Evaluation of the immunoregulatory activity of intraepithelial lymphocytes in a mouse model of chronic intestinal inflammation

D. V. Ostanin1,2, C. M. Brown1, L. Gray1, S. Bharwani3 and M. B. Grisham1

1Department of Molecular and Cellular Physiology, 2Department of Medicine, Center of Excellence for Arthritis and Rheumatology and 3Department of Pathology, Louisiana State University Health Sciences Center, 1501 Kings Highway, PO Box 33932, Shreveport, LA 71130-3932, USA

Correspondence to: D. Ostanin; E-mail: dostan@lsuhsc.edu

Received 27 June 2010, accepted 1 October 2010

Abstract

Intraepithelial lymphocytes (IELs) represent the first line of lymphocyte defense against the intestinal bacteria. Although previous studies have demonstrated a protective role of IELs in the development of colitis, the data supporting a regulatory role for IELs are limited. The objective of this study was to examine the suppressive activity of IELs in vitro and in vivo using a mouse model of chronic small and large bowel inflammation. Adoptive transfer of CD8α+ IELs isolated from small intestines of wild-type (WT) mice into TCRβxδ-deficient (TCRβxδ−/−) recipients did not prevent or delay the onset of the disease induced by WT CD4+CD45RBhigh T cells. On the contrary, we observed a more rapid onset of wasting and clinical signs of intestinal inflammation when compared with animals injected with CD4+CD45RBhigh T cells alone. Histopathological scores of small and large bowel did not differ significantly between the two groups. Transfer of IELs alone did not produce any pathological changes. Real-time PCR analysis of intestinal tissues showed up-regulation of message for Tnf, 1- and macrophage-derived cytokines in colon and small bowel. Using Foxp3-GFP reporter mice, we were unable to detect any Foxp3+ cells within the CD8α+ IELs but did find a small population of Foxp3+CD4+ IELs in the small and large bowel. Using in vitro suppression assay, we found that neither TCRαβ+CD8αα+, TCRαβ+CD8αβ− nor TCRγδ−CD8αα+ IELs were capable of suppressing CD4+ T-cell proliferation. Taken together, our data do not support an immunoregulatory role for CD8α+ IELs in a mouse model of small and large bowel inflammation.

Keywords: adaptive immune system, animal models, cytokines, inflammation, inflammatory bowel disease, intraepithelial lymphocytes, TCRβxδ-deficient mice

Introduction

The intestinal epithelium constitutes the largest surface area within the body that acts to physically separate our internal organs from the noxious and potentially harmful enteric environment. In addition to preventing microorganisms from gaining access into the underlying lamina propria (LP), the epithelium contains a subset of lymphocytes located between the epithelial cells (ECs) where they are anchored to their basolateral side. This heterogenous population of lymphocytes is called intraepithelial lymphocytes (IELs) (1). The ratio of ECs to IELs ranges from 5 to 10 to 1, respectively, depending on the region of the intestine (2, 3). Although IELs undoubtedly possess a variety of different functions, it is clear that these cells play a major role in protection against invasion and systemic dissemination of enteric pathogens and commensal bacteria (4, 5).
IELs and intestinal inflammation

express CD4 or CD8α co-receptors, whereas ~60% of IELs in small intestine and ~5% in the large intestine express the CD8αα homodimer, a phenotype not typically found in the periphery (11). These unconventional CD8αα+ IELs contain approximately equal numbers of TCRζβ and TCRγδ lymphocytes. Noteworthy is that virtually all the TCRγδ T cells are CD8αα+ (11). This differentiation into the two IEL subsets goes well beyond their phenotypical differences but also reflects their antigen-recognition ability as well as functional differences. Conventional IELs are thought to be activated in the gut-associated lymphoid tissues, such as Peyer’s patches (PP) or mesenteric lymph nodes (MLNs), which imprint a gut-homing phenotype (12, 13) on these cells allowing them to gain entry into intestinal LP. These are thymus-dependent effector cells that respond to antigens presented by classical MHC class I and II molecules. Recent work by Poussier et al. led to the appearance of Tr1-like cells in the small intestine, which was correlated with increased IL-10 production by IELs and, possibly, other intestinal T cells (28). Recent work by Maynard et al. demonstrated that adoptive transfer of both IELs with CD4+CD45RBhi+ T cells remarkably suppressed the development of chronic colitis in T- and B-cell-deficient recipients. These investigators found that the suppressive activity of IELs resided within the TCRζβ+CD4+CD8αα− subset. Interestingly, this same subset isolated from IL-10-deficient donors did not confer protection. Our laboratory confirmed the studies by Poussier et al. demonstrating that co-transfer of CD8+ IELs with naive T cells into T- and B-cell-deficient RAG-1−/− recipients suppressed the development of chronic colitis (32). A study by Das et al. (33) showed that adoptive transfer of Tn1-2-polarized cells into lymphopenic recipients acquire CD4+CD8αα+ double-positive (DP) phenotype in the intestinal epithelium and show a Tr1,Treg-like pattern of cytokine production with high levels of IFN-γ and IL-10. These DP cells were shown to inhibit colonic inflammation induced by transfer of in vitro polarized Tn1 cells into RAG-1−/− mice in an IL-10-dependent manner.

