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Intestinal Epithelial Cells Modulate CD4 T Cell Responses via the Thymus Leukemia Antigen

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The intestinal epithelium is comprised of a monolayer of intestinal epithelial cells (IEC), which provide, among other functions, a physical barrier between the high Ag content of the intestinal lumen and the sterile environment beyond the epithelium. IEC express a nonclassical MHC class I molecule known as the thymus leukemia (TL) Ag, TL is known to interact with CD8α-expressing cells, which are abundant in the intestinal intraepithelial lymphocyte compartment. In this report, we provide evidence indicating that expression of TL by IEC modulates the cytokine profile of CD4+ T cells favoring IL-17 production. We show in an adoptive transfer model of colitis that donor-derived cells become more pathogenic when TL is expressed on IEC in recipient animals. Moreover, TL+IEC promote development of IL-17–mediated responses capable of protecting mice from Citrobacter rodentium infection. We also show that modulation of IL-17–mediated responses by TL+IEC is controlled by the expression of CD8α on CD4+ T cells. Overall, our results provide evidence for an important interaction between IEC and CD4+ T cells via TL, which modulates mucosal immune responses. The Journal of Immunology, 2011, 187: 4051–4060.

The intestinal mucosa constitutes an important barrier against microorganisms and plays a critical role in maintaining the balance between beneficial commensal bacteria and pathogens. Residing between the Ag-rich intestinal lumen and the sterile environment of the body are intestinal epithelial cells (IEC). In addition to providing a physical barrier against the migration of pathogens and commensal bacteria, emerging evidence suggests that IEC may also modulate mucosal immune responses. For example, IEC express MHC class II molecules, indicating a role for these cells as APC (1). Additionally, IEC function as stimulators in MLRs (2, 3) and can process and present Ags to CD4+ T cells in an environment in which IFN-γ is present (4). Other reports indicate that normal human IEC promote CD4+ T cell proliferation and IFN-γ secretion, and this effect is augmented when IEC from inflammatory bowel disease (IBD) patients are employed in CD4+ T cell cultures (5). Moreover, IEC are capable of inducing expansion of CD4+Foxp3+ regulatory T cells (Treg) in an Ag-specific manner (6). However, despite all these reports, a full understanding of the interaction between CD4+ T cells and IEC is still missing.

The thymus leukemia (TL) Ag is a nonclassical MHC class I molecule encoded by a locus within the MHC (7). TL lacks the capacity to present Ags due to the occlusion of its protein domain with homology to the Ag-binding groove of classical MHC class I molecules (8). Interestingly, the expression pattern of TL is primarily confined to IEC of both the small and large intestine (9–11). Although the functions of TL remain poorly understood, it has been shown that TL binds with high affinity to CD8α homodimers (8, 12–16). Considering that CD8α is a prevalent surface marker of intraepithelial lymphocytes (IEL) and that these cells reside in close proximity to TL-expressing IEC, it is believed that TL regulates the effector functions of IEL. Indeed, recent studies have indicated that TL can modulate IEL proliferation, cytokine production, and cytotoxicity (10, 14). Moreover, we have demonstrated that TL deficiency results in increased susceptibility to the development of spontaneous colitis induced in TCRα-deficient mice (10). Each of these observations points toward an important role for IEC in the activation and modulation of T lymphocyte effector functions.

In the current study, we demonstrate a novel function of IEC as modulators of CD4+ T cell responses in the intestinal mucosa. We show that IEC expressing TL influence the in vivo and in vitro cytokine response of CD4+ T cells, as TL-competent IEC promote development of IL-17–mediated responses capable of protecting mice from Citrobacter rodentium infection.

Materials and Methods

Mice

T3-deficient mice (TL−/−) on a C57BL/6 background have been described (10). As wild-type (WT) mice, we used C57BL/6 mice derived from our own colony. These mice were bred and maintained in similar conditions as TL-deficient mice. RAG-2-deficient, CD8α-deficient, and β2-microglobulin–

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Abbreviations used in this article: IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte; LP, lamina propria; MLN, mesenteric lymph node; TL, thymus leukemia; Treg regulatory T cell.
deficient mice were purchased from The Jackson Laboratory. TL−/− and RAG-2−/− mice were bred to obtain TL−/− RAG-2−/− and TL−/+ RAG-2−/− mice. Mice were maintained in accordance with the Institutional Animal Care and Use Committee at Vanderbilt University.

Isolation of lymphocytes from spleen, lymph nodes, lamina propria, and intestinal epithelium

Lymphocytes from spleen and lymph nodes were isolated following conventional procedures. CD4+ T cell purification and CD8α+ cell depletion were performed using Miltenyi beads following the manufacturer’s instructions (Miltenyi Biotec). Colonic IEL were isolated following an established protocol (10). Briefly, after flushing the intestinal contents with cold PBS, the intestine was cut longitudinally, and excess mucus was removed with a pipette tip. Small pieces of intestine (∼1 cm long) were cut and shaken for 45 min at 37°C in HBSS supplemented with 5% FBS. Supernatant was passed through a glass wool column, and the cells were recovered by centrifugation. Cells were resuspended and centrifuged in a 40/70% Percoll (General Electric) discontinuous gradient, and IEL were recovered at the interface. IEL purity was >70% with mostly IEC contaminants. Colonic lamina propria (LP) lymphocytes were recovered from small pieces of intestine that were incubated and shaken twice for 20 min in HBSS supplemented with 5% FBS and 2 mM EDTA. The tissue was incubated in the presence of 1.5 mg/ml collagenase VIII (Sigma-Aldrich) and 100 U DNAse 1 (Sigma-Aldrich) for two consecutive 20-min incubations at 37°C. After digestion, cells were recovered by Percoll centrifugation as described above.

