Regulatory T Cells Modulate Staphylococcal Enterotoxin B-Induced Effector T-Cell Activation and Acceleration of Colitis

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Oral administration of bacterial superantigen Staphylococcus aureus enterotoxin B (SEB) activates mucosal T cells but does not cause mucosal inflammation. We examined the effect of oral SEB on the development of mucosal inflammation in mice in the absence of regulatory T (Treg) cells. SCID mice were fed SEB 3 and 7 days after reconstitution with CD4+ CD45RBhigh or CD4+ CD45RBhigh plus CD4+ CD45RBlow T cells. Mice were sacrificed at different time points to examine changes in tissue damage and in T-cell phenotypes. Feeding SEB failed to produce any clinical effect on SCID mice reconstituted with CD4+ CD45RBhigh and CD4+ CD45RBlow T cells, but feeding SEB accelerated the development of colitis in SCID mice reconstituted with CD4+ CD45RBhigh T cells alone. The latter was associated with an increase in the number of CD4+ Vβ8+ T cells expressing CD69 and a significantly lower number of CD4+ CD25+ Foxp3+ T cells. These changes were not observed in SCID mice reconstituted with both CD45RBhigh and CD45RBlow T cells. In addition, SEB impaired the development of Treg cells in the SCID mice reconstituted with CD4+ CD45RBhigh T cells alone but had no direct effect on Treg cells. In the absence of Treg cells, feeding SEB induced activation of mucosal T cells and accelerated the development of colitis. This suggests that Treg cells prevent SEB-induced mucosal inflammation through modulation of SEB-induced T-cell activation.

Inflammatory bowel disease (IBD) is a chronic inflammatory condition associated with alteration of immunoregulatory mechanisms responsible for the control of immune responses to commensal microbiota and their products (15, 34). Under normal conditions, commensal microbiota influences the development and function of local and systemic immune responses limiting an overactive inflammatory response (23, 38, 44). Conversely, bacterial pathogens or their products can stimulate both innate and acquired immune responses, resulting in overt acute and chronic mucosal inflammation (22).

Superantigens (SAgs) are microbial proteins that activate large subsets of T or B lymphocytes. Staphylococcus enterotoxins, toxic shock syndrome toxin 1, streptococcal SAgs, and Mycoplasma arthritidis mitogen are examples of T-cell SAgs (24, 26, 28). T-cell SAgs bind to the variable region of the T-cell receptor (TCR) β or γ chain and cross-link with the major histocompatibility complex class II molecules (11, 13, 18, 29). Oral administration of Staphylococcus aureus enterotoxin B (SEB) induces a transient mucosal T-cell activation followed by persistent anergy and deletion of T cells bearing the SEB-reactive Vβ8 TCR for up to 4 weeks after the treatment (33, 47). Given the large number of SAg-producing microbial agents in the gut flora, it is probable that the mechanism involved in regulation of mucosal immune T-cell responses to microbial SAgs is critical to the prevention of commensal bacterium-induced chronic inflammation (32). Furthermore, SAgs have been implicated in immune-mediated diseases such as rheumatoid arthritis, multiple sclerosis, psoriasis, and IBD (25, 26, 39, 40, 50, 51). Skewed TCR repertoires have been identified in patients with IBD (5, 37, 46), and a SAg-like protein derived from Pseudomonas fluorescens, I2, was identified in colonic lesions of over 50% of Crohn’s disease patients in a study (8, 10, 14). However, the exact mechanism defining how SAgs may contribute to inflammation in the intestinal mucosa is unknown.

Here we investigated the role of regulatory T (Treg) cells in the effect of orally administered SEB on T-cell subsets and on the development of mucosal inflammation. SCID mice were fed SEB after mice were reconstituted with CD4+ CD45RBhigh T cells alone or CD4+ CD45RBhigh T cells plus CD4+ CD45RBlow T cells. While feeding SEB had no clinical effect on SCID mice reconstituted with both CD45RBhigh and CD45RBlow T cells, feeding SEB accelerated the development of colitis in SCID mice reconstituted with CD4+ CD45RBhigh T cells alone. This was associated with activation and expansion of SEB-reactive CD4+ Vβ8+ T cells and prevention of the development of T cells expressing Foxp3. These results suggest that Treg cells modulate effector T-cell responses to enteric bacterium-derived SAgs, preventing excessive activation of mucosal T cells and preserving the normal intestinal structure and function.