What wasn’t clear from this study as well as the Poussier study was that whether IL-10 production by IELs is critical for their protective effects or whether the presence of IL-10 affects the development of specific IEL subset(s) that possess protective properties, thus pointing to an indirect role of this cytokine in prevention of colitis by IELs. Indeed, Poussier et al. and well as others (3) did not detect IL-10 in the supernatant following in vitro activation of these IELs. Because all the studies demonstrated a regulatory role for IELs were performed in mice that lack both T and B cells and develop only colonic inflammation, we wished to reevaluate the regulatory activity of different populations of IELs in a more complex animal model that contains functional B cells and develops both small and large bowel inflammation. To do this, we made use of the TCR βxδ double-deficient (TCR βxδ−/−) mice that lack IELs and peripheral T cells but retain functional B cells. We have demonstrated that adoptive transfer of naive CD4+ T cells induces chronic small and large bowel inflammation in these recipients (34). In addition, we evaluated the ability of various subsets of IELs isolated from the small intestines of wild-type (WT) mice to suppress the activation of CD4+ T cells in vitro.

**Methods**

**Animals**

Female or male C57Bl/6 WT mice 8–10 weeks old as well as TCR βxδ−/− (B6.129P2-Tcrβ−/−C0/J) on a C57Bl/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were maintained on 12 h/12 h light/dark cycles in standard animal cages with filter tops under specific pathogen-free conditions in our animal care facility at Louisiana State University Health Sciences Center, Shreveport. All animals were given standard laboratory rodent chow and regular tap water ad libitum. All experimental procedures involving the use of animals were reviewed and approved by the Institutional Animal Care and Use Committee of Louisiana State University Health Sciences Center and performed according to the criteria outlined by the National Institute of Health.
**Antibodies**

All antibodies were purchased either from BD Biosciences (San Diego, CA, USA) or eBioscience (San Diego, CA, USA). The following antibodies were used for cell sorting: FITC-conjugated anti-CD45R/B220, anti-CD8α and anti-CD11b, biotin-conjugated anti-CD4 mAb followed by streptavidin-670 (Invitrogen, Carlsbad, CA, USA) and PE-conjugated anti-CD45RB mAb (BD PharMingen). For analysis, the following antibodies were used: for calculation of T cell numbers: CD3-PECy5, CD4-allophycocyanin (APC); for determining activation antibodies were used: for calculation of T cell numbers: CD3-PECy5, CD4-APC and CD25-PE. Appropriate isotype controls were used for every experimental procedure. Samples were acquired on BD FACSCalibur flow cytometer from BD Biosciences.

**Induction of chronic gut inflammation**

Transfer of naïve CD4+CD45RBlow T cells into TCR βx-/- mice was performed as previously described (34, 35). Briefly, spleens were removed from donor C57Bl6 female mice and teased into single-cell suspensions in PBS containing 4% FCS using frosted sides of glass micro slides. Erythrocytes were removed by hypotonic lysis. For enrichment of CD4+ T cells, the MACS system from Miltenyi Biotec (Auburn, CA, USA) was employed for negative selection by magnetic cell sorting according to manufacturer’s instructions. Cells were incubated with FITC-conjugated anti-CD45R/B220, anti-CD8α and anti-CD11b mAbs and subsequently labeled with anti-FITC microbeads (Miltenyi Biotec). Unlabeled cells were separated from labeled cells on a depletion column (column type CS; Miltenyi Biotec) assembled into the magnetic separator (VarioMACS; Miltenyi Biotec). Enriched CD4+ T cells were labeled with anti-CD4 and anti-CD45RB mAbs. Following the staining, cells were separated into CD4+CD45RBhigh fraction by two color sorting on a FACS Vantage (Becton-Dickinson, San Jose, CA, USA). The CD45RBhigh population was defined as the brightest staining 40% of CD4+ T cells and were found to be >98% pure on post-sort analysis. To induce the disease, recipient TCR βx-/- mice were injected [Intra-peritoneal (i.p.)] with either 5 x 10^5 CD45RBhigh T cells (either alone or in combinations) re-suspended in a total volume of 0.5 ml of 1 x PBS. Clinical evidence of disease (e.g. body weight loss and loose stool/diarrhea) was followed and recorded weekly from the time of the injection for up to 11 weeks. Clinical evidence of disease (e.g. body weight loss and loose stool/diarrhea) was followed and recorded weekly from the time of the injection for up to 11 weeks.