Isolation of IEC

To obtain an enriched population of IEC, small and large intestines from TL−/+ RAG-2−/− or TL−/− RAG-2−/− mice were dissected, flushed of intestinal contents, and cut into small pieces. Tissue was shaken twice for 40 min in HBSS supplemented with 5% FBS and 2 mM EDTA. Recovered supernatant was centrifuged and the pellet resuspended in a 40/70% Percoll discontinuous gradient. Cells recovered from the interface were washed, and IEC were sorted according to forward and sideways scatter patterns using an FACS Aria sorter (BD Biosciences). Microscopic visualization and staining with anti-g8.8 Ab indicated >95% purity. IEC were used immediately.

Reagents and flow cytometry

Fluorochrome-coupled anti-CD3, -CD28, -CD4, -CD8α, -CD45RB, -IFNγ, -IL-17, -Foxp3, and -rat anti-Ig Abs and uncoupled anti-IL-2, -IL-12, -IL-4, -IL-7, and -g8.8 Abs were purchased from BD Biosciences. For intracellular staining, cells were stimulated with PMA/ionomycin (1 ng/ml and 1 nM, respectively) for 5 h in the presence of GolgiPlug (BD Biosciences). Cells were surface stained, fixed, and permeabilized with Wash/Perm solution (BD Biosciences) followed by intracellular staining. All staining samples were acquired using an FACS Calibur Flow System (BD Biosciences) and data analyzed using FlowJo software (Tree Star).

Coculture of CD4+ T cells and IEC

Purified CD4+ T cells and IEC were incubated at a 1:3 ratio in flat-bottom 96-well plates at a maximum density of 3 × 105 cells/well. CD4+ T cells were stimulated with plate-bound anti-CD3 Ab (3 μg/ml) and soluble anti-CD28 Ab (2.5 μg/ml). Four days later, cells were recovered, washed, and stained for surface and intracellular markers. CD8α-depleted T cells were obtained by magnetically labeling total Th17 cell cultures (2) after activation with anti-CD8α beads (Miltenyi Biotec) and running the cells through a depletion column (Miltenyi Biotec) according to the manufacturer’s instructions. Efficiency of CD8α cell depletion was usually at least 75%. Cells were used immediately in the coculture systems.

Generation of Th17 and Th1 cells

Th17 cells were generated as described (17) with some modifications. Briefly, total splenocytes were lysed of RBCs and plated at a density of 2.5 × 106 cells/well (12-well plate) in RPMI 1640 10% FCS in the presence of plate-bound anti-CD3 Ab (5 μg/ml), soluble anti-CD28 Ab (2.5 μg/ml), anti-IFNγ Ab (10 μg/ml), anti–IL-4 Ab (10 μg/ml), recombinant human TGF-β1 (5 ng/ml; R&D Systems), and recombinant murine IL-6 (20 ng/ml; BD Biosciences) supplemented with sodium pyruvate and nonessential amino acids (Life Technologies). Two days after culture, cells received 1 ml fresh medium containing anti-IFNγ Ab (10 μg/ml), anti–IL-4 Ab (10 μg/ml), recombinant human TGF-β1 (5 ng/ml), and recombinant murine IL-6 (20 ng/ml) supplemented with sodium pyruvate and nonessential amino acids. Cells were monitored for growth and, if needed, transferred to a larger plate. At day 4 and/or 6, cells were analyzed for cytokine production. For Th1 cells, RBC-lysed splenocytes were plated at a density of 3 × 105 cells/well (12-well plate) in RPMI 1640 10% FCS in the presence of plate-bound anti-CD3 Ab (5 μg/ml), soluble anti-CD28 Ab (2.5 μg/ml), anti–IL-4 Ab (10 μg/ml), IL-2 (20 U/ml), and IL-12 (5 ng/ml) and supplemented with sodium pyruvate and nonessential amino acids. Two days after culture, cells received 1 ml fresh medium containing anti-IL-12 Ab, IL-2, IL-12, sodium pyruvate, and nonessential amino acids. Cells were monitored for growth and, if needed, transferred to a larger plate. At day 4 and/or 6, cells were analyzed for cytokine production and CD4+ T cells purified as above and used for experimentation.

Adoptive transfer experiments

Because intestinal inflammation and IBD development depends on the intestinal flora, we used donor and recipient animals that were housed in our animal colony for several months under identical conditions to normalize the influence that the flora may have both in the donor pathogenic cells as well as in the intestinal environment of the recipient mouse. Total splenocytes from donor mice were lysed of RBCs and depleted of B cells using magnetic beads and columns as described by the manufacturer (Miltenyi Biotec). Cells were stained with anti-CD4 and anti-CD45RB Abs and sorted to obtain a fraction (∼99% purity) of CD4+CD45RB+ cells as previously described (18). Sorted cells were thoroughly washed with sterile PBS, and 1.75 × 107 cells were adoptively transferred i.v. into each recipient mouse.

