An imbalance of esophageal effector and regulatory T cell subsets in experimental eosinophilic esophagitis in mice

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Zhu X, Wang M, Crump CH, Mishra A. An imbalance of esophageal effector and regulatory T cell subsets in experimental eosinophilic esophagitis in mice. Am J Physiol Gastrointest Liver Physiol 297: G550–G558, 2009. First published July 1, 2009; doi:10.1152/ajpgi.00148.2009.—We recently reported a critical role for T cells in the induction of eosinophilic esophagitis (EE) in mice; however, the role of specific T cell subsets in disease pathogenesis is not yet understood. In the current study, we tested the hypothesis that allergen-induced EE develops in response to the disproportionate of functionally different effector and regulatory T cells in the esophagus. Fluorescence-activated cell sorter analysis was performed to examine activated T cell subsets using the cell surface activation markers CD25 and CD69. A significant increase in activated CD4+ and CD4− T cells was observed in the total esophageal cells isolated from the mouse model of EE. Furthermore, an imbalance in the effector and regulatory T cells was observed in the esophagus. The esophageal CD4+CD45RBlow effector T cells in allergen-challenged mice increased compared with saline-challenged mice (65.4 ± 3.6 × 10³ to 44.8 ± 4.2 × 10³), whereas CD4+CD45RBhigh mostly regulatory T cells decreased in allergen-challenged mice compared with saline-challenged mice (5.8 ± 0.9 × 10³ from 10.2 ± 1.7 × 10³). The functional characteristics were examined by analysis of the pro- and anti-inflammatory cytokine profile of purified low and high CD4+CD45RB subsets from the spleen. Additionally, a significantly reduced interleukin (IL)-2 production by CD4+CD45RBlow cells in allergen-challenged mice compared with saline-challenged mice was observed. The reduced IL-2 in the CD4+CD45RBlow subset may be associated with reduction of CD4+CD45RBlow subset. In conclusion, our results suggest that local regulatory interaction of CD45RBhigh and CD45RBlow CD4+ T cells may be required for protective and pathogenic immunity in EE.

Eosinophilic esophagitis (EE) is a chronic inflammatory disease that mimics gastroesophageal reflux disease but does not respond to acid suppressive therapy, but rather responds to anti-inflammatory agents and measures designed to reduce allergen-triggered responses (9, 16, 35). EE is thought to occur as a result of Th2-dominated immune responses to otherwise innocuous food and environmental allergens (20–22). Recently, it has been reported that EE pathogenesis is dependent upon adaptive T cell immunity (23). A greater understanding of the mechanism of regulation of T cell activity in disease pathogenesis is needed to develop effective therapeutic strategies for the treatment of EE in humans. Regulatory T cells have been identified as important in the prevention of autoimmune disease and other immunopathologies (1). Studies in mice and humans have demonstrated that a novel transcription-repressor protein, FOXP3, is exclusively expressed by naturally occurring and induced regulatory T cells (6, 7, 12, 28, 40); therefore, FOXP3 is the best marker to identify these cells (5, 13, 32). Regulatory T cells are functionally distinguished from effector T cells by their capacity to limit T cell proliferation and function (3, 26). Evidence shows that regulatory T cells control autoimmune responses (8, 14, 34).

The effector and regulatory T cells can be divided into different subsets based on the expression of cell surface markers, production of cytokines, and their mechanism of action. Notably, a number of earlier studies demonstrated that effector and regulatory T cells both express CD45RB (19, 27, 42) and the level of CD45RB expression differentiates T cell pathogenic and regulatory function (19, 27). Regulatory T cells are predominantly found within the CD4+CD45RBlow subset (19, 27, 42), and they have a role in the control of immune response to infectious agents, allografts, cancer, and graft-versus-host reactions (2, 4, 24, 33, 39). Regulatory T cells have been implicated in mediating oral tolerance, and indirect evidence suggests that they may control the development of allergen-induced diseases in mice (11, 15, 38, 41). Therefore, we focused our current study on testing the hypothesis that EE may involve an imbalance in functionally different effector and regulatory T cells in response to allergen exposure.