**Macroscopic and histopathological analyses of small and large intestines**

At 11 weeks following T-cell reconstitution, or when animals lost 15–20% of their original body weight, mice were euthanized, colons removed, cleaned of fecal material, divided into proximal and distal sections and scored for macroscopic evidence of inflammation using our published scoring criteria (35). Briefly, normal colonic morphology was assessed a score of 0; mild bowel wall thickening in the absence of visible hyperemia was assigned a score of 1; moderate bowel wall thickening and hyperemia were given a score of 2; severe bowel wall thickening with rigidity and marked hyperemia were assigned a score of 3 and severe bowel wall thickening with rigidity, hyperemia and colonic adhesions were given a score of 4. In addition, a small piece of each section was placed in 10% PBS formalin and fixed overnight at 4°C. The fixed tissue was then rinsed with PBS, partially dehydrated in ethanol and embedded in JB-4 (Polysciences, Inc.). These samples were sectioned (5 μm) using glass knives and processed for standard hematoxylin and eosin (H and E) staining for histopathology.

Swiss rolls of the proximal and distal portions of the small intestine were prepared as follows: the entire small intestine was cut into two equal parts, ~ 10 to 15 cm, and labeled as proximal and distal. Using scissors and a wet cotton swab, intestines were carefully opened longitudinally and spread onto a piece of cardboard and lumen flushed with saline using a plastic pipette. Excess saline was carefully blotted with a paper towel and 10% formalin solution was dripped along the entire length of the intestine to cover it completely. Each piece was prefixed in this manner for 5–10 min. Excess fixative was carefully blotted and both intestinal segments rolled luminal side up without stretching onto a saline-dipped wooden stick starting with the most proximal end to the distal so that the proximal end of each segment is always in the middle of each roll. Ends of each roll were fixed in place with a pin or 26G hypodermic needle. Each roll was placed in small jar filled with 10% formalin and fixed overnight. After fixation, rolls were placed in cassettes, paraffin embedded and 5-μm cross-sections were cut followed by staining with H and E. Blinded histopathological analyses of colons were assigned using our previously published methods (34, 35). The severity of the inflammatory changes in the proximal and the distal colon was based on the sum of the scores reported for each parameter. Blinded histopathological scoring of small intestines was done using our previously published criteria (34) using the following parameters: (i) inflammatory infiltrate score ranging from 0 to 3; (ii) extent of inflammation, given a score ranging from 0 to 3; (iii) crypt damage with the score ranging from 0 to 3; (iiii) percent involvement, given a score ranging from 0 to 3 and (v) villus atypia, distortion, branching, atrophy and blunting, given a score ranging from 0 to 3. The severity of the inflammatory changes was based on the sum of the scores reported for each parameter.

**Lymphocyte isolation from spleen, MLNs, small intestine and colon**

Lymphocytes were obtained from spleen, small intestine and colon and analyzed by flow cytometry as previously described (35–37). Briefly, spleens were removed from reconstituted TCR βx-/- mice and teased into a single-cell suspension using the frosted ends of two glass slides in 4% FACS buffer on ice. The suspension was then passed through a 26G syringe to obtain a single-cell suspension. After pelleting, red blood cells were removed by hypotonic lysis and the resulting leukocytes were washed to remove lysis solution. Cells were re-suspended in the FACS buffer containing anti-Fc receptor mAb and viable cells were counted using 0.4% trypan blue dye/PBS solution.

Analysis of intestinal IELs was performed using previously described method (36, 37). Briefly, small and large intestines were removed from mice flushed of luminal contents and...
IELs and intestinal inflammation

trimmed of excess fat and connective tissue. Small and large intestines were opened longitudinally and cut into small (0.5 cm) pieces in PBS on ice. Pieces were then incubated in prewarmed (37°C) PBS/4%FCS/0.2 mM EDTA/10 mM d-glucose on a rotating shaker for 20 min at 250 r.p.m. at 37°C. After incubation, intestinal pieces were vortexed on a medium setting for 3-5 s. Supernatants from individual animals were collected into a separate 50 ml conical tubes and kept on ice. Incubations were repeated for a total of three times to insure complete removal of epithelium. Intestinal pieces from individual animals were processed separately and never mixed. The resultant pooled supernatants were pelleted by centrifugation and re-suspended in 30 ml of 40% Percoll. IELs were further purified by centrifugation over a 40% Percoll gradient for 25 min, 1000 × g at room temperature. After centrifugation, the pellet of IELs was washed and re-suspended in FACS buffer containing anti-Fc receptor mAb. Viable cells were counted using 0.4% tryphan blue dye/1 × PBS solutions.