IBD score

Recipient mice were followed twice a week for weight change and signs of colitis, including diarrhea, rectal bleeding, and scruffiness. Each of these signs of disease was scored as 1 point. Pathology of the colons was scored in a blinded fashion as previously described (18): infiltration (0–3 points), lack of goblet cells (0–3 points), and ulcers (0–3 points). Scores from histological examination and IBD signs were added to obtain the final disease score.

RNA extraction and real-time RT-PCR

RNA was isolated from the colon using the TRizol isolation protocol (Invitrogen) with slight modifications. Colon tissue was homogenized in 1 ml TRIzol reagent and chloroform was then added and shaken. Following an isopropanol precipitation, the RNA was washed with 70% ethanol and treated with RNase Inhibitor (Applied Biosystems) for 45 min. Following resuspension of the RNA at 65°C for 15 min, RNA preparations were further purified using the Quagen RNAeasy isolation kit (Qiagen). RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For real-time RT-PCR, we used the TaqMan gene expression method (19). GAPDH served as the normalizer, and tissue from uninfected or untreated mouse colons (from the respective background) served as the calibrator. All cDNA samples were analyzed in triplicate, along with no reverse transcriptase controls, using a Step One Plus real-time PCR instrument (Applied Biosystems). Levels of cytokine expression are indicated as relative units, based on comparison of tissue from treated or infected mice with tissue from untreated or uninfected mice (calibrator tissue) (19). Primer and probe sets were purchased as TaqMan Gene Expression Assays from Applied Biosystems.

C. rodentium infections

Mice were orally inoculated with WT C. rodentium as described previously (20). Briefly, bacteria were grown overnight in Luria broth, and mice were infected by oral gavage with 0.1 ml broth containing ∼108 CFU C. rodentium. Control mice received sterile broth. Weight was monitored during the length of the experiment. After the selected end point, animals were sacrificed, and colons were removed, cleaned, and Swiss rolled for histology. Proximal and distal samples were taken for RNA and colonization studies. Sections were scored following these parameters: acute (neutrophilic), chronic (lymphocytic), and depth of inflammation. We used the following scale: 1, mucosa; 2, submucosa; and 3, muscular propria. The percentage of colon involved was also determined. The injury score was determined as: (chronic inflammation + acute inflammation) × percent involvement.

Statistical analysis

Statistical significance between the groups was determined by application of an unpaired two-tailed Student t test. A p value <0.05 was considered significant. In the experiments involving C. rodentium infection, Mann–Whitney U and log-rank (Mantel–Cox) tests were performed. A p value <0.05 was considered significant.
Results

**TL promotes an environment that is favorable for the development of colitis by CD4+CD45RBhi T cells**

Our previous report indicated that TL expression on IEC is important for protection in a spontaneous model of colitis caused by an unconventional population of CD4 lymphocytes expressing TCRβ homodimers (10), leading us to investigate whether TL is also protective in colitis induced by conventional CD4+ T cells. For this reason, we took advantage of the well-established IBD model in which the adoptive transfer of CD4+CD45RBhi T cells into immunodeficient hosts results in the development of IBD (18). This system allowed us to determine the influence of IEC on a specific lymphocyte population (donor CD4+ T cells) and permitted us to determine if TL expressed on IEC of recipient animals could modulate CD4+ T cell-mediated inflammation and colitis. For this purpose, we used TL wild-type (TL+/+) and TL−/− RAG-2−/− mice as recipients of CD4+CD45RBhi T cells from WT donors. We observed that TL wild-type mice began to lose weight at 2 wk posttransfer, after which they developed other signs of colitis such as diarrhea, rectal bleeding, and scruffiness (Fig. 1A). Although colitis in this model generally develops 4 wk after cell transfer, an early disease onset has also been noted in reports by other groups (21). We believe the early onset of colitis observed under our experimental conditions is due either to the number of cells transferred or to the microbiota present in our animal facility. Surprisingly, TL−/− RAG-2−/− mice were largely resistant to weight loss and developed only mild signs of colitis (Fig. 1A).

Histological analysis of the colons of recipient mice at 2 wk after transfer showed severe cellular infiltration and disruption of the epithelial architecture of TL wild-type mice, whereas TL−/− RAG-2−/− recipient mice showed modest pathological changes (Fig. 1B, top panel). Overall, the disease scores of TL wild-type mice were significantly higher than TL−/− RAG-2−/− mice (Fig. 1B, bottom panel). Importantly, donor-derived CD4+ T cells were detected in both types of recipient mice. However, due to the ongoing inflammatory process in TL wild-type mice, donor cell numbers recovered from these mice, as compared with cells

![FIGURE 1](http://www.jimmunol.org/)