MATERIALS AND METHODS

Mice. Congenic C.B-17 SCID mice and BALB/c mice were obtained from Harlan (Indianapolis, IN). DO1.10 breeders were purchased from Charles River Laboratories (Wilmington, MA). Female mice between 8 and 12 weeks of age were used for all experiments. Blood samples were collected for serum production. For lymphocyte isolation, mice were sacrificed by cervical dislocation. Lymphocytes were isolated from the spleens and mesenteric lymph nodes as described previously (26). T cells were isolated by negative selection using the Pan T-Cell Isolation Kit (BD Biosciences, San Jose, CA).
age were used in these studies. All animal experiments were performed in accordance with institutional guidelines as approved by the Animal Care Review Board of McMaster University. All mice were housed under specific-pathogen-free conditions at the central animal facility at McMaster University. Donor and recipient mice in our colony were routinely screened for *Helicobacter* species infection by PCR capable of detecting ribosomal sequences common to all *Helicobacter* species and were free of infection (48).

Isolation and purification of CD45RBlow and CD45RBlow CD4+ spleen cells. CD4+ T-cell subsets from the spleens of BALB/c and DO11.10 mice were isolated and sorted as described previously (36). Briefly, single-cell suspensions were depleted of B220+, MAC1+, and CD8+ cells by negative selection using M-850 sheep anti-rat immunoglobulin G-coated Dynabeads (Dynal Biotech, Oslo, Norway). Purified anti-CD8α, anti-CD11b, and anti-MAC1 antibodies were obtained from BD PharMingen (Mississauga, Ontario, Canada). CD4+ CD45RBlow and CD4+ CD45RBlow T-cell fractions were sorted on a FACSAvant SE cell sorter (BD Biosciences, San Jose, CA) under sterile conditions. The purity of each subpopulation was >98%.

Reconstitution of SCID-bg mice with T-cell subsets and SEB treatment. BALB/c and DO11.10-derived CD4+ CD45RBlow and CD4+ CD45RBlow T cells were washed and resuspended at 2 × 10^6 cells/ml in sterile phosphate-buffered saline (PBS). Eight- to 12-week-old SCID mice each received either CD4+ CD45RBlow T cells (4 × 10^5 cells/mouse, intraperitoneally) alone or combined with CD45RBlow CD4+ T cells (2 × 10^6 cells/mouse) from BALB/c mice or CD4+ CD45RBlow and CD45RBlow CD4+ T cells from BALB/c and DO11.10 mice or DO11.10 and BALB/c mice, respectively. At 3 and 7 days after T-cell reconstitution and before the onset of colitis in SCID mice that received CD4+ CD45RBlow cells, recipient SCID mice were fed 10 μg of SEB (Sigma, St. Louis, MO) by gavage (intragastrically) in 200 μl of PBS with 400 μg of soybean trypsin inhibitor (Sigma) or soybean trypsin inhibitor alone in PBS. Mice were euthanized at different points of time after the second administration of SEB.

Histological examination. To determine if there was a difference in or an effect on the development of chronic colonic inflammation, which usually takes about 6 to 8 weeks, mice were euthanized at the moment at which most animals receiving SEB reached end point or maximum weight loss (i.e., 6 weeks). At the time of harvesting, the abdomen was opened longitudinally and separated into ascending, transverse, and descending colon and cecum. Tissues were fixed in 10% buffered formalin and sectioned and stained with hematoxylin and eosin. Each segment was examined for the severity of intestinal inflammation and graded by a gastrointestinal pathologist (C.J.S.) on a scale from 0 (no change) to 4 (most severe), as described previously (20). The scores at each segment were combined to provide an overall score of inflammation with a maximum score of 16.

