inflammation, they are not required for progressive airway remodeling that occurs following the acute lung inflammatory response.

DISCUSSION

Allergic asthma is characterized by type 2 inflammatory responses in the lung, presumed to be orchestrated by CD4 T cells. Here, we used a chronic Th2-driven airway model of allergic asthma to define the role of CD4+ cells, including CD4 T cells, during the chronic phase of response separable from any action in regulating the acute phase of inflammation. We found that CD4+ cells continue to contribute significantly to eosinophilic lung inflammation after the establishment of acute inflammation, throughout the period of chronic antigen exposure as well as several weeks after the initiation of chronic allergen challenge. Interestingly, the depletion of CD4+ cells...
CD4 depletion will also eliminate the CD4 T cells, possibly the maintenance of populations of memory T cells. Studies have hypothesized that they might regulate development of tertiary lymphoid structures in inflamed tissue and possibly the maintenance of populations of memory T cells. CD4 depletion will also eliminate the CD4+ subset of dendritic cells, although less than 2% of lung CD4+ cells were CD3 negative (data not shown), suggesting that these dendritic cells represent a minor fraction of the lung infiltrate. However, we found that CD4 depletion strongly results in a reduction in the total numbers of all CD11c+ dendritic cells that accumulate in the lung. Lung dendritic cells expressing CD11c most likely rely on CD4 T cells for homeostasis and proliferation, and signaling through CD40 and the lymphotoxin β-receptor (LTBR) during the interaction of dendritic cells with CD4 T cells may mediate these processes (37, 41). Thus, the range of cell types affected by CD4 depletion is great, but we conclude that these varying populations are not needed at all, or are not needed in great numbers, for the progressive remodeling that occurs over 4 wk of chronic allergen challenge.

We also observed a significant decrease in CD8 T cell lung populations after administration of anti-CD4 for 1 or 4 wk. In different model systems, CD8 T cell responses are known to require CD4 T cell help and may explain this result (2, 18). Interestingly, CD8 T cells have been shown to play a role in airway inflammation and hyperresponsiveness (32, 38) and hence their reduced numbers in the lungs may have contributed to the reduction in inflammation we observed. On the other hand, the remaining CD8 T cells might be required for the continued eosinophilic inflammation that we observed in the absence of CD4+ cells, and this needs to be investigated in future studies depleting this subset of cells.

One last cell type that needs to be considered is the CD4+ regulatory T cell. CD4+CD25+Foxp3+ Tregs generally represent 5–15% of the total CD4+ population in a mouse, and CD4 depletion will eliminate these cells as well. A study in OVA-sensitized rats revealed that CD25+ αβ T cells in the tracheal mucosa begin to increase after the first intranasal challenge of OVA.
OVA and remained elevated after 10 daily challenges (36). These cells were able to suppress OVA-specific T cell proliferation ex vivo. Other reports have shown that depletion of CD4+CD25+ cells before acute airway allergen challenges exacerbated lung eosinophilia, and adoptive transfer of these cells can also significantly protect against developing lung inflammation (26, 29). Not surprisingly, mice deficient in Foxp3 have increased lung inflammation and smooth muscle mass after repetitive antigen challenge (10). Additionally, a study employing adoptive transfer of OVA-specific CD4+CD25+ cells into mice, after acute lung inflammation was established, revealed that these cells could significantly decrease airway inflammation and remodeling brought about by subsequent repetitive airway challenges (21). Interestingly, when these cells were transferred at a later time point after remodeling was established, there was no reversal of remodeling features or inflammation. However, there is little direct data showing that any endogenous Treg cells continue to be active in a more physiological chronic inflammatory response in the lung, and, in particular, during the time period we targeted here after acute inflammation was established. Thus, although previous studies suggest that these cells have the potential to be suppressive in a chronic response, the effect of depleting CD4+ regulatory T cells is unclear in our model system. Given the reduction in measures of airway inflammation after CD4 depletion, it seems likely that the CD4 effector population is playing a larger or more dominant role than CD4 regulatory cells. Collectively, these results then support a model whereby lung inflammation and airway remodeling become disconnected at a certain point during a chronic asthmatic response. Consistent with this, a recent report demonstrated that IL-13 had persistent proremodeling effects in the lung long after inflammation had resolved (13). Furthermore, in human asthmatics, airway expression of procollagen I and α-smooth muscle actin were found to increase 7 days after allergen challenge when inflammatory cells had returned to baseline (20). Perhaps, after initiation of the remodeling process, which collectively requires CD4 T cells, other CD4+ cells, and eosinophilic inflammation, a number of growth factors and cytokines, including TGF-β1 and VEGF, produced by CD4-negative cell types such as macrophages or activated epithelial cells, are sufficient for maintenance of remodeling as implied from other reports (25, 31). Although our data support an uncoupling of inflammation and remodeling similar to prior observations (13, 20, 27, 31), the mechanisms that lead to such a divergence have yet to be fully elucidated.

In summary, this study demonstrates that depletion of CD4+ cells, after the establishment of acute Th2-driven airway inflammation, has no effect on mucus metaplasia, bronchial fibrosis, or smooth muscle thickness. However, CD4 cells continue to contribute to eosinophilic inflammation, even at late times after chronic exposure to allergen has occurred. Thus, successful therapies of chronic asthma might need to use combination approaches that target airway remodeling distinct from those that target Th2 cells and inflammation.

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