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J Immunol. Author manuscript; available in PMC 2012 October 15.

Published in final edited form as:

J Immunol. 2011 October 15; 187(8): 4051–4060. doi:10.4049/jimmunol.1101052.

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

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Abstract

The intestinal epithelium is comprised of a monolayer of intestinal epithelial cells (IEC) which provide, among other functions, a physical barrier between the high antigen content of the intestinal lumen and the sterile environment beyond the epithelium. IEC express a non-classical MHC class I molecule known as the thymus leukemia (TL) antigen. TL is known to interact with CD8αα-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.

INTRODUCTION

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 antigen-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 major histocompatibility (MHC) class II molecules, indicating a role for these cells as APC (1). Additionally, IEC function as stimulators in mixed lymphocyte reactions (2, 3), and can

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process and present antigens to CD4$^+$ T cells in an environment where 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 IBD patients are employed in CD4$^+$ T cell cultures (5). Moreover, IEC are capable of inducing expansion of CD4$^+$Foxp3$^+$ regulatory T cells in an antigen-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) antigen is a non-classical MHC class I molecule encoded by a locus within the MHC (7). TL lacks the capacity to present antigens due to the occlusion of its protein domain with homology to the antigen-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 CD8αα is a prevalent surface marker of 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 present 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 favor IL-17 production by lowering the IFN-γ to IL-17 ratio. Consequently, *in vivo*, TL-deficiency results in reduced colitis in an adoptive transfer model and increased sensitivity to *Citrobacter rodentium* infection.

MATERIAL AND METHODS

Mice

T3-deficient mice (TL$^{-/-}$) on a C57BL/6 background have been described (10). As 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 β_2-microglobulin-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. 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 minutes at 37°C in Hanks' balanced saline solution 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 LP lymphocytes were recovered from small pieces of intestine that were incubated and shaken twice for 20 minutes in Hanks' balanced saline solution supplemented with 5% FBS and 2 mM EDTA. The tissue was incubated in the presence of 1.5 mg/ml of collagenase VIII (Sigma) and 100 units of DNase I (Sigma) for two consecutive 20 min incubations at 37°C. After digestion, cells were recovered by Percoll centrifugation as described above.

Isolation of intestinal epithelial cells

In order to obtain an enriched population of IEC, small and large intestines from $TL^{+/+}RAG\text{-}2^{-/-}$ or $TL^{-/-}RAG\text{-}2^{-/-}$ mice were dissected, flushed of intestinal contents and cut into small pieces. Tissue was shaken twice for 40 minutes in Hanks' balanced saline solution 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 a FACSAria sorter. Microscopic visualization and staining with anti-g8.8 antibody indicated >95% purity. IEC were used immediately.

Reagents and flow cytometry

Fluorochrome-coupled anti-CD3, -CD28, -CD4, -CD8α, -CD45RB, -TCRβ, -IFN-γ, -IL-17, -Foxp3, and anti-rat IgG antibodies, and uncoupled anti-IL-2, -IL-12, -IL-4, -IL-17, and -g8.8 antibodies were purchased from BD Biosciences. For intracellular staining, cells were stimulated with PMA/Ionomycin (1 ng/ml and 1 nM, respectively) for 5 hours 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 a FACSCalibur Flow System (BD) and data analyzed using FlowJo software (TreeStar).

Co-culture of $CD4^+$ T cells and IEC

Purified $CD4^+$ T cells and IEC were incubated at 1:3 ratio in flat-bottomed 96-well plates at a maximum density of 3×10^5 cells per well. $CD4^+$ T cells were stimulated with plate-bound anti-CD3 antibody (3 μg/ml) and soluble anti-CD28 antibody (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 days after activation) with anti-CD8α beads (Miltenyi) and running the cells through a depletion column (Miltenyi) according to the manufacturer's instructions. Efficiency of CD8α cell depletion was usually at least 75%. Cells were used immediately in the co-culture systems.