TL promotes IL-17 expression and induction of colitis by adoptively transferred CD4+CD45RBhi T cells. Naive CD4+CD45RBhi T cells (0.75 × 10⁶/mouse) were adoptively transferred into TL+/+RAG-2−/− or TL−/− RAG-2−/− mice. A, Weight change (top panel) and disease course (bottom panel) of treated mice. Control mice refer to animals that received unsorted CD4+ T cells. Data represent one representative experiment of at least three independent experiments with three to four mice per group. B, Pathology of treated mice 2 wk after transfer (top panel). Scale bars, 100 μm. Disease score of TL+/+RAG-2−/− or TL−/− RAG-2−/− mice at 2 wk after transfer (bottom panel). See Materials and Methods for full description of pathological and disease scores. C, Two weeks after transfer, donor-derived cells were cultured in the presence (stimulated) or absence (unstimulated) of PMA/ionomycin for analysis of IFN-γ and IL-17 by intracellular staining in the indicated organs (left panel). Plots show gating on TCRβ+CD4+ cells. Summary of dot plots is represented as bars (right panels). Data represent at least three independent experiments with three to four mice per group. D, Similar experiments as in C analyzed at 4 wk after transfer.
recovered from TL−/−RAG-2−/− mice, were consistently higher in the spleen, mesenteric lymph nodes (MLN), LP, and the IEL compartment (Supplemental Fig. 1).

We also analyzed the levels of IFN-γ and IL-17, two important cytokines for the development of colitis, in donor-derived cells obtained from spleen, MLN, colonic LP, and colonic IEL compartment from TL+/+RAG-2−/− and TL−/−RAG-2−/− mice 2 wk after transfer. As shown in Fig. 1C, donor-derived cells from TL+/+RAG-2−/− or TL−/−RAG-2−/− hosts had similar proportions of IFN-γ–producing cells in the spleen, MLN, LP, and IEL compartment when stimulated in vitro with PMA/ionomycin. However, donor cells isolated from spleens and IEL of TL+/+RAG-2−/− recipients repeatedly had statistically significant lower percentages of IL-17–producing cells when compared with TL+/+RAG-2−/− hosts (Fig. 1C). Consistently, IFN-γ levels were similar in donor-derived cells from TL+/+RAG-2−/− and TL−/−RAG-2−/− hosts in all lymphoid compartments analyzed at 4 wk after transfer (data not shown), whereas donor cells recovered from the IEL compartment of TL+/+RAG-2−/− recipients had significantly higher IL-17 proportions than cells isolated from TL−/−RAG-2−/− recipients (Fig. 1D) when stimulated in vitro with PMA/ionomycin. Our data suggest that TL promotes CD4+ T cells to produce IL-17, a hallmark Th17 cytokine. To determine whether TL influences other Th17-related cytokines, we investigated cytokine gene expression in the colons of recipient mice 2 wk after CD4+CD45RBhi T cell transfer. We observed that colons from TL−/−RAG-2−/− recipients had reduced levels of IL-17A and IL-21 mRNA (although not statistically significant for IL-17A) but similar levels for IL-22, IFN-γ, and TNF-α mRNA when compared with TL+/+RAG-2−/− recipients (Fig. 2). Because IL-17A and IL-21 have been implicated in the development of Th17 effector cell responses, these results raise the possibility that adoptively transferred cells fail to acquire the appropriate pathogenic phenotype in TL−/−RAG-2−/− recipient mice. Because of the relationship between IL-17–producing T cells (Th17) and Treg (22), we investigated whether loss of IL-17–producing CD4+ T cells correlated with the development of Treg. Therefore, we analyzed whether donor derived cells in recipient TL−/−RAG-2−/− mice had an expanded Treg population. Indeed, we observed that the proportion of Foxp3+ donor-derived T cells was greater in the spleen and LP of TL−/−RAG-2−/− recipient mice compared with TL+/+RAG-2−/− recipients (Fig. 3). Recently, it has been reported that a fraction of regulatory CD4+Foxp3+ T cells are capable of producing IL-17 both in human and mice (23, 24), which may be involved in mucosal inflammatory responses. To determine whether Foxp3+IL-17+ T cells develop from donor-derived cells during colitis induction in TL+/+RAG-2−/− and TL−/−RAG-2−/− mice, we used a similar approach as described above, and we observed that the proportion of Foxp3+IL-17+ T cells was increased in the spleen of TL−/−RAG-2−/− recipients compared with TL+/+RAG-2−/− mice (Fig. 4). Together, these results suggest that TL promotes the development of Th17 cells and suppresses the development of Treg, leading to an imbalance in the Th17/Treg ratio and development of colitis.
TL−/−RAG-2−/− recipient mice, we determined the proportion of these cells in our experimental system. We observed that the proportion of donor-derived Foxp3+IL-17+ T cells was very low in all immune compartments analyzed, and there were no statistically significant differences for cells recovered from the spleen, MLN, and IEL compartment of TL+/+RAG-2−/− versus TL−/−RAG-2−/− recipient mice (in the spleen, 0.7 ± 0.2 versus 0.7 ± 0.2; MLN, 0.4 ± 0.4 versus 0.2 ± 0.2; IEL, 0.02 ± 0.02 versus 0.01 ± 0.02, respectively). However, we observed that in the LP, there was a higher and statistically significant difference in the Foxp3+/IL-17+ T cell population present in TL+/+RAG-2−/− recipients compared with TL−/−RAG-2−/− recipients (0.12 ± 0.02 versus 0.03 ± 0.02, respectively; p = 0.001). However, because the proportion of Foxp3+/IL-17+ T recovered from the LP was usually very low, the physiological significance of this finding is difficult to determine at this time.