To examine this, we identified and examined the subsets of pathogenic effector and anti-inflammatory regulatory T cells in a murine model of experimental EE. A significant increase of effector T cells was observed in the esophagus, but the number of regulatory T cells decreased. Furthermore, we observed reduced interleukin (IL)-2 production by splenic CD4+CD45RBlow cells following allergen challenge, providing evidence for an imbalance in pathogenic CD45RBhigh and regulatory CD45RBlow CD4+ T cells following EE induction.

MATERIALS AND METHODS

Mice. Specific pathogen-free BALB/c mice were obtained from Jackson Laboratory (Bar Harbor, ME). All the experiments were performed on age- and gender-matched, 6- to 8-wk-old mice. The mice were maintained in a pathogen-free barrier facility, and animals were handled according to institutional animal care and use committee (IACUC) and National Institutes of Health guidelines. The studies reported were approved by IACUC.

Experimental EE induction. A mouse model of allergic EE was established using methods described previously (20). In brief, mice were lightly anesthetized with isoflurane (Iso-Flo; Abbott Laboratories, North Chicago, IL), and 100 µg (50 µl) of Aspergillus fumigatus (Bayer Pharmaceuticals, Spokane, WA) or 50 µl of normal saline alone was applied to the nares using a micropipette with the mouse held in the supine position. After instillation, mice were held upright

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until alert. After three treatments per week for three weeks, mice were killed between 18 and 20 h after the last intranasal allergen and saline challenge.

**Esophageal cell isolation.** The allergen-challenged mice were euthanized, and immediately thereafter the esophagus from proximal to distal was removed and thoroughly rinsed with PBS, pH 7.2. The esophagus was opened longitudinally, washed with Ca- and Mg-free PBS, pH 7.2, and cut into small pieces that were subsequently pooled. The pooled tissue was incubated 40 min in 2 ml of RPMI tissue culture media (GIBCO) with 0.5 mg/ml Liberase Chloride (Rush Biochemicals) in 0.5% CO2 at 37°C. After digestion, single cells were isolated and filtered through a 70-μm cell strainer followed by a 40-μm cell strainer (BD Falcon). The cells were centrifuged, and red blood cells were removed from the cell pellet by using RBC lysis buffer (Sigma). Cells were centrifuged at 250 g for 5 min, and cell pellets were resuspended in PBS and counted.

**Antibodies and flow cytometric analysis.** The total esophageal cells were stained with cell surface-specific antibodies for flow cytometric analysis. The following reagents were used for specific antigen analysis; anti-CD3, -CD4, -CD8, -CD45 (pan marker), -B220, -CD45RB, -CD25, -CD69, and respective isotype controls obtained from eBiosciences. FcR block (anti-CD16/CD32) was added to all surface-staining mixtures. 7ADD was used to exclude dead cells. The cells were incubated for the specific antigens with the required combination of antibodies at 4°C for 45 min followed by two washes. Flow cytometry was performed using a FACSCaliber or LSRII (BD Biosciences) and analyzed using CellQuest software (BD Sciences).

**Isolation of CD45RB<sup>low</sup> and CD45RB<sup>high</sup> cells.** A single cell suspension was prepared in cold PBS from the spleen of BALB/c mice. The total cells were labeled with APC-conjugated CD4 and phycoerythrin conjugated CD45RB for the sorting the CD45RB<sup>low</sup> and CD45RB<sup>high</sup> T cell population. All of the analyses were performed in sterile conditions. The CD45RB<sup>low</sup> and CD45RB<sup>high</sup> T cell fractions were obtained using multicolor cell sorting on a fluorescence-activated cell sorter (FACS) Vantage (BD Sciences). The CD45RB<sup>low</sup> and CD45RB<sup>high</sup> T cell populations were defined as the highest staining 40–50% and the dullest staining 15–20% CD4<sup>+</sup> T cells, respectively. Intermediate staining populations were discarded. The sorted populations were 99% pure on analysis.