Generation of Th17 and Th1 cells

Th17 cells were generated as described (17) with some modifications. Briefly, total splenocytes were lysed of red blood cells and plated at a density of 2.5×10^6 cells per well (12-well plate) in RPMI-10% FCS in the presence of plate-bound anti-CD3 antibody (5 μg/ml), soluble anti-CD28 antibody (2.5 μg/ml), anti-IFN-γ antibody (10 μg/ml), anti-IL-4 antibody (10 μg/ml), rhTGF-β1 (5 ng/ml; R&D), rmIL-6 (20 ng/ml; BD Biosciences), and supplemented with sodium pyruvate and non-essential amino acids (GIBCO). Two days after culture, cells received 1 ml of fresh medium containing anti-IFN-γ antibody (10 μg/ml), anti-IL-4 antibody (10 μg/ml), rhTGF-β1 (5 ng/ml), rmIL-6 (20 ng/ml), and supplemented with sodium pyruvate and non-essential 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, $CD4^+$ T cells purified using magnetic beads (Miltenyi), and used for experimentation. For Th1 cells, red blood cell-lysed splenocytes were plated at a density of 3×10^6 cells per well (12-well plate) in RPMI-10% FCS in the presence of plate-bound

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anti-CD3 antibody (5 μg/ml), soluble anti-CD28 antibody (2.5 μg/ml), anti-IL-4 antibody (10 μg/ml), IL-2 (20 units/ml), IL-12 (5 ng/ml), and supplemented with sodium pyruvate and non-essential amino acids. Two days after culture, cells received 1 ml of fresh medium containing anti-IL-4 antibody, IL-2, and IL-12, sodium pyruvate and non-essential 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, $CD4^+$ 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 in order 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 mice. Total splenocytes from donor mice were lysed of red blood cells and depleted of B cells using magnetic beads and columns as described by the manufacturer (Miltenyi). Cells were stained with anti-CD4 and anti-CD45RB antibodies, and sorted to obtain a fraction (~99% purity) of $CD4^+CD45RB^{hi}$ cells as previously described (18). Sorted cells were thoroughly washed with sterile PBS and 0.75×10^6 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 rtPCR

RNA was isolated from the colon using the TRIZOL isolation protocol (Invitrogen) with slight modifications. Colon tissue was homogenized in 1 ml of TRIZOL reagent and then two chloroform extractions were performed. Following an isopropanol precipitation, the RNA was washed with 70% ethanol and treated with RNase Inhibitor (Applied Biosystems) for 45 minutes. Following resuspension of the RNA at 65°C for 15 minutes, RNA preparations were further purified using the Qiagen RNAeasy isolation kit (Qiagen Inc.). RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For real-time rtPCR, we used the relative gene expression method (19). Glyceraldehyde 3-phosphate dehydrogenase (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.

Citrobacter rodentium infections

Mice were orally inoculated with wild type *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 of broth containing 1×10^8 CFU of *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

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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, 3 = muscular propria. The percentage of colon involved was also determined. The injury score was determined as: (chronic inflammation + acute inflammation) × % involvement.

Statistical analysis

Statistical significance between the groups was determined by application of an unpaired 2-tailed Student's *t* test. $P<0.05$ was considered significant. In the experiments involving *C. rodentium* infection Mann Whitney U and Log-rank (Mantel-Cox) tests were performed. $P<0.05$ was considered significant.