LP lymphocytes were prepared by digestion of finely minced intestinal pieces remaining after IEL isolation and prior to Percoll enrichment step, cell suspension was passed over nylon wool, which was loosely bound in a solution of 2% ultrapure formaldehyde (Polysciences, Inc., Warrington, PA, USA) in FACS buffer for 20 min on ice, washed twice and analyzed the following day on the FACS Calibur (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Adoptive transfer of IELs
IELs were isolated from small intestines of 8–10 male WT C57Bl/6 mice using previously published protocols (6, 36) with minor modifications. To ensure removal of mucus, 1 mM of dithiothreitol was added to the EDTA buffer. Following IEL isolation and prior to Percoll enrichment step, cell suspension was passed over nylon wool, which was loosely packed into a 10-ml syringe to remove highly adherent cells and large clumps. Viable cells were counted using 0.4% tryphan blue dye/1 × PBS solution, stained with anti-CD8-FITC antibody and sorted immediately on FACS Vantage cell sorter (BD Biosciences). To avoid aggregation, DNase was added (Sigma; final concentration 10 U ml⁻¹) to a cell suspension prior to sorting followed by filtration through a 70-µm nylon cell strainer. If cell clumping became evident at anytime during sorting, cells were re-suspended by gentle pipetting and filtered through a 70-µm cell strainer again. On post-sort analysis, CD8⁺ cells were found to be >95% pure. After sorting, 3.5–5 × 10⁶ cells were injected i.p. into TCR β⁻/⁻ recipients and mice were allowed to become reconstituted with IELs for 2 weeks prior to receiving CD45RB⁺⁺ T cells. IEL transfer experiments were performed twice and included mice for ‘IEL only’ group and 10 mice that received IELs and CD45RB⁺⁺ cells. In the latter group, one mouse was reconstituted with 3.5 × 10⁶ IELs, two mice were reconstituted with 4 × 10⁶ IELs and the rest were reconstituted with 5 × 10⁶ IELs.

RNA isolation and quantitative reverse transcriptase–polymerase chain reaction
Small pieces of colon were removed from animals, cleansed of fecal material and snap-frozen in liquid nitrogen. Each small intestine was divided into proximal and distal halves, gently cleaned with cotton swab and snap-frozen in liquid nitrogen. Tissue samples were stored at −80°C until further processing. Total RNA was isolated from tissues of three representative samples in each group using TRIzol Reagent (Gibco-BRL, Grand Island, NY, USA) according to manufacturer's instructions. RNA was re-suspended in RNase-free water and stored at −80°C. One microgram of DNase-treated RNA was converted to cDNA by reverse transcriptase–polymerase chain reaction (RT–PCR) using GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA) in 20 µl total reaction volume as previously described (34). Briefly, quantitative PCR reactions for murine IL-1β, IL-10, tumor necrosis factor (TNF)-α, IFN-γ and TGF-β cytokines were performed using predeveloped primers with 200 ng of input cDNA in 50 µl total reaction volume using iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA, USA) in the iCycler iQ System (Bio-Rad Laboratories). Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control. Each sample was run in duplicate wells. Samples were analyzed using my IQ software (Bio-Rad, ver. 1.0) and the cytokine levels were expressed as fold changes over baseline, which were matching tissue samples isolated from normal WT mice.

Statistics
Data are presented as mean ± SEM. Statistical significance between more than two groups was evaluated using one-way analysis of variance. Statistical significance between selected groups was evaluated using Bonferroni post-hoc test. A probability (P value) of P < 0.05 was considered significant.