**Cytokine analysis.** Cytokine production from CD4<sup>+</sup>CD45RB<sup>low</sup> subsets of saline- and allergen-challenged mice were measured following the 72 h in vitro cell culture at 37°C in the humidified chamber containing 5% CO2 with 5 μg/ml anti-mouse CD3 and 2 μl/ml anti-mouse CD28. The supernatant was collected, and cytokine levels were determined by using BD OptEIA enzyme-linked immunosorbent assay (ELISA) set (BD Biosciences, San Diego, CA) as per the manufacturer’s protocol. Briefly, cell culture supernatant was applied to a cytokine-specific monoclonal antibody precoated 96-well ELISA plates. The absolute numbers in the esophagus of activated CD4<sup>+</sup> and CD4<sup>+</sup> T cells were calculated and are shown in B, F, and H. Data are expressed as means ± SD; n = 3 experiments, and each experiment contains 6–8 mice/group.

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**Fig. 1.** Activated T cells are increased in the esophagus following Aspergillus challenge. Flow cytometric (fluorescence-activated cell sorter (FACS)) analysis was performed to examine activated T cells in the esophagus using the lymphocyte activation marker antibody anti-CD25. The total esophageal cells from saline- and allergen-challenged mice were stained with anti-CD45 (pan marker), anti-CD25, anti-CD69, and 7ADD. Total live leukocytes (CD45<sup>-</sup> and 7ADD<sup>-</sup>) were gated and analyzed for activated T cells (data not shown). The analysis indicates a significant increase of CD4<sup>-</sup>CD25<sup>+</sup>, CD4<sup>-</sup>CD25<sup>-</sup>, CD4<sup>+</sup>CD69<sup>-</sup>, and CD4<sup>+</sup>CD69<sup>+</sup> subsets in the esophagus of allergen-challenged mice compared with saline-challenged mice (A–H). A representative histogram analysis of saline- and allergen-challenged esophageal cells are shown for CD4<sup>-</sup>/CD25<sup>+</sup> (A), CD4<sup>-</sup>/CD25<sup>-</sup> (C), CD4<sup>+</sup>/CD69<sup>-</sup> (E), and CD4<sup>+</sup>/CD69<sup>+</sup> (G). The absolute numbers in the esophagus of activated CD4<sup>+</sup> T cells were calculated and are shown in B, D, F, and H. Data are expressed as means ± SD; n = 3 experiments, and each experiment contains 6–8 mice/group.
plate after blocking nonspecific protein binding with 10% FBS. The plate was incubated for 2 h at room temperature and washed with 0.05% Tween 20-PBS, and biotinylated cytokine-specific monoclonal antibody was applied to each well followed by avidin-horseradish peroxidase conjugate reagent. Finally, TMB substrate solution (BD Biosciences PharMingen) was added to each well, the color was developed in the dark at room temperature, and the optical density was read at 450 nm immediately. The cytokine concentration of each sample was calculated by using a standard curve.

Real-time PCR analysis. The purified CD45RB cell RNA samples (500 ng) were subjected to reverse transcription analysis using BioScript reverse transcriptase (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. The cytokine mRNA levels were quantified by real-time PCR using the LightCycler instrument and LightCycler FastStart DNA master SYBR green I as a ready-to-use reaction mix (Roche, Indianapolis, IN). Results were then normalized to β-actin amplified from the same cDNA mix and expressed as fold induction compared with the controls. cDNA were amplified using the primers obtained from SuperArray Bioscience (Frederick, MD).

Statistical analysis. Data are expressed as means ± SD. Statistical significance comparing different sets of mice was determined by unpaired InStat GraphPad t-test, and P < 0.05 is considered statistically significant.