RESULTS

TL promotes an environment that is favorable for the development of colitis by $CD4^+CD45RB^{hi}$ 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 $CD4^+CD45RB^{hi}$ 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 $TL^{+/+}RAG\text{-}2^{-/-}$ and $TL^{-/-}RAG\text{-}2^{-/-}$ mice as recipients of $CD4^+CD45RB^{hi}$ T cells from WT donors. We observed that $TL^{+/+}RAG\text{-}2^{-/-}$ began to lose weight at two weeks post transfer, after which they developed other signs of colitis such as diarrhea, rectal bleeding, and scruffiness (Fig. 1a). Although colitis in this model generally develops four weeks 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, $TL^{-/-}RAG\text{-}2^{-/-}$ 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 weeks after transfer showed severe cellular infiltration and disruption of the epithelial architecture of $TL^{+/+}RAG\text{-}2^{-/-}$ mice, whereas $TL^{-/-}RAG\text{-}2^{-/-}$ recipient mice showed modest pathological changes (Fig. 1b, top). Overall, the disease scores of $TL^{+/+}RAG\text{-}2^{-/-}$ mice were significantly higher than $TL^{-/-}RAG\text{-}2^{-/-}$ mice (Fig. 1b, bottom). Importantly, donor-derived $CD4^+$ T cells were detected in both types of recipient mice. However, due to the ongoing inflammatory process in $TL^{+/+}RAG\text{-}2^{-/-}$ mice, donor cell numbers recovered from these mice, as compared with cells recovered from $TL^{-/-}RAG\text{-}2^{-/-}$ mice, were consistently higher in the spleen, mesenteric lymph nodes (MLN), LP, and the IEL compartment (Fig. S1).

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 $TL^{+/+}RAG\text{-}2^{-/-}$ and $TL^{-/-}RAG\text{-}2^{-/-}$ mice two weeks after transfer. As shown in Figure 1c, donor-derived cells from $TL^{+/+}RAG\text{-}2^{-/-}$ or $TL^{-/-}RAG\text{-}2^{-/-}$ 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 $TL^{-/-}RAG\text{-}2^{-/-}$ recipients repeatedly had statistically significant lower percentages of IL-17-producing cells when compared with

$TL^{+/+}RAG\text{-}2^{-/-}$ hosts (Fig. 1c). Consistently, IFN-γ levels were similar in donor-derived cells from $TL^{+/+}RAG\text{-}2^{-/-}$ and $TL^{-/-}RAG\text{-}2^{-/-}$ hosts in all lymphoid compartments analyzed at 4 weeks after transfer (data not shown), whereas donor cells recovered from the IEL compartment of $TL^{+/+}RAG\text{-}2^{-/-}$ recipients had significantly higher IL-17 proportions than cells isolated from $TL^{-/-}RAG\text{-}2^{-/-}$ recipients (Fig. 1d) when stimulated in vitro with PMA/ionomycin. Our data suggests 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 two weeks after $CD4^+CD45RB^{hi}$ T cell transfer. We observed that colons from $TL^{-/-}RAG\text{-}2^{-/-}$ 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 to $TL^{+/+}RAG\text{-}2^{-/-}$ 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 $TL^{-/-}RAG\text{-}2^{-/-}$ recipient mice.