Results
Co-transfer of CD8⁺⁺ IELs and CD4⁺⁺CD45RB⁺⁺ cells does not suppress the induction of chronic intestinal inflammation in TCR β⁻/⁻ mice
Previous work from our laboratory has demonstrated that adoptive transfer of naive T cells into TCR β⁻/⁻ mice devoid of T cells but possessing functional B cells develop moderate-to-severe colitis (34). In order to determine whether adoptive transfer of IELs may suppress the induction of chronic colitis in these mice, we reconstituted a group of TCR β⁻/⁻ mice with flow-purified CD8⁺⁺ IELs isolated from the small intestines of syngeneic donor mice 2 weeks prior to transfer of naive CD4⁺⁺CD45RB⁺⁺ T cells. A control...
group of mice included TCR βxΔ−/− mice that received IELs only. We found that adoptive transfer of CD8α+ IELs alone did not induce outward signs of disease with recipient mice gaining weight over the 11-week observation period (Fig. 1A). In contrast, adoptive transfer of CD4+CD45RBhigh (naive) T cells induced a progressive loss of body weight over the 11-week period with significant weight loss occurring in the last week when compared with the group that received IELs alone (Fig. 1A). Interestingly, when naive T cells were injected into TCR βxΔ−/− mice that had been engrafted with IELs 2 weeks earlier, animals exhibited signs of accelerated disease and more rapid weight loss that became significant by week 4 post-reconstitution compared with TCR βxΔ−/− mice that received naive T cells alone (Fig. 1A). At sacrifice, we observed significantly greater weight-to-length ratios for TCR βxΔ−/− mice that received either naive T cells alone or both naive T cells plus IELs when compared with TCR βxΔ−/− mice that received IELs alone (Fig. 1B). We found that although there was a trend for blinded histopathological scores of colons obtained from the IEL/CD45RBhigh→TCR βxΔ−/− to be greater than those from the CD45RBhigh→TCR βxΔ−/− mice, statistical significance was not achieved (P = 0.074, Fig. 1C). Histopathological evaluation revealed moderate-to-severe inflammation characterized by extensive transmural infiltration of polymorphonuclear and mononuclear leukocytes, goblet cell dropout and crypt abscesses (Fig. 2A). No evidence of disease was detected in IEL→TCR βxΔ−/− mice.

In addition to inducing chronic colitis, we have shown in a previous study that adoptive transfer of CD45RBhigh cells into TCR βxΔ−/− mice induces chronic inflammation of the proximal small bowel, primarily, in the duodenum and upper jejunum (34). When TCR βxΔ−/− mice received CD8α+ IELs and CD45RBhigh cells, we observed the development of inflammation in both the proximal and the distal small intestine (Fig. 2B–D). Disease was manifested by an even greater hyperplasia of the crypts and a more pronounced inflammatory infiltrate into LP in the duodenum/jejenum compared with the other groups (Figs. 1D and 2B). In addition, histopathological evaluation of the distal small bowel revealed the presence of substantial numbers of polymorphonuclear leukocytes and mononuclear cells in the LP of the tissue resulting in distortion of normal crypt architecture and separation of crypts from muscularis propria layer (Fig. 2D). We observed a trend for higher histopathological scores for the distal small intestine in IEL/CD45RBhigh→TCR βxΔ−/− mice compared with the CD45RBhigh→TCR βxΔ−/− mice; however, these differences were not statistically significant (Fig. 1E). Together, our findings suggest that transfer of CD8α+ IELs does not induce colitis nor does it suppress the induction of disease but rather leads to an accelerated onset of disease in TCR βxΔ−/− recipients.

Engraftment of IELs and CD4+ T cells within the small and large bowel

The lack of suppression of gut inflammation by transfer of IELs into TCR βxΔ−/− mice was surprising in view of recent work by our laboratory as well as others demonstrating that certain subsets of IELs are capable of mediating protection in T-cell transfer model of gut inflammation (8, 32). One possible reason for this discrepancy could be due to the presence of small numbers of disease-producing (naive) T cells contaminating the CD8α+ IEL population. However, when we transferred IELs alone, no disease was induced in these mice, suggesting that these cells by themselves are not capable of inducing disease (Figs. 1 and 2). It may also be that the lack of protection was due to the poor engraftment of the transferred IELs within the intraepithelial cell compartment of the gut in the recipient mice. However, when we analyzed the different lymphocyte populations residing within the small bowel and colons of IEL/CD45RBhigh→TCR βxΔ−/− mice at 11 weeks post-transfer, we found that IELs were indeed capable of repopulating the intraepithelial compartment of small intestine and, to a lesser extent, the colon (Fig. 3A and B). Interestingly, we also observed a relatively large population of the CD4+CD8α+ DP cells within the small intestinal IEL compartment. Analysis of CD4+ T cells revealed peripheral numbers in the spleen that were not significantly different between groups that received CD45RBhigh and IEL/CD45RBhigh; 7.3 ± 1.2 × 10^6 and 3.5 ± 1.7 × 10^6, respectively (Fig. 3B), suggesting comparable engraftment and expansion of transferred CD4+ T cells in the T-cell-deficient recipients. Numbers of CD4+ T cells recovered from the intraepithelial compartment of small and large intestines were also not significantly different between the two groups (Fig. 3B). Interestingly, within the small intestines of the IEL→TCR βxΔ−/− mice, we could identify a small but distinct population of CD4+CD8α+ cells (Fig. 3A); some of the CD8α+ IELs are also capable of up-regulating CD4 co-receptor. This may indicate that cells residing at this site may be stimulated by different environmental signals to co-express CD4 and CD8α molecules. This may also correspond with some of the unique features and regulatory functions that are attributed to this subset of IELs (22, 33). Finally, surface expression of CD4+ cells revealed a phenotype consistent with antigen-experienced activated/memory cells as indicated by low levels of expression of CD45RB (data not shown). Taken together, the lack of protection by transferred CD8α+ IELs cannot be attributed to defects in engraftment within the recipient mice.