RESULTS

T cells are increased in the esophagus following intranasal allergen exposure. We previously showed that Aspergillus-induced experimental EE is a T cell-dependent manner (23); therefore, we tested the hypothesis that EE occurs in response to the local induction of activated T cells. Accordingly, we examined the number of CD25+ and CD69+ T cells in a CD45+ total leukocyte population of totally isolated esophageal cells from saline- and allergen-challenged mice. The T cell marker antibodies (i.e., anti-CD25 and anti-CD69) were used to identify T cells. Our analysis indicated a significant increase in the percent and absolute numbers of both CD4+ and CD4− activated T cells (Fig. 1, A–H). The total number of CD4+ cells in the esophagus was used to calculate absolute numbers of CD4+ and CD4− activated T cells. The absolute numbers of CD4+CD25+ and CD4−CD25− cells in the allergen-challenged mouse esophagus were 5.8 ± 0.8 × 10^3 and 6.9 ± 0.9 × 10^3 compared with 3.3 ± 0.4 × 10^3 and 4.9 ± 0.9 × 10^3, respectively (mean ± SD, n = 3) in saline-challenged mice (Fig. 1, B and D), and the absolute numbers of CD4+CD69+ and CD4−CD69− in allergen-challenged mice esophagus are 5.9 ± 0.7 × 10^3 and 12.1 ± 1.9 × 10^3 compared with 3.2 ± 0.2 × 10^3 and 8.1 ± 1.9 × 10^3, respectively (mean ± SD, n = 3), in saline-challenged mice (Fig. 1, F and H). Interestingly, a comparable level of total CD3+, CD8+, and B220+ cell subsets were observed in isolated esophageal cells from saline- and allergen-challenged mice (data not shown).

**Fig. 2.** CD4+CD45rbhigh FOXP3− is increased in the esophagus in experimental eosinophilic esophagitis (EE). The total esophageal cells from saline- and allergen-challenged mice were analyzed for effector T cells (CD4+CD45rbhighFOXP3−) using flow cytometric (FACS) analysis. The CD4+CD45rbhigh population was gated (A), and their numbers were examined following saline and allergen challenge (B). C: CD4+CD45rbhigh cells were quantified, and a significant increase is observed in Aspergillus-challenged mice compared with the saline-challenged mice. Anti-FOXP3 staining was performed to confirm that CD4+CD45rbhigh cells are not regulatory T cells; >99% of the CD4+CD45rbhigh cells are FOXP3−. Data are expressed as means ± SD; n = 3 experiments, and each experiments contain 6–8 mice/group.
challenged mice compared with saline-challenged mice (Fig. 2A), and a comparable number of CD4⁺CD45RB<sup>high</sup> cells were observed in allergen-challenged and saline-challenged mice (Fig. 2A). The absolute numbers of CD4⁺CD45rb<sup>high</sup> cells in allergen- and saline-challenged mice were 65.4 ± 3.6 × 10<sup>2</sup> and 44.8 ± 4.2 × 10<sup>2</sup> and 28.6 ± 4.2 × 10<sup>2</sup> and 24.9 ± 6.4 × 10<sup>2</sup> in CD4⁺CD45rb<sup>high</sup> cells, respectively (mean ± SD, n = 3). Furthermore, to confirm that CD4⁺CD45RB<sup>high</sup> cells were FOXP3⁻ in the esophagus, we analyzed these cells for FOXP3 expression. Our analysis revealed that >99% of CD⁺CD45RB<sup>high</sup> cells were FOXP3⁻ in saline- and allergen-challenged mice (Fig. 2C).