LPL isolation. Lamina propria lymphocytes (LPL) were prepared as previously described (9). Briefly, the small intestines from a group of four to five mice were removed and the Peyer's patches were carefully excised. For removal of epithelial cells and intraepithelial lymphocytes, the intestines were washed and cut into small pieces, and then the pieces were incubated with calcium- and magnesium-free Hanks' balanced salt solution supplemented with 10% bovine calf serum and 5 mM EDTA (Sigma-Aldrich) on a magnetic stirrer at 37°C for 30 min. This process was repeated three times. The tissues were then incubated with RPMI 1640 containing 10% bovine calf serum, antibiotics, 25 mM HEPES, and 1.5 mg/ml collagenase A (Roche Diagnostics, Indianapolis, IN) at 30 min at 37°C with stirring. The digestion was repeated three times. The isolated cells were pooled and separated on a 40/75% discontinuous Percoll gradient (Pharmacia, Piscataway, NJ) centrifuged at 600 × g and 25°C for 20 min.

Phenotypic analysis by flow cytometry. For flow cytometry analysis, suspensions of 5 × 10^6 mononuclear cells were suspended in PBS-0.2% (wt/vol) bovine serum albumin supplemented with 0.1% (wt/vol) sodium azide and then incubated with relevant monoclonal antibody for 30 min at 4°C and washed. Three- or four-color flow cytometry analysis was performed on a FACSscan sorter (BD Biosciences). The following reagents and antibodies were obtained from BD PharMingen: fluorescein isothiocyanate-conjugated anti-CD3e (145-2C-11), phycoerythrin (PE)- and CyChrome-conjugated anti-CD4 monoclonal anti-body (L3T4), PE-conjugated CD25 (interleukin-2 [IL-2] receptor α chain, p55), and PE- and fluorescein isothiocyanate-conjugated anti-F23.1 (V8β-1.3). Alexa Fluor (488)-conjugated anti-mouse Foxp3 and allophycocyanin-conjugated anti-CD4 antibody were obtained from Biolegend (San Diego, CA), and PE-Cy5.5-conjugated anti-mouse DO11.10 TCR (KJ1-26) antibody was from ebioscience (San Diego, CA). A total of 5 × 10^6 events gated on lymphocytes were collected by a FACSscan sorter using the CellQuest software, and the data were analyzed with WinList version 5.0 (Verity Software House, Topsham, ME).

T-cell proliferation assays. For the T-cell proliferation assay, 5 × 10^5 cells, feeding SEB has no clinical effect on SCID mice reconstituted with CD4+ CD45RBlowT cells accelerated onset of colitis. Feeding SEB causes rapid activation and cytokine production by T cells in murine gut-associated lymphoid tissues (47). To examine whether feeding SEB can influence the development of intestinal inflammation, we fed SEB to SCID mice during the first week after reconstitution with BALB/c-derived CD4+ CD45RBlowT cells. Control PBS-fed SCID mice reconstituted with CD4+ CD45RBlowT cells developed a gradual and persistent weight loss with signs of diarrhea starting around 4 to 5 weeks after cell transfer. These mice developed bloody diarrhea and rectal prolapse by 8 to 10 weeks after reconstitution. The histology of the colon in these mice showed significant epithelial cell hyperplasia, lymphocyte infiltration, goblet cell depletion, and the occasional ulceration and crypt abscesses as previously described (27). In contrast, SCID mice reconstituted with BALB/c CD4+ CD45RBlowT cells and fed SEB at days 3 and 7 after reconstitution developed significant weight loss beginning 24 h after the second feeding. The majority of these mice lost 20% of their original body weight within 4 to 6 weeks (Fig. 1A). The differences in body weight gain between SEB-fed and PBS-fed CD4+ CD45RBlow T-cell recipients were statistically significant (P < 0.05 by ANOVA).