Because of the relationship between IL-17-producing T cells (Th17) and regulatory T cells (T_{regs}) (22), we investigated whether loss of IL-17-producing $CD4^+$ T cells correlated with the development of T_{reg}. Therefore, we analyzed whether donor derived cells in recipient $TL^{-/-}RAG\text{-}2^{-/-}$ mice had an expanded T_{reg} population. Indeed, we observed that the proportion of $Foxp3^+$ donor-derived T cells was greater in the spleen and LP of $TL^{-/-}RAG\text{-}2^{-/-}$ recipient mice compared with $TL^{+/+}RAG\text{-}2^{-/-}$ 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. In order to determine whether $Foxp3^+IL\text{-}17^+$ T cells develop from donor-derived cells during colitis induction in $TL^{+/+}RAG\text{-}2^{-/-}$ and $TL^{-/-}RAG\text{-}2^{-/-}$ recipient mice, we determined the proportion of these cells in our experimental system. We observed that the proportion of donor-derived $Foxp3^+IL\text{-}17^+$ 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 $TL^{+/+}RAG\text{-}2^{-/-}$ vs. $TL^{-/-}RAG\text{-}2^{-/-}$ recipient mice (spleen, 0.7 ± 0.2 vs. 0.7 ± 0.2; MLN, 0.4 ± 0.4 vs. 0.2 ± 0.2; IEL, 0.02 ± 0.02 vs. 0.01 ± 0.02, respectively). However, we observed that in the LP there was a higher and statistically significant difference in the $Foxp3^+IL\text{-}17^+$ T cell population present in $TL^{+/+}RAG\text{-}2^{-/-}$ recipients compared with $TL^{-/-}RAG\text{-}2^{-/-}$ recipients (0.12 ± 0.02 vs. 0.03 ± 0.02, respectively; $p = 0.001$). However, because the proportion of $Foxp3^+IL\text{-}17^+$ 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). In order to rule out a possible effect of TL present in the $CD4^+CD45RB^{hi}$ T cell fraction that may influence disease development, we adoptively transferred $CD4^+CD45RB^{hi}$ T cells derived from $TL^{-/-}$ donor mice into $TL^{+/+}RAG\text{-}2^{-/-}$ and $TL^{-/-}RAG\text{-}2^{-/-}$ mice. Similar to donor cells derived from WT mice, $TL^{+/+}RAG\text{-}2^{-/-}$ mice recipients of donor cells from $TL^{-/-}$ mice developed severe colitis, whereas $TL^{-/-}RAG\text{-}2^{-/-}$ recipients did not (Fig. S2), indicating that 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 $TL^{+/+}RAG\text{-}2^{-/-}$ or $TL^{-/-}RAG\text{-}2^{-/-}$ recipient mice showed no significant difference in the levels of CD8αα expression (Fig. S3a).

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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., RAG$^+$) 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 (Fig. S4). 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 where IEC express TL

Th17 and Th1 cells are important CD4$^+$ T cell subsets in colitis development. In order 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 two weeks post-transfer, TL$^{+/+}$RAG-2$^{-/-}$ mice receiving Th17 cells lost more weight and developed increased disease scores when compared to 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). 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 than cells recovered from TL$^{-/-}$RAG-2$^{-/-}$ recipient mice (Fig. 4c). 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

In order 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 co-cultured 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 co-culture TL$^+$IEC or TL$^-$IEC with either differentiated Th17 or Th1 cells, with or without TCR stimulation. Four days after co-culture, CD4$^+$ T cells were recovered and IL-17 and IFN-γ production was determined by intracellular staining following PMA/ionomycin stimulation. In all experimental groups, re-activated 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 co-cultured in the presence of TL$^+$IEC, in comparison to Th17 cells cultured with TL$^-$IEC or in the absence of IEC (Fig. 5a, dot plots and middle bar graph). In contrast, Th17 cells cultured alone or with

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TL^+IEC exhibited a decrease in the percentage of cells producing IFN-γ compared with Th17 cultured with TL^-IEC (Fig. 5a, dot plots and upper bar graph). 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 graph). 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, dot plots and bar graph).

Th17 cells that were not restimulated with anti-CD3 antibodies maintained similar levels of IFN-γ production in the presence or absence of TL^+IEC or TL^-IEC (Fig. 5c, dot plots and upper bar graph). In contrast to TCR-restimulated cells, Th17 cells that were not restimulated lost their capacity to produce IL-17 when co-cultured with TL^+IEC, as compared with Th17 cells cultured alone and, to a lesser extent, cultured in the presence of TL^-IEC (Fig. 5c, dot plots and middle bar graph). 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 bar graph). 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 to Th17 cells cultured alone, this was not due to cell viability since 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 naïve $CD4^+$ T cells towards 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 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 to 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 $CD4^+CD8\alpha^+$ T cell population from Th17 cultures by magnetically sorting $CD8\alpha^+$ cells (see Fig. S3b), and culturing the remaining $CD4^+CD8\alpha^-$ 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 co-culture with TL^+IEC or TL^-IEC (Fig. 6b, right plots). 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 (Fig. 6b; compare with Fig. 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 CD8αα 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 and b). When the bacterial load in the colon was measured,

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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 to 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 suggests 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 CD8αα, the cytokine response of $CD4^+$ T cells.