Regulatory T cells (CD4⁺CD45RB<sup>low</sup>FOXP3⁺) are decreased in the esophagus following intranasal allergen exposure. It has been well established that CD4⁺CD45RB<sup>low</sup> cells in the gastrointestinal tract are predominately anti-inflammatory cytokine-producing regulatory T cells (29, 37). Therefore, we next tested the hypothesis that an imbalance of pathogenic and regulatory T cells occurs in the esophagus following the induction of experimental EE. Accordingly, the esophageal cells were examined for CD4⁺CD45RB<sup>low</sup> and CD4⁺CD45RB<sup>low</sup> subsets in saline- and allergen-challenged mice (Fig. 3A). A significantly reduced number of CD4⁺CD45RB<sup>low</sup> subset in the esophagus of allergen-challenged mice compared with saline-challenged mice (Fig. 3B) and a comparable number of CD4⁺CD45RB<sup>low</sup> subsets in saline- and allergen-challenged mice were observed (data not shown). Consistent with these findings, a significant decrease in FOXP3⁺ cells in the esophagus of allergen-challenged mice compared with saline-challenged mice was observed (Fig. 3C). The quantification of esophageal FOXP3⁺CD4⁺CD45RB<sup>low</sup> cells in allergen-challenged mice showed a significant reduction compared with saline-challenged mice (Fig. 3D), whereas a comparable number of FOXP3⁺CD4⁺CD45RB<sup>low</sup> cells was observed in saline- and allergen-challenged mice (Fig. 3E). The absolute number of CD4⁺CD45RB<sup>low</sup>FOXP3⁺ and CD4⁺CD45RB<sup>low</sup>FOXP3⁻ cells in saline- and allergen-challenged mice was 31.5 ± 3.1 × 10<sup>2</sup> and 69.9 ± 8.3 × 10<sup>2</sup> compared with 9.1 ± 7.9 × 10<sup>2</sup> and 51.7 ± 9.2 × 10<sup>2</sup> (mean ± SD, n = 3). Interestingly, a significant number of FOXP3⁺ cells in the CD4⁺CD45RB<sup>low</sup> subset was observed in the esophagus, but their numbers were comparable in saline- and allergen-challenged mice. The absolute number of CD4⁺CD45RB<sup>low</sup>FOXP3⁺ cells in saline- and allergen-challenged mice was 0.3 ± 0.2 × 10<sup>2</sup> and 0.4 ± 0.2 × 10<sup>2</sup> (mean ± SD, n = 3).

The spleen of allergen- and saline-challenged mice has a comparable number of activated T cells. Next, we tested the hypothesis that the imbalance in effector and regulatory T cells...
in the esophagus was specific for this tissue. Therefore, we further examined CD25, CD69, CD45RB, and FOXP3 positive cells in CD4\(^+\) and CD4\(^-\) cell subsets in the spleen of mice following allergen and saline challenge. The splenocyte analysis showed comparable numbers of both CD4\(^+\) and CD4\(^-\) activated T cells (Fig. 4, A–D). The absolute number of CD4\(^+\)CD25\(^+\) and CD4\(^+\)CD25\(^-\) cells in the spleen of allergen-challenged mice was 19.8 ± 8.2 \times 10^3 and 4.8 ± 3.6 \times 10^3 compared with 14.8 ± 8.2 \times 10^3 and 4.1 ± 1.3 \times 10^3, respectively (mean ± SD, n = 3), in saline-challenged mice, and CD4\(^+\)CD69\(^+\) and CD4\(^+\)CD69\(^-\) in allergen-challenged mouse esophagus was 13.8 ± 0.6 \times 10^3 and 11.8 ± 2.1 \times 10^3 compared with 13.6 ± 2.4 \times 10^3 and 9.4 ± 1.6 \times 10^3, respectively (mean ± SD, n = 3), in saline-challenged mice. Further analysis indicated that the spleen also has comparable CD4\(^+\)CD45RBlowFOXP3\(^-\) cells, but CD4\(^+\)CD45RBlowFOXP3\(^+\) numbers decrease in allergen-challenged mice compared with saline-challenged mice. Interestingly, mediastinal lymph nodes showed a similar increase of activated T cells and decrease of regulatory T cells as we observed in the esophagus, except an increase of CD4\(^-\)CD45RBlow subset. The absolute numbers of both CD4\(^+\) and CD4\(^-\) lymphocytes identified by the activation marker CD25 and CD69 were increased in the mediastinal lymph nodes following allergen challenged in mice (Fig. 5, A and B). Additional analysis showed that CD4\(^+\)CD45RB\(^{high}\)FOXP3\(^-\) regulatory T cells were decreased, and no significant change in the absolute numbers of CD4\(^+\)CD45RB\(^{high}\)FOXP3\(^-\) effector T cells was observed in allergen-challenged mice compared with saline-challenged mice (Fig. 5, E and F).