Microscopic examination of the colon showed a significant amplification of colonic inflammation in SEB-fed mice with extensive epithelial hyperplasia, massive lymphocyte infiltration, and numerous crypt abscesses (Fig. 2). Overall histological evaluation of all the segments of the colon showed that feeding SEB was associated with a significant increase in the severity of colitis. The mean histological score was 6.5 ± 1.0 for PBS-fed SCID mice reconstituted with CD4+ CD45RBlow T cells, whereas the average score for SEB-fed SCID recipients was 11.0 ± 2.0 (Fig. 1B, P < 0.01). Thus, in the absence of Treg cells, feeding SEB to CD45RBlow T-cell-reconstituted SCID mice caused a more severe colitis.

SEB feeding has no clinical effect on SCID mice reconstituted with both CD4+ CD45RBlow and CD4+ CD45RBlow T cells. SCID mice reconstituted with CD4+ CD45RBlow and CD4+ CD45RBlow T cells do not develop colitis (41). Feeding SEB to SCID mice that received both CD4+ CD45RBlow and CD4+ CD45RBlow T cells also failed to induce weight loss (Fig. 1A) or histological evidence of inflammation (Fig. 1B). Similar results were observed in SCID mice reconstituted with unseparated CD4+ T cells (data not shown). In fact, repeated oral administration of SEB (10 μg per mouse, twice a week for 4 weeks) to SCID mice reconstituted with combined CD4+ CD45RBlow and CD4+ CD45RBlow T cells failed to cause weight loss or colitis (data not shown).
Feeding SEB to SCID mice reconstituted with CD4+ CD45RB<sup>high</sup> T cells leads to early activation of T cells in the absence of Treg cells. The significant weight loss and clinical signs of colitis seen immediately after the second SEB feeding in SCID mice reconstituted with CD4+ CD45RB<sup>high</sup> T cells suggested that oral SEB caused early T-cell activation. We examined the induction of T-cell early-activation marker CD69 on CD4+ CD45RB<sub>high</sub> T cells and CD4<sup>+</sup> CD45RB<sub>low</sub> T cells during the first 72 h after the second SEB feeding. In SEB-fed BALB/c mice, a significant increase in the expression of CD69<sup>+</sup> was detected on T cells in the LPL and a small but not statistically significant increase was detected in the MLN (Fig. 3A). On the other hand, feeding SEB to SCID mice reconstituted with CD4<sup>+</sup> CD45RB<sub>high</sub> T cells alone led to an increase in the percentage of CD4<sup>+</sup> V<beta>8<sup>+</sup> T cells in the spleen, MLN, and LPL by 12 h after the second feeding, and this remained significantly elevated for at least 4 weeks, while the percentage of CD4<sup>+</sup> V<beta>8<sup>+</sup> T cells in the PBS-fed SCID recipients was unchanged (Fig. 4).