We found that $CD4^+CD45RB^{hi}$ T cells adoptively transferred into $TL^{-/-}RAG\text{-}2^{-/-}$ recipient mice have a diminished capacity to induce colitis that otherwise is evident in TL-competent RAG-2-deficient recipients (Fig. 1a and b). 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 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 these cytokines 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 $CD4^+CD8\alpha\alpha^+$ 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, where CD8αα expression has not been previously detected (39). However, we confirmed that, under our experimental conditions, splenic $CD4^+$ T cells upregulated CD8α homodimers, albeit transiently and at a very low frequency (~0.2 to 0.9% of total $CD4^+$ T cells) (Fig. 6a). These results suggest that, *bona fide* $CD4^+CD8\alpha\alpha^+$ 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 CD8α homodimers, we demonstrated that depletion of the small population of $CD4^+CD8\alpha\alpha^+$ 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

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interaction between IEC and $CD4^+CD8\alpha\alpha^+$ IEL or $CD4^+CD8\alpha\alpha^{transient}$ 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 (Fig. S4). 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 antibody staining in unactivated or activated dendritic cells or monocytes (D.O-V and L.V.K. unpublished results).

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 $TL^{-/-}TCR\alpha^{-/-}$ mice (10). Why does TL expression protect against colitis in the $TCR\alpha^{-/-}$ model but renders mice more susceptible to colitis in the adoptive transfer model used here? The spontaneous colitis that develops in the $TCR\alpha^{-/-}$ model is characterized as a Th2-like disease mediated predominantly by IL-4 produced by a rare population of $CD4^+$ T cells expressing TCRβ-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/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 $CD4^+TCR\beta\beta^+$ T cells in TCRα-deficient 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.

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Supplementary Material

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Acknowledgments

Grant support: D.O-V, L.V.K, V.V.P., and K.R. were supported by Vanderbilt-Meharry Center for AIDS Research (CFAR) Pilot Program (funded by NIH grant P30AI54999), Pilot and Feasibility Project from the Vanderbilt Digestive Disease Research Center (funded by NIH grant P30DK058404), and NIH grant AI072417. H.S.M.A. was supported by a Career Development Award from the Department of Veterans Affairs (CDA-2), and K.S., M.B.P and K.T.W. were supported by NIH grants AT004821, AT004821-02S1, and DK053620.