Cytokine expression in the small and large intestine

Quantitative determinations of cytokine message levels isolated from the colons and small intestine of CD45RBhigh→TCR βxΔ−/− and IEL/CD45RBhigh→TCR βxΔ−/− recipients revealed substantial but comparable increases in mRNA expression of TNF-γ, TNF-α, and IL-1β (Fig. 4). More specifically, colonic TNF-γ message levels were increased ~50- and 30-fold in the IEL/CD45RBhigh→TCR βxΔ−/− versus 20- and 25-fold in the CD45RBhigh→TCR βxΔ−/− proximal and distal colons, respectively (Fig. 4A and B). mRNA levels for IL-1β were up-regulated ~8-fold in the IEL/CD45RBhigh→TCR βxΔ−/− colons, whereas IL-1β levels were increased 20- and 45-fold in their proximal and distal colons, respectively, in the CD45RBhigh→TCR βxΔ−/− recipients. The increases in TNF-α message levels of ~3- to 5-fold were comparable in both groups of recipient mice.
Cytokine mRNA expression profiles in the proximal and distal regions of the small intestine revealed substantial increases in the IFN-\(\gamma\) mRNA levels. We observed 12- and 27-fold increases in the proximal and distal small bowel, respectively, in the IEL/CD45RB\(^{\text{high}}\)/TCR\(\beta\)\(x\)\(\mu\)/\(\delta\)-CD mice compared with a 10- and 6- fold increase in the proximal and distal small intestines, respectively, for the CD45RB\(^{\text{high}}\)/IEL mice (Fig. 4C and D). In addition, we observed a large and significant increase of \(\sim 4\)-fold in TNF-\(\alpha\) message levels in the distal but not in the proximal small bowel in the IEL/CD45RB\(^{\text{high}}\)→ TCR\(\beta\)\(x\)\(\mu\)/\(\delta\)-CD mice. We also found that the mRNA levels of regulatory cytokines, such as IL-10 and TGF-\(\beta\), were not significantly different between the colons and small intestines of the two groups. Altogether, real-time PCR data showing higher levels of the IFN-\(\gamma\) message levels in the colons of the IEL/CD45RB\(^{\text{high}}\)→...
Fig. 2. Representative images showing development of chronic colitis and small intestinal inflammation following the transfer of CD4+CD45RBhigh T cells alone or together with CD8α+ IELs into TCR βδ−/− mice. (A) Representative histological images of the distal colon (shown at ×100 magnification) from colitic mice show greatly enlarged colons with crypt hyperplasia, extensive transmural inflammation, infiltrating polynucleated and mononucleated cells and loss of goblet cells. On the other hand, normal size and crypt architecture of distal colon 11 weeks after the transfer of CD8α+ IELs alone. Representative images of the proximal (B) and distal (C and D) small intestines isolated from mice that received indicated subsets of cells. Transfer of CD45RBhigh alone or together with CD8α+ IELs but not IELs alone resulted in proximal small bowel inflammation (B). In the TCR βδ−/−/CD45RBhigh and TCR βδ−/−/IEL/CD45RBhigh, inflammation was observed in the proximal and was characterized by villus blunting, crypt hyperproliferation, infiltration of inflammatory cells and slight increase in muscle layer thickness. In some cases, crypt abscesses filled with mononuclear and polymorphonuclear cells were evident. (C and D) Distal small intestinal inflammation was grossly evident only in TCR βδ−/− mice that were transferred with CD45RBhigh together with CD8α+ IELs and was characterized by infiltrating mononucleated and polymorphonucleated cells into the villus LP. Images in panels (B) and (C) are taken at a ×100 magnification and in panel (D) at a ×400 magnification to show the nature of inflammatory infiltrate.
TCR βδ−/− group correlated well with our observation of faster onset of wasting and weight loss and a slightly higher histopathological colon scores as compared with the CD45RBhigh/TCR βδ−/− mice.