**Functional characteristics of CD4\(^+\)CD45RB\(^{cell} subset**

Next, we determined the pro- and anti-inflammatory characteristic of FACS-sorted low and high CD45RB CD4\(^+\) T cell subsets isolated from the spleen of naive mice. RNA from the FACS sorted CD4\(^+\)CD45RB\(^{high}\) and CD4\(^+\)CD45RB\(^{low}\) subsets (>95% pure) were isolated (Fig. 6, A and B), and the transcription profile of IL-5, IL-13, IL-10, and transforming growth factor (TGF)-\(\beta\) was examined using quantitative real-time PCR analysis. A high mRNA level of IL-5 and IL-13 was observed in the CD4\(^+\)CD45RB\(^{high}\) subset compared with non-detectable in CD4\(^+\)CD45RB\(^{low}\) cells (Fig. 6, C and D), whereas a higher mRNA level of IL-10 and TGF-\(\beta\) was observed in the CD4\(^+\)CD45RB\(^{low}\) subset compared with CD4\(^+\)CD45RB\(^{high}\) cells (Fig. 6, E and F).

**IL-2 mRNA and protein levels are decreased in CD4\(^+\)CD45RB\(^{low}\) subsets in experimental EE.** IL-2 is the survival factor for T cells; therefore, we examined the mRNA
and protein levels in CD4+CD45RB\textsuperscript{low} subsets of T cells to test the hypothesis that the allergen challenge may affect IL-2 production by this T cell subset. Accordingly, we determined mRNA and protein levels of IL-2 in CD4+CD45RB\textsuperscript{low} FACS sorted CD3/CD28 stimulated cells along with the IL-4 and IL-10. The IL-2 mRNA and protein levels in sorted CD4+CD45RB\textsuperscript{low} cells following allergen challenge were decreased more than eightfold compared with the saline-challenged mice (Fig. 7, A and B; quantification of FACS analysis indicates a significant increase in the absolute numbers of CD4+CD25\textsuperscript{+}, CD4+CD25\textsuperscript{+}, or CD4+CD69\textsuperscript{+} and CD4+CD69\textsuperscript{+} subsets in the mediastinal lymph node of allergen-challenged mice compared with saline-challenged mice. Additionally, low and high populations of effector and regulatory T cells were analyzed by staining total mediastinal lymph node cells with anti-CD4, anti-CD45RB, and anti-FOXP3 antibodies. C: total lymphocytes were gated and analyzed for low and high subsets of CD4+CD45RB\textsuperscript{+}FOXP3 as shown in D. E: a significant decrease in CD4+CD45RB\textsuperscript{+}FOXP3. F: no change in CD4+CD45RB\textsuperscript{+}FOXP3 cells. Data are expressed as means ± SD; n = 3 experiments, and each experiment contains 6–8 mice/group.