**CD45RB<sub>low</sub> T cells limited CD45RB<sub>high</sub> T-cell expansion in reconstituted SCID mice regardless of SEB administration.** To determine if SEB induced a chronic effect on expansion of RB<sub>high</sub>-derived T cells and if RB<sub>low</sub> was effective at controlling expansion of RB<sub>high</sub> T cells in SCID mice receiving both types (2), we examined the effect of SEB on the expansion of CD45RB<sub>high</sub> T cells in vivo, in the presence or absence of CD45RB<sub>low</sub> Treg cells after 7 weeks. SCID mice were reconstituted with combined transfers of CD45RB<sub>high</sub> and CD45RB<sub>low</sub> T-cell subsets from DO11.10 and BALB/c donor mice. CD4<sup>+</sup> T cells from DO11.10 mice possess the transgenic TCR (KJ1-26<sup>+</sup>) specific for ovalbumin. This TCR has the V<beta>8 chain that is reactive with SEB (52) and can be differentiated from BALB/c TCR by the clonotype-specific monoclonal antibody KJ1-26. The effect of SEB on CD4<sup>+</sup> CD45RB<sub>high</sub> T cells from BALB/c (BALB/c RB<sub>high</sub>) donors transferred into SCID mice was compared with the effect in mice receiving both BALB/c RB<sub>high</sub> donor cells plus DO11.10 CD4<sup>+</sup> CD45RB<sub>low</sub> donor cells (DO11.10 RB<sub>low</sub>) or DO11.10 RB<sub>high</sub> donor cells combined with BALB/c RB<sub>low</sub> donor cells (Fig. 5). SCID recipient mice were fed SEB or PBS at 3 and 7 days after T-cell reconstitution, and the number of CD4<sup>+</sup> T cells was estimated using the total number of mononuclear cell counts obtained after collection of spleen and the percentage of CD4<sup>+</sup> cells detected in flow cytometry analysis. SEB did not have a chronic effect on total CD4<sup>+</sup> T-cell expansion in spleen or MLN from SCID mice reconstituted with RB<sub>high</sub> cells alone or RB<sub>high</sub> and RB<sub>low</sub> cells as observed at 7 weeks posttransfer. Furthermore, RB<sub>low</sub> cells significantly limited expansion of RB<sub>high</sub> T cells in
both SEB- and PBS-fed SCID mice receiving both cell types compared with SCID mice receiving only RB<sup>high</sup> cells.

Feeding SEB impaired CD<sup>4</sup><sup>+</sup> CD<sup>25</sup><sup>-</sup> Foxp<sup>3</sup><sup>-</sup> T-cell development in SCID mice reconstituted with CD<sup>4</sup><sup>+</sup> CD45RB<sup>high</sup> T cells alone. In order to determine if the presence of Treg cells modulates the development or function of effector T cells in mice fed SEB, we examined the expression of Treg cell markers in spleens from SCID mice receiving donor cells. Feeding SEB significantly decreased the percentages of CD<sup>25</sup><sup>-</sup> CD<sup>4</sup><sup>+</sup>, Foxp<sup>3</sup><sup>-</sup> CD<sup>4</sup><sup>+</sup>, and CD<sup>25</sup><sup>-</sup> Foxp<sup>3</sup><sup>-</sup> CD<sup>4</sup><sup>+</sup> T cells in spleens of SCID mice that received BALB/c CD<sup>4</sup><sup>+</sup> CD45RB<sup>high</sup> T cells alone (P < 0.05), suggesting that SEB prevented or delayed development of CD45RB<sup>high</sup>-derived Treg cells (Fig. 6; only CD25<sup>-</sup> Foxp3<sup>-</sup> double-positive CD4<sup>+</sup> T cells are shown). However, SEB failed to alter the percentages of T cells expressing these Treg markers in spleens from SCID mice reconstituted with both RB<sup>high</sup> and RB<sup>low</sup> T cells, indicating that Treg RB<sup>low</sup> T cells modulated the effect of SEB on RB<sup>high</sup>-derived Treg cells. Moreover, SEB did not affect RB<sup>low</sup>-derived Treg cells (data not shown).

**DISCUSSION**

The results presented here demonstrated that in the absence of Treg cells, mucosal exposure to a bacterially derived product with
SAg activity, i.e., SEB, significantly activated the development of chronic intestinal inflammation. Specifically, oral administration of SEB to SCID mice reconstituted with CD4<sup>+</sup>/H11001 CD45RB<sup>high</sup> T cells was associated with activation of effector CD4<sup>+</sup>/H11001 T cells and expansion of SEB-reactive V<sub>β</sub>8<sup>+</sup>/H11001 T cells. Therefore, in the absence of Treg cells, bacterially derived SAg induced activation and expansion of effector T cells that may have accelerated the onset of colitis. In addition, oral administration of SEB to SCID mice reconstituted with CD4<sup>+</sup>/H11001 CD45RB<sup>high</sup> T cells impaired development of T cells expressing the Treg phenotypes, i.e., T cells expressing CD25<sup>+</sup>/H11001 Foxp3<sup>+</sup>.