It has been reported that activated T cells can express TL (25) or snatch TL from IEC and express it on their surface membranes (26). To rule out a possible effect of TL present in the CD4+ T cell fraction that may influence disease development, we adoptively transferred CD4+CD45RBhi T cells derived from TL−/− donor mice into TL+/+RAG-2−/− and TL−/−RAG-2−/− mice. TL+/RAG-2−/− recipients of donor cells from TL−/− mice developed severe colitis, whereas TL−/−RAG-2−/− recipients did not (Supplemental Fig. 2). This set of data is similar to results of experiments in which donor cells were derived from WT mice. Thus, the status of TL expression on donor T cells is dispensable for disease development in this model.

CD4+ T cells upregulate CD8αα expression upon entering the IEL compartment (27). We considered that absence of TL might reduce CD8αα expression in T cells entering the mucosa, which, in turn, could impact T cell activation, inflammation, and colitis development. However, analysis of T cells obtained from the IEL compartment of TL+/+RAG-2−/− or TL−/−RAG-2−/− recipient mice showed no significant difference in the levels of CD8αα expression (Supplemental Fig. 3A).

Because adoptively transferred CD4+ T cells lost IL-17 production in mice lacking TL expression in IEC, we investigated whether TL-deficient mice with a fully competent immune system (i.e., RAGζ) displayed a deficiency in the production of this cytokine. Therefore, we analyzed the production of IL-17 by IEL and LP T cells from the colon and small intestine of WT and TL−/− mice. We found that both groups of animals had similar percentages of IL-17–producing cells in these mucosal compartments (Supplemental Fig. 4). Therefore, TL does not impact resident CD4+ T cells in the mucosa.

Adoptive transfer of Th17 cells results in higher colitis severity, but not weight loss, than Th1 cell transfer in an environment in which IEC express TL

Th17 and Th1 cells are important CD4+ T cell subsets in colitis development. To determine the influence of TL over these cell types, we transferred in vitro-differentiated Th17 and Th1 cells into TL−/−RAG-2−/− and TL−/−RAG-2−/− recipient mice. Starting at 2 wk posttransfer, TL+/RAG-2−/− mice receiving Th17 cells lost more weight and developed increased disease scores compared with TL−/−RAG-2−/− recipient mice (Fig. 4A). Similarly, TL+/RAG-2−/− mice receiving Th1 cells lost substantially more weight than TL−/−RAG-2−/− recipients, although without apparent difference in the disease score (Fig. 4B).

![FIGURE 4](http://www.jimmunol.org/)

Colitis severity, but not weight loss, is more pronounced for Th17 cells than Th1 cells introduced into an environment in which IEC express TL. Differentiated Th17 cells (A, C) or Th1 cells (B, D) were adoptively transferred into TL+/+RAG-2−/− or TL−/−RAG-2−/− mice (0.25 × 10⁶ cells/recipient). Hosts were followed for weight change (A, B, top panels), and disease scores were analyzed 3 wk after transfer (A, B, bottom panels). Intracellular cytokine production by donor cells recovered from the IEL compartment was analyzed in the presence (stimulated) or absence (unstimulated) of PMA/ionomycin (C, D). Data are from one of two independent experiments of three to four mice per group.
It is possible that Th1 cells affect other organs, impacting the weight of TL+/+RAG-2−/− animals; however, the reasons for this effect are unknown. Cells recovered from the IEL compartment of TL+/+RAG-2−/− and TL−/−RAG-2−/− recipient mice that received Th17 cells lost production of IL-17 and gained production of IFN-γ (the original population adoptively transferred contained 20–25% IL-17 producers and 1–3% IFN-γ producers) when stimulated in vitro with PMA/ionomycin. Loss of IL-17 production and gain of IFN-γ secretion by differentiated Th17 cells has been previously observed (17). However, despite these alterations in cytokine distribution, cells recovered from TL-competent recipient mice produced lower levels of IFN-γ but higher levels of IL-17 in response to in vitro stimulation with PMA/ionomycin. Loss of IL-17 production and gain of IFN-γ secretion by differentiated Th17 cells has been previously observed (17). However, despite these alterations in cytokine distribution, cells recovered from TL-competent recipient mice produced lower levels of IFN-γ but higher levels of IL-17 in response to in vitro stimulation with PMA/ionomycin. A similar pattern was observed when Th1 cells were transferred into TL+/+RAG-2−/− or TL−/−RAG-2−/− recipient mice (Fig. 4D). Thus, in terms of disease, the interaction of Th17 cells with IEC-expressing TL resulted in significantly more colitis than the interaction of Th17 cells with IEC lacking TL expression, an effect not observed when Th1 cells were transferred.