Fig. 5. Quantification of allergen-induced CD4\textsuperscript{+} T cell subsets in mediastinal lymph nodes. The mediastinal lymph node cells from saline- and allergen-challenged mice were analyzed for activated T cells and FOXP3 positive and negative CD4\textsuperscript{+}CD45RB\textsuperscript{+} cells. The activated CD4\textsuperscript{+} and CD4\textsuperscript{−} cells were detected by staining the cells with anti-CD25, anti-CD69, and 7ADD. Total live leukocytes (7ADD\textsuperscript{−}) were gated and analyzed (data not shown). A and B: quantification of FACS analysis indicates a significant increase in the absolute numbers of CD4+CD25\textsuperscript{+}, CD4+CD25\textsuperscript{+}, or CD4+CD69\textsuperscript{+} and CD4+CD69\textsuperscript{+} subsets in the mediastinal lymph node of allergen-challenged mice compared with saline-challenged mice. Additionally, low and high populations of effector and regulatory T cells were analyzed by staining total mediastinal lymph node cells with anti-CD4, anti-CD45RB, and anti-FOXP3 antibodies. C: total lymphocytes were gated and analyzed for low and high subsets of CD4+CD45RB\textsuperscript{+}FOXP3 as shown in D. E: a significant decrease in CD4+CD45RB\textsuperscript{+}FOXP3. F: no change in CD4+CD45RB\textsuperscript{+}FOXP3 cells. Data are expressed as means ± SD; n = 3 experiments, and each experiment contains 6–8 mice/group.

**DISCUSSION**

EE is a chronic inflammatory disease associated with marked eosinophil accumulation and eosinophilic microabscesses in the esophageal epithelial mucosa (25, 30, 31). It has been shown that esophageal T cell subsets are increased in human and experimental EE, and adaptive T cell immunity is critical in the development of EE (23, 25); however, the role of specific T cell subsets is still not understood in disease pathogenesis. Several reports indicate that effector and regulatory T cell subsets have an important role in the protective and pathogenic process in allergic and autoimmune diseases (10, 18, 27, 29, 37). Therefore, we explored the status of the pathogenic effector and regulatory T cell subsets in the pathogenesis of experimental EE. We induced experimental EE in mice as per the protocol described earlier (20) and first examined the number of CD25 and CD69\textsuperscript{+} T cells in the spleen and esophagus following the induction of experimental EE. Second, we examined the types of activated T cells on the basis of the expression levels of CD4, CD45RB, and FOXP3 in saline- and allergen-challenged mice. Third, we examined the pathogenic and anti-inflammatory characteristics of high and low populations of splenic CD45RB T cells by performing quantitative PCR analysis on isolated subsets from naïve mice.

We demonstrate that activated CD4\textsuperscript{+} and CD4\textsuperscript{−} T cells are increased in the mediastinal lymph nodes and esophagus, but not in the spleen following allergen exposure. This indicates that lymphocytes are recruited in esophagus via draining lymph nodes, where they proliferate and activate in response to allergen. Furthermore, analysis showed a substantial disproportionate in the frequency of CD4\textsuperscript{+}CD45RB\textsuperscript{high} and CD4\textsuperscript{+}CD45RB\textsuperscript{low} subset in the esophagus of allergen-
challenged mice compared with saline-challenged mice. An increase of CD4⁺CD45RB<sup>high</sup> subsets was observed in the esophagus and no change in the spleen and mediastinal lymph nodes, whereas the number of CD4⁺CD45RB<sup>low</sup> cells was decreased in the spleen, mediastinal lymph nodes, and esophagus. Moreover, no significant change was observed in the esophageal CD4⁺CD45RB<sup>low</sup> or high subsets in allergen-challenged mice compared with saline-challenged mice. These data demonstrate that an inequity of low and high subsets of CD4⁺CD45RB cells specifically occurs in the esophagus that may have a critical role in disease pathogenesis.