CD8+ IELs do not express Foxp3 and are not capable of suppressing T-cell activation in vitro

Although Foxp3 expression is primarily confined to the CD4+ population, a small population of CD8α+ T cells has been shown to express this classical regulatory T-cell marker/transcription factor (38, 39). To determine whether Foxp3-expressing T cells are present within the intraepithelial compartment, we isolated cells from Foxp3-GFP reporter mice wct 2(kindly provided to us by Alexander Rudensky (38)) (38) and analyzed small and large intestine IELs using flow cytometry. In agreement with previous reports (40), we found a distinct population of TCRβCD4+Foxp3+ cells in the spleen, MLN and PP of reporter mouse (Fig. 5A ). Interestingly, we also found a small population of these cells within the intraepithelial compartment of the small and large intestines. We did not observe any Foxp3-positive staining within the CD8α+ cells or in the TCRβδ+ population of T cells in all tissues analyzed (Fig. 5B). This finding agrees with the recent publication by Denning et al. showing very low levels of Foxp3 mRNA in the IELs using real-time PCR and microarray (41). Despite the fact that these cells lacked the expression of Foxp3, there are several reports suggesting that Foxp3-negative T cells are capable of in vitro and in vivo suppressive activity. Among these are Tr1 cells that are generated by stimulation of T cells in the presence of immunosuppressive drugs and vitamin D3 (42, 43). In addition, IL-10-expressing CD8α+ T cells are highly enriched in the small intestinal IEL compartment compared with other tissues (28, 29). Therefore, we wished to determine whether different IEL subsets are capable of suppressing effector T-cell activation and proliferation despite their lack of Foxp3 expression. Because it proved to be impossible to obtain sufficient numbers of CD4+Foxp3+ cells from the intraepithelial compartment of the small and large intestines.

Fig. 3. Analysis of T cells obtained from indicated tissues of reconstituted mice 9–11 weeks following the transfer of CD45RBhigh T cells. (A) Cells were isolated as described in detail in the Methods. Representative contour plot graphs were gated on lymphocyte population based on their forward and side scatter. Numbers indicate percentages of CD8α+ and CD4+ T cells isolated from indicated tissues. (B) Absolute numbers of CD4+ (left columns) and CD8α+ (right columns) T cells obtained from indicated tissues. These numbers were calculated by multiplying total numbers of viable cells isolated from tissue by the percentages of total CD4+ or CD8α+ cells as determined by flowcytometric analysis. At least three non-pooled individual mice per group were analyzed. Significant differences, where applicable, are indicated. *P < 0.05; **P < 0.01; ***P < 0.001.
compartment of healthy mice, we used freshly isolated CD4$^{+}$Foxp3$^{+}$ T cells (Tregs) from spleens of Foxp3-GFP reporter mice as our positive control. We found that co-culture of TCR$^{a}$CD8$^{a+}$, TCR$^{a}$CD8$^{b+}$ or TCR$^{b}$CD8$^{a+}$ IELs with CD4$^{+}$Foxp3/C0 responder T cells in the presence of irradiated accessory cells and soluble anti-CD3 mAb did not suppress T cell proliferation, whereas freshly isolated splenic CD4$^{+}$Foxp3$^{+}$ cells exhibited potent suppressor activity (Fig. 6).

**Discussion**
Over the past few years, several studies have suggested that certain subsets of IELs may function as a first line of defense in regulating mucosal immune responses in a variety of mouse models of chronic gut inflammation (8, 22, 32, 33, 44). Because virtually all the previous studies demonstrating a regulatory role for IELs were performed in mice that lack both T and B cells and developed only colonic inflammation, we wished to reevaluate the regulatory activity of different populations of IELs in a more complex animal model that contains functional B cells and develops both small and large bowel inflammation. To do this, we transferred naive T cells into TCR$^{b}$x$^{5+}$ IEL/CD45RBhigh recipients that had received CD8$^{a+}$ IELs 2 weeks previously. We found that transfer of IELs failed to attenuate the induction of colitis and small bowel inflammation in these mice in contrast to what we as well as others have reported in immunodeficient RAG-1$^{-/-}$ mice (8, 32). On the contrary, the presence of both CD8$^{a+}$ IELs and CD4$^{+}$ T cells accelerated the development of the well-characterized wasting syndrome (e.g. weight loss, loose stools/diarrhea, piloerection, lethargy) when compared with the CD45RBhigh IELs alone (Fig. 1). Despite the accelerated systemic disease, blinded histopathological colon and small bowel scores of the IEL/CD45RBhigh/TCR$^{b}$x$^{5+}$ mice were not significantly greater than the CD45RBhigh/TCR$^{b}$x$^{5+}$ group at the end of the 11-week observation period. These data suggest that the enhanced wasting in the IEL/CD45RBhigh/TCR$^{b}$x$^{5+}$ mice may be due to extraintestinal manifestations in these mice. Lack of suppression of intestinal inflammation by IELs did not appear to be due to the presence of pathogenic cells within the transferred IELs as TCR$^{b}$x$^{5+}$/IELs alone did not develop disease (Fig. 1).