Overall, in terms of disease severity, we conclude that TL expressed by IEC modulates colitis caused by in vitro-differentiated Th17 cells, whereas TL appears to lack an effect on Th1 cells.

**IEC modulate the effector functions of CD4+ T cells in a TL/CD8αα-dependent manner**

To confirm that the interaction between TL+IEC and CD4+ T cells promoted IL-17 production, we took advantage of an in vitro assay in which TL+IEC or TL−IEC were cocultured in the presence of CD4+ T cells, followed by analysis of cytokine profiles. Because IEL have an activated phenotype and because Th17 and Th1 cells are important cell populations involved in mucosal immunity, we decided to coculture TL+IEC or TL−IEC with either differentiated Th17 or Th1 cells, with or without TCR stimulation. Four days after coculture, CD4+ T cells were recovered, and IL-17 and IFN-γ production of IL-17 and IFN-γ were assessed.

**FIGURE 5.** TL+IEC promote IL-17 production in differentiated Th17 cells. A. Differentiated Th17 cells were cultured in the absence (top left panel) or in the presence of highly purified IEC (1:3 ratio) from TL-competent (middle left panel) or TL-deficient (bottom left panel) donors. Cells were activated with plate-bound anti-CD3. Four days after coculture, cells were analyzed for intracellular cytokine production by incubation in the presence (stimulated) or absence (unstimulated) of PMA/ionomycin. Bar graphs (right panels) represent stimulated cells. B. Similar experiments as in A using differentiated Th1 cells. C. Similar experiments as in A using Th17 cells but without secondary anti-CD3 activation. All plots show gating on TCRβ+CD4+ cells. All data are representative of at least two independent experiments.
production was determined by intracellular staining following PMA/ionomycin stimulation. In all experimental groups, reactivated Th17 cells lost the capacity to produce IL-17 (after primary activation, ~25% of cells produced IL-17; data not shown), likely due to the absence of cytokines such as IL-23 that maintain the Th17 profile (Fig. 5A). However, this decrease in IL-17 production was less pronounced when Th17 cells were cocultured in the presence of TL+IEC, in comparison with Th17 cells cultured with TL−IEC or in the absence of IEC (Fig. 5A, left and middle right panels). In contrast, Th17 cells cultured alone or with TL+IEC exhibited a decrease in the percentage of cells producing IFN-γ compared with Th17 cultured with TL−IEC (Fig. 5A, left and upper right panels). Overall, our results indicated that TL+IEC shift the balance between IFN-γ and IL-17 production by Th17 cells in favor of IL-17 production (Fig. 5A, bottom right panel). In sharp contrast to the effects of TL expression observed for in vitro-differentiated Th17 cells, the cytokine profile of activated Th1 cells was not influenced by TL expression (Fig. 5B).

Th17 cells that were not restimulated with anti-CD3 Abs maintained similar levels of IFN-γ production in the presence or absence of TL+IEC or TL−IEC (Fig. 5C, left and top right panels). In contrast to TCR-restimulated cells, Th17 cells that were not restimulated lost their capacity to produce IL-17 when cocultured with TL+IEC, as compared with Th17 cells cultured alone and, to a lesser extent, cultured in the presence of TL−IEC (Fig. 5C, left and middle right panels). However, in this set of experiments, the IFN-γ to IL-17 ratio was not modified by the presence or absence of TL+IEC or TL−IEC (Fig. 5C, bottom right panel). Although there was a tendency for an overall reduction in the percentages of IFN-γ+ and IL-17+ cells when Th17 cells were incubated in the presence of IEC in comparison with Th17 cells cultured alone, this was not due to cell viability because the recovery of CD4+ T cells was similar among all groups (data not shown). Therefore, our results indicate that activation of Th17 cells in the presence of TL+IEC is required for modulation of cytokine production. Interestingly, the presence of IEC, either from TL+/+ or TL−/− mice, did not affect the differentiation of naive CD4+ T cells toward the Th1 or Th17 cell phenotype (data not shown), indicating that IEC primarily influence differentiated CD4+ T cells.

CD8αα is the only known ligand for TL. Because CD4+ T cells reaching the mucosa upregulate CD8αα (27–30), we tested whether recently activated CD4+ T cells upregulate CD8αα in an in vitro activation protocol. Indeed, under both Th0 and Th17 cell-culture conditions, we observed expression of CD8αα mainly at day 2 after culture (Fig. 6A). We next explored whether IEC had an effect on in vitro-generated Th17 cells derived from CD8α-deficient mice. As shown in Fig. 6B, IL-17 expression by Th17 cells derived from CD8α-deficient mice was unaffected by the