Previous studies suggested that SAg-induced mucosal T-cell stimulation may be implicated in the development of IBD as evidenced by skewed TCR V<sub>β</sub> usage in IBD patients (1, 20). Indeed, systemic administration of the bacterial SAg SEB to mice induces a self-limiting enteropathy (6, 32) with stimulation of CD4<sup>+</sup> and CD8<sup>+</sup>/H11001 T cells (19, 43) and release of tumor necrosis factor alpha, IL-1, IL-2, IL-6, and gamma interferon (26, 28). SAg can also increase proinflammatory cytokine production from both healthy and inflamed colonic mucosa, suggesting that SAg could be an important initiator of the inflammatory cascade through direct T-cell activation (12). Therefore, our finding that feeding SEB accelerated and aggravated colitis in SCID mice reconstituted with RB<sup>high</sup> T cells alone and that oral SEB induced expansion of responsive CD4<sup>+</sup>/V<sub>β</sub>8<sup>+</sup> T cells in SCID mice reconstituted with CD4<sup>+</sup>/CD45RB<sup>high</sup> T cells provides one mechanism by which mucosal exposure to a SAg can induce mucosal inflammation.

Under normal circumstances interactions between the intestinal microflora and the host immune system are tightly regulated, preventing excessive local inflammation (15, 34). Feeding SAg to immunocompetent mice with normal gut flora fails to induce mucosal inflammation, suggesting that the normal mucosal environment also prevents or modulates the immune response to luminal SAg exposure (16, 31, 35). In addition, direct mucosal administration, e.g., intrarectal administration, of SEB to normal immunocompetent mice failed to induce inflammation (30). These findings support our results showing that feeding SEB to SCID mice reconstituted with combined CD4<sup>+</sup> CD45RB<sup>high</sup> T cells impaired development of T cells expressing the Treg phenotypes, i.e., T cells expressing CD25<sup>+</sup> Foxp3<sup>+</sup>.

Several immune mechanisms, including Treg cells, restrict and regulate the response of the mucosal immune system to mucosal bacterial antigens (16, 35). An alteration of immune regulatory mechanisms in response to microbial products may result in the development of chronic inflammatory diseases such as Crohn’s disease and ulcerative colitis (7, 49). Our results showed that SEB impaired development of RB<sup>high</sup>-derived Treg cells. However, SEB did not affect Treg cells from the RB<sup>low</sup> subset or RB<sup>high</sup>-derived Treg cells in the presence of RB<sup>low</sup>. This would suggest that in addition to direct T-cell
activation, SEB may alter development and function of \textit{RB}\textsuperscript{high}\textsuperscript{-}derived Treg cells and that this effect is also modulated by the presence of Treg cells. It is not known how SEB affects the development of Treg cells, but it is possible that SEB altered the conversion of naïve T cells into effector T cells, while somehow blocking the expression of Foxp3 and the development of Treg cells. However, it is also likely that the microenvironment (e.g., cytokine and chemokine milieu) promoted by the conversion of naïve T cells into effector T cells might not have allowed for the development of Treg cells (45).

Finally, \textit{RB}\textsuperscript{high}\textsuperscript{-}T-cell proliferation or expansion in vivo was limited by the presence of \textit{RB}\textsuperscript{low}\textsuperscript{-}T cells. In SCID mice reconstituted with \textit{CD4}\textsuperscript{+} \textit{CD25}\textsuperscript{-} \textit{Foxp3}\textsuperscript{-} T cells, the number of \textit{CD4}\textsuperscript{+} T cells recovered from spleen lymphocytes and MLN at the time of colitis was three to six times higher than that found in SCID mice reconstituted with both \textit{CD4}\textsuperscript{+} \textit{CD45RB}\textsuperscript{high} and \textit{CD4}\textsuperscript{+} \textit{CD45RB}\textsuperscript{low} T-cell subsets (20). Previous studies showed that administration of regulatory cytokines (e.g., cytokine and chemokine milieu) promoted by the conversion of naïve T cells into effector T cells might not have allowed for the development of Treg cells (45).

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