The reasons for the differences between the current study and other studies from our own laboratory as well as others (8, 32, 33, 44) are not readily apparent, but several possible explanations may account for these disparities. One possibility may be due to method of delivery of the different lymphocyte populations. We used i.p. administration of naive T cells and IELs, whereas others (8, 32) have used the intravenous route for delivery of IELs and CD4$^{+}$ T cells. Another possible reason for the discrepant results may relate to the fact that all the previous studies demonstrating a protective...
effect of IELs used RAG-1⁻/⁻ or SCID recipients that are deficient in both T and B cells, whereas in the current study, we used mice devoid of all T cells but retain functional B cells (TCRβ⁻/⁻/⁻/⁻/⁻/⁻ mice).

It is possible that the presence of B cells in the mice used in the current study may affect the ability of IELs to suppress enteric antigen-dependent activation of naïve T cells to yield colitogenic TCR βxδ⁻/⁻/⁻ mice. Indeed, B cells have been shown to exacerbate the development of chronic ileitis and colitis induced in SCID mice following adoptive transfer of both T and B cells obtained from SAMP/Yit when compared with disease induced by transfer of CD4⁺ T cells alone (45).

Another difference between data obtained in the current study and studies that used SCID or RAG-1⁻/⁻/⁻/⁻/⁻/⁻ recipients is that the presence of B cells may reduce engraftment of transferred IELs in the small but not the large bowel in recipient mice. If this tissue-specific reduction in IEL engraftment accounts for the lack of suppressive activity of exogenously administered IELs to TCR βxδ⁻/⁻/⁻/⁻/⁻/⁻ recipients is that unfractionated CD8α⁺ IELs were used rather than flow-purified subsets of IELs, thereby ‘diluting’ the effective numbers of one or more IEL subsets thought to possess regulatory activity. This does not appear to be a problem for two reasons. First, we have previously demonstrated that adoptive transfer of similar numbers of unfractionated CD8α⁺ IELs into RAG-1⁻/⁻/⁻/⁻/⁻/⁻ mice suppressed the development of chronic colitis (32). Second,
Half are CD8⁺ IELs capable of suppressing colitis induced by CD4⁺ T cells. If, in fact, the actual regulatory IEL subset are the CD45RB⁺ T cells, then we estimate that we injected 1.5–2 million Foxp3⁺ IELs per mouse of which approximately 50–70% of IELs express TCRβ⁺. We found that TCRβ⁺ CD4⁺ CD8⁺ IELs may interfere with the putative regulatory activity of TCRβ⁺ CD4⁺ CD8⁻ β⁻ IELs in our experimental design.

In addition to evaluating the suppressive activity of IELs in vitro, we also examined the abilities of the different IEL subsets to suppress the activation of CD4⁺ responder T cells using a standard suppression assay in vivo. We found that TCRβ⁺ CD8⁺ IELs were unable to suppress proliferation of responder CD4⁺ T cells even at a very high ratio of IEL: T-cell responders (1:1). On the other hand, freshly isolated nTregs isolated from Foxp3-GFP reporter mice expressed potent suppressive activity (Fig. 6).

An important point to mention is the possible indirect effects that IELs have on inducing regulatory mechanisms within the gut via their interaction with surrounding ECs. It was found, for example, that interaction between the IELs and intestinal ECs through non-traditional MHC complexes, such as MICA RAs in an expansion of TCRβ⁺ CD4⁺ CD8⁺ IELs that attenuated epithelial injury and inflammation induced by oral dextran sodium sulfate administration (22). These same DP cells were shown to produce high levels of IL-10 and IFN-γ and prevent colonic inflammation induced by adaptive transfer of in vitro polarized T₃₁ cells into immunodeficient Rag-1⁻/⁻ mice at the ratio of DP IEL to T₃₁ cells of 1:3 (33). Furthermore, Wei et al. (44) reported that protection from colitis in two different transfer models by MLN-derived B cells correlated with increases in CD3⁺ CD4⁺ CD8⁺ (DP) IEL subset in small and large intestines. We also observed that a small but significant percentage of IELs isolated from the small bowels of CD45RB⁺→ TCR β⁻/⁻ as well as of IEL/CD45RB⁺→ TCR β⁻/⁻ displayed the CD4⁺ CD8⁻ DP phenotype (Fig. 3A).

Taken together, our data suggest that adoptive transfer of IELs obtained from the small bowel of healthy donors are unable to attenuate the development of both small and large bowel inflammation in a mouse that possesses functional B cells. These data suggest that our knowledge of the complex interactions between the multiple cell types that contribute to the intestinal homeostasis is still very limited.

**Funding**

Work was supported by PO1 DK43785 (Project 1, Animal Models Core and Histopathology Core).

**References**


IELs and intestinal inflammation


41 Barret, F. J., Cua, D. J., and Boonstra, A. et al. 2002. In vitro generation of interleukin 10-producing regulatory CD4+ T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1) and Th2-inducing cytokines. J. Exp. Med. 195:603.