**FIGURE 6.** The TL/CD8αα interaction is critical for modulation of CD4+ T cell responses by IEC. A, Activated CD4+ T cells transiently express CD8αα. To avoid background staining from conventional CD8+ T cells, we used total splenocytes from β2-microglobulin–deficient mice. Cells were activated in vitro (anti-CD3/CD28) under either Th0 or Th17 cell differentiation conditions or cultured without TCR stimulation (unstimulated). Two and 3 d after activation, cells were stained for surface markers. Results are shown for cells gated on CD8+ cells as an irrelevant marker for noise reduction. Similar results were obtained when splenocytes from WT mice were used. Data are representative of two independent experiments using two to three mice per condition. B, Purified, fully differentiated Th17 cells from CD8αα-deficient mice or Th17 cells from WT mice depleted of the transient CD4+CD8αα+ T cell population (see Materials and Methods for procedure and Supplemental Fig. 3B for purification efficiency) were cocultured in the absence (top left panels) or in the presence of purified IEC from TL-competent (middle left panels) or -deficient (bottom left panels) donor animals. Four days after coculture, cells were analyzed for intracellular cytokine production by incubation in the presence (stimulated) or absence (unstimulated) of PMA/ionomycin. Bar graphs (right panels) represent stimulated cells. The remainder of the experiment was performed as in Fig. 5A. A summary of the results is indicated in the right panels. Data represent results from two independent experiments. *p > 0.03, comparing CD8α staining of Th0 or Th17 versus unstimulated cells.
presence or absence of TL expression on IEC. However, the presence of IEC (regardless of TL status) in the cultures increased IFN-γ production when compared with Th17 cells cultured alone. To further establish that CD8α expression in CD4+ T cells is important for the interaction with TL+IEC, we depleted the CD4+CD8α+ T cell population from Th17 cultures by magnetically sorting CD8α+ cells (Supplemental Fig. 3B) and culturing the remaining CD4+CD8α− Th17 cells in the presence or absence of TL+IEC or TL-IEC. We observed that the production of IL-17 and IFN-γ was unaffected when Th17 cells were depleted of cells expressing CD8α prior to coculture with TL+IEC or TL-IEC (Fig. 6B, right panel). Importantly, the IFN-γ/IL-17 ratio was unaffected when CD4+ T cells were unable to express CD8α or when the CD4+ T cells expressing CD8α were removed (compare Fig. 6B with 5B).

Taken together, these results indicate that the mechanism by which TL promotes IL-17 production over IFN-γ is mediated by its interaction with CD8α expressed on CD4+ T cells.

**TL expression confers resistance to Citrobacter rodentium infection**

Because Th17 cell responses have been implicated in protective immunity against *C. rodentium* (31), and because our results suggested that TL-deficiency causes a decrease in IL-17 cell responses in the mucosa, we infected WT and TL−/− mice with *C. rodentium*. Infected WT mice lost little weight, and the majority survived the infection. Infected TL−/− mice, however, lost substantial amounts of weight, and ∼40% failed to recover and succumbed to infection (Fig. 7A, 7B). When the bacterial load in the colon was measured, we observed that surviving TL−/− mice were less capable than WT mice in clearing the infection (Fig. 7C). Consistent with this finding, there was more histologic evidence of colonic inflammation in TL−/− mice than in WT animals (Fig. 7D). Real-time PCR analysis from colons revealed that WT mice had a greater expression of IL-17A and IL-21, but similar IL-22 and IFN-γ expression, in comparison with TL-deficient mice (Fig. 7E). In summary, we postulate that TL expression promotes an environment conducive to the development of a protective Th17 response against *C. rodentium* infection.

**Discussion**

IEC are in the frontline between the immensely high antigenic load of the intestinal lumen and the sterile environment beyond the basal side of the epithelium. It is well known and established that priming of conventional T lymphocytes associated with the mucosa occurs primarily in the MLN, LP, or Peyer’s patches, and after this initial activation, some T cells migrate to the epithelium to become a fraction of the IEL population. The intimate location and relationship between IEL and IEC suggest that the latter cells provide regulatory signals to activated conventional T lymphocytes migrating to the epithelium and/or residing as IEL. Using a combination of in vivo and in vitro systems, we have demonstrated in this report that IEC modulate, via TL and CD8α, the cytokine response of CD4+ T cells.

We found that CD4+CD45RBhi T cells adoptively transferred into IL-2−/− RAG-2−/− recipient mice have a diminished capacity to induce colitis that otherwise is evident in TL-competent RAG-2−deficient recipients (Fig. 1A, 1B). In this model of colitis, it is well known that Th1/IFN-γ responses are important for disease development (18) and that IL-17 has an important role for disease development (19, 20).

**FIGURE 7.** TL expression by IEC protects against *C. rodentium* infection. WT and TL−/− mice were infected orally with 1 × 10^9 CFU of *C. rodentium*. Weight (A) and survival (B) were monitored for 12 d. WT, n = 18; TL−/−, n = 21. WT ctl and TL−/− ctl refer to mice treated with vehicle (broth). C, Bacterial load in the colons of infected mice 12 d postinfection. Representative data are shown. D, Left panel, Injury scores of colons at 12 d postinfection. See Materials and Methods for a full description of injury score. Right panel, Representative H&E staining from dissected colons (original magnification ×4 and ×20). E, Real-time PCR analysis from colons isolated at 6 d postinfection.
induction (32), although other authors have reported that T cell-derived IL-17A is protective in this experimental model (33). Moreover, it has been shown that patients with Crohn’s disease express higher levels of IL-17 and IFN-γ in the intestine than normal individuals (34, 35), implicating these two cytokines in colitis development in humans. Our findings recapitulate the importance of IL-17 as a key cytokine in intestinal inflammation in the adoptive transfer model, but moreover, our data indicate that IEC have the capacity to modulate this cytokine in a manner that is influenced by TL expression.