The CD4⁺CD45RB<sup>low</sup> cells are capable of producing more anti-inflammatory cytokines than the proinflammatory cytokines, and the present data confirmed their characteristics. The proinflammatory and anti-inflammatory cytokine-producing capability of both CD45RB subsets functionally differentiates their characteristics. Interestingly, further analysis revealed that the reduction of CD4⁺CD45RB<sup>low</sup> cell subsets is due to a decrease of FOXP3<sup>+</sup> cells, since CD4⁺CD45RB<sup>low</sup>FOXP3<sup>+</sup> cells have comparable numbers in the esophagus and mediastinal lymph nodes of saline- and allergen-challenged mice. Regulatory T cells have been increasingly defined as important in the prevention of adaptive and innate immunity. In the allergen-induced experimental model of EE, CD4⁺CD45RB<sup>low</sup>FOXP3<sup>+</sup> regulatory T cells are decreased in all of the examined tissues, including draining mediastinal lymph nodes. This decrease may be because of increase of heterogeneity of CD45RB unstable expression in response to the allergen or CD4⁺CD45RB<sup>low</sup> cells may undergo apoptosis. Furthermore, to understand the possible mechanism of CD4⁺CD45RB<sup>low</sup> cell reduction, we examined IL-2 expression in this subset. A loss of IL-2-producing capability in sorted CD4⁺CD45RB<sup>low</sup> cells was noticed in allergenchallenged mice. This loss of IL-2-producing capability may promote apoptosis of CD4⁺CD45RB<sup>low</sup> cells, and their number decreases following allergen challenge. Our data also indicate the importance of CD4⁺CD45RB<sup>low</sup> cells during disease progression in EE, since they are a rich source of IL-10 in allergen-challenged mice. These results are consistent with the other studies that show T regulatory cells have an important role in Th2 cytokine-induced immune responses (14, 27, 29, 37).

These data provide the possibility of a role for CD4⁺CD45RB T cells in EE pathogenesis. Previously, it has been shown that CD45RB<sup>low</sup> and CD45RB<sup>high</sup> populations of CD4 T cells are functionally different and have a protective and pathogenic activity (36, 37). The cytokine transcription profile of FACS sorted CD4⁺CD45RB<sup>high</sup> and CD4⁺CD45RB<sup>low</sup> provides evidence that indeed these cells have pathogenic and protective characteristics although these cells were isolated from the spleen and not the esophagus (the latter did not have sufficient numbers for ready analysis).

Our data are in accordance with earlier reports that demonstrated that low and high subsets of CD4⁺CD45RB cells are the source of anti-inflammatory and proinflammatory cytokines in the gastrointestinal tract (27, 37). Collectively, we report an imbalance of anti-inflammatory and proinflammatory T cell subsets in the esophagus and suggest a pathogenic and protective role for CD45RB<sup>high</sup> and CD45RB<sup>low</sup> CD4⁺ T cells.

Fig. 6. Characterization of sorted CD4⁺CD45RB cell subsets. Representative dot-blot histogram of total CD45RB cell subsets (A), the histogram plot showing the levels of CD45 expression on gated CD4⁺ cells that were sorted for the phenotype characterization. B: representative histogram of purified CD4⁺CD45RB<sup>low</sup> and CD4⁺CD45RB<sup>high</sup> T cell subsets; the purity of these subsets was >95%. The pathogenic cytokine transcript levels of interleukin (IL)-5 (C) and IL-13 (D) and regulatory cytokine transcript levels of IL-10 (E) and transforming growth factor (TGF)-β (F) in CD4⁺CD45RB<sup>low</sup> and CD4⁺CD45RB<sup>high</sup> T cell subsets were determined to examine the subsets characteristics before transferring into the mice. Data are expressed as means ± SD; n = 3 experiments, and each experiment contains 6–8 mice/group.
in EE pathogenesis. Taken together, the present findings indicate that regulatory interaction between CD45RBhigh and CD45RBlow CD4 T cells may be critical in promoting protective and pathogenic immunity in EE. Further studies are required to define the in vivo role of CD45RBlow CD4+ T and CD45RBhigh CD4+ T subsets in experimental EE.

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GRANTS

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Fig. 7. Cytokine profile of CD4+CD45RBlow subsets following allergen challenge. Cytokine production of sorted CD4+CD45RBlow subsets of saline- and allergen-challenged mice was measured after CD3/CD28 stimulation for 72 h. The mRNA expression and protein levels of the cytokines IL-2 (A and B), IL-4 (C and D), and IL-10 (E and F) following saline and Aspergillus challenge. Data were obtained 18–20 h after the last treatment and are expressed as means ± SD; n = 9–11 mice/group, and each experiment contains 6–8 mice/group. ND, not detectable.