Our results suggest that the interaction between TL+/IEC and what we believe are donor-derived CD4+CD8αα+ T cells promotes the production of IL-17. In turn, IL-17 induces chemokine expression and secretion by IEC or endothelial cells, which subsequently recruit inflammatory cells promoting the development of a pathogenic response (36), probably IFN-γ/Th1 mediated. These findings are in agreement with previous reports indicating that IL-17-Th17 responses are relevant for the development of subsequent Th1 responses both during vaccine development (37) and protection against intracellular bacterial infections (38).

Considering that CD8αα is a high-affinity ligand for TL, it should be noted that our in vitro experiments were performed using CD4+ T cells derived from spleen, in which CD8αα expression has not been previously detected (39). However, we confirmed that, under our experimental conditions, splenic CD4+ T cells upregulated CD8αα homodimers, albeit transiently and at a very low frequency (~0.2–0.9% of total CD4+ T cells) (Fig. 6a). These results suggest that bona fide CD44+CD8αα+ IEL and other CD4+ T cells transiently expressing CD8αα can interact with TL expressed in the mucosa. Although it is unclear whether all or only subsets of CD4+ T cells can upregulate CD8αα homodimers, we demonstrated that depletion of the small population of CD4+ CD8αα+ splenic T cells was sufficient to overcome the effect conferred by TL+ IEC on CD4+ T cell responses (Fig. 6b). We speculate that this population may be more likely to migrate to the mucosa and/or be involved in intestinal immunity. Our findings raise the possibility that one outcome of the interaction between IEC and CD44+CD8αα+ IEL or CD44+CD8ααtransient+ T cells in the mucosa is to assure the maintenance of IL-17 production or a Th17 cell phenotype.

It is interesting to note that the numbers and proportions of resident mucosal CD4+ T cells producing IL-17 in the LP or IEL compartments were similar in unmanipulated TL-deficient and WT animals (Supplemental Fig. 4). This observation indicates that TL does not affect the development or steady state of IL-17–producing cells in the mucosa, but suggests that TL may only be responsible for modulating CD4+ T cells that were either activated in the intestinal mucosa or recently migrated into the epithelium.

Th17 cells are an important subset of CD4+ T cells in the intestinal mucosa that are characterized by production of IL-17, IL-21, and IL-22 and are believed to be generated in response to commensal bacteria, suggesting a role for these cells in host-microflora homeostasis (40, 41). Additionally, recent publications by several groups have postulated a critical role for Th17 cells in clearing pathogenic bacteria from the mucosa of both the intestinal and respiratory tracts (31, 42, 43). In our report, we have also shown that TL expression is important for mounting an appropriate Th17 cell response against C. rodentium, underscoring the relevance of the interaction between effector CD4+ T cells and IEC for bacterial clearance.

It has been reported that TL is also expressed in activated monocytes and dendritic cells (39), and therefore, it remains possible that the effects we observed were due to mucosal TL+ APC interacting with effector CD4+ T cells. However, we have been unable to detect TL expression either by real-time PCR technology or by Ab staining in unactivated or activated dendritic cells or monocytes (D. Olivares-Villagómez and L. Van Kaer, unpublished observations).

In our previous report, we have shown that expression of TL in the gut mucosa protects TCRα/-deficient mice from a severe form of colitis characterized by increased penetrance, earlier disease onset, and more severe signs observed in TCRα/-/- mice (10). Why does TL expression protect against colitis in the TCRα/-/- model but renders mice more susceptible to colitis in the adoptive transfer model used in this study? The spontaneous colitis that develops in the TCRα/-/- model is characterized as a Th2-like disease mediated predominantly by IL-4 produced by a rare population of CD4+ T cells expressing TCRB homodimers (44, 45), whereas colitis generated by adoptively transferring naïve cells into immunodeficient mice is the result of a Th1-like disorder, most likely accompanied by Th17/L-IL-17 responses (18). Therefore, we propose that TL may also function as a temporal immune regulatory switch capable of modifying the Th profile of a subset of CD4+ T cells (i.e., Th17 and possibly Th2 cells) while leaving others undisturbed (e.g., Th1 cells as shown in this report). Thus, TL may delay the pathogenic potential of CD4+ Tcells in TCRα/-/- mice by inducing a non-optimal cytokine microenvironment for disease development, whereas in the adoptive transfer model, TL expression directs the cytokine microenvironment to one that is favorable for development of Th17/Th1-mediated inflammation and colitis.

In summary, we report an important role for the interaction of IEC with CD4+ T cells in regulating CD4+ T cell responses. Our results indicate that CD4+ T cells require interaction of IEC to affirm their effector functions when migrating into the mucosa and that this interaction modifies the outcome of adaptive immune responses and intestinal inflammation. Thus, in some instances, a full mucosal immune response is not promoted until IEC provide the final clearance. Further understanding of the mechanisms by which IEC modulate mucosal immune responses should prove useful for developing new treatments for diseases associated with the intestinal tract.

Disclosures

The authors have no financial conflicts of interest.

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