Abbreviations

IEC intestinal epithelial cells

IEL intraepithelial lymphocytes

TL thymus leukemia

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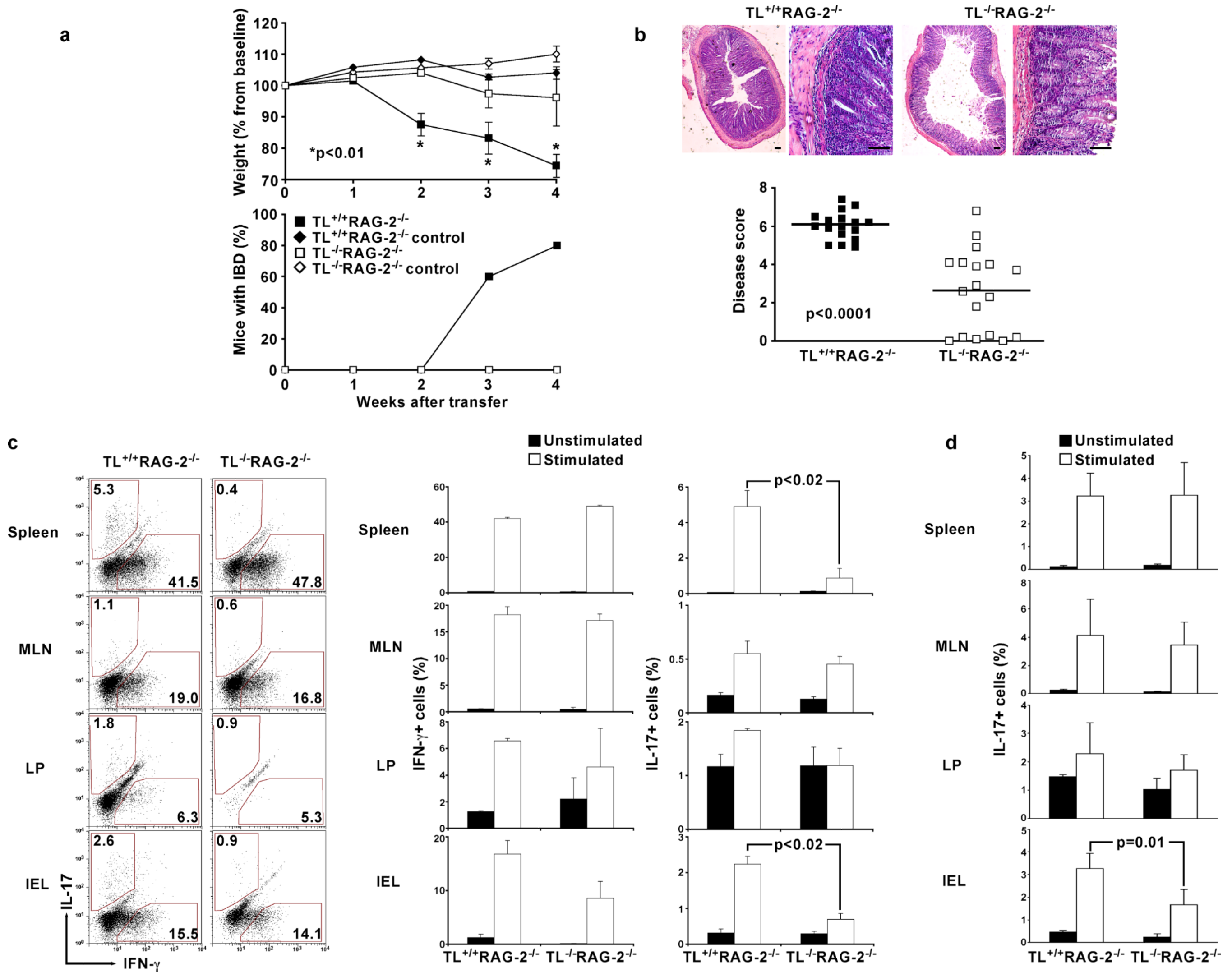

Figure 1. TL promotes IL-17 expression and induction of colitis by adoptively transferred $CD4^+CD45RB^{hi}$ T cells

Naïve $CD4^+CD45RB^{hi}$ T cells (0.75×10^6/mouse) were adoptively transferred into $TL^{+/+}RAG\text{-}2^{-/-}$ or $TL^{-/-}RAG\text{-}2^{-/-}$ mice. (a) Weight change (top) and disease course (bottom) of treated mice. Control mice refer to animals that received unsorted $CD4^+$ T cells. Data represents one representative experiment of at least three independent experiments with 3–4 mice per group. (b) Pathology of treated mice two weeks after transfer (top); the bar scale represents 100 μm. Disease score of $TL^{+/+}RAG\text{-}2^{-/-}$ or $TL^{-/-}RAG\text{-}2^{-/-}$ mice at 2 weeks after transfer (bottom). See Materials and Methods section 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). Plots show gating on $TCR\beta^+CD4^+$ cells. Summary of dot plots is represented as bars (right). Data represents at least 3 independent experiments, with 3–4 mice per group. (d) Similar experiments as in (c) analyzed at 4 weeks after transfer.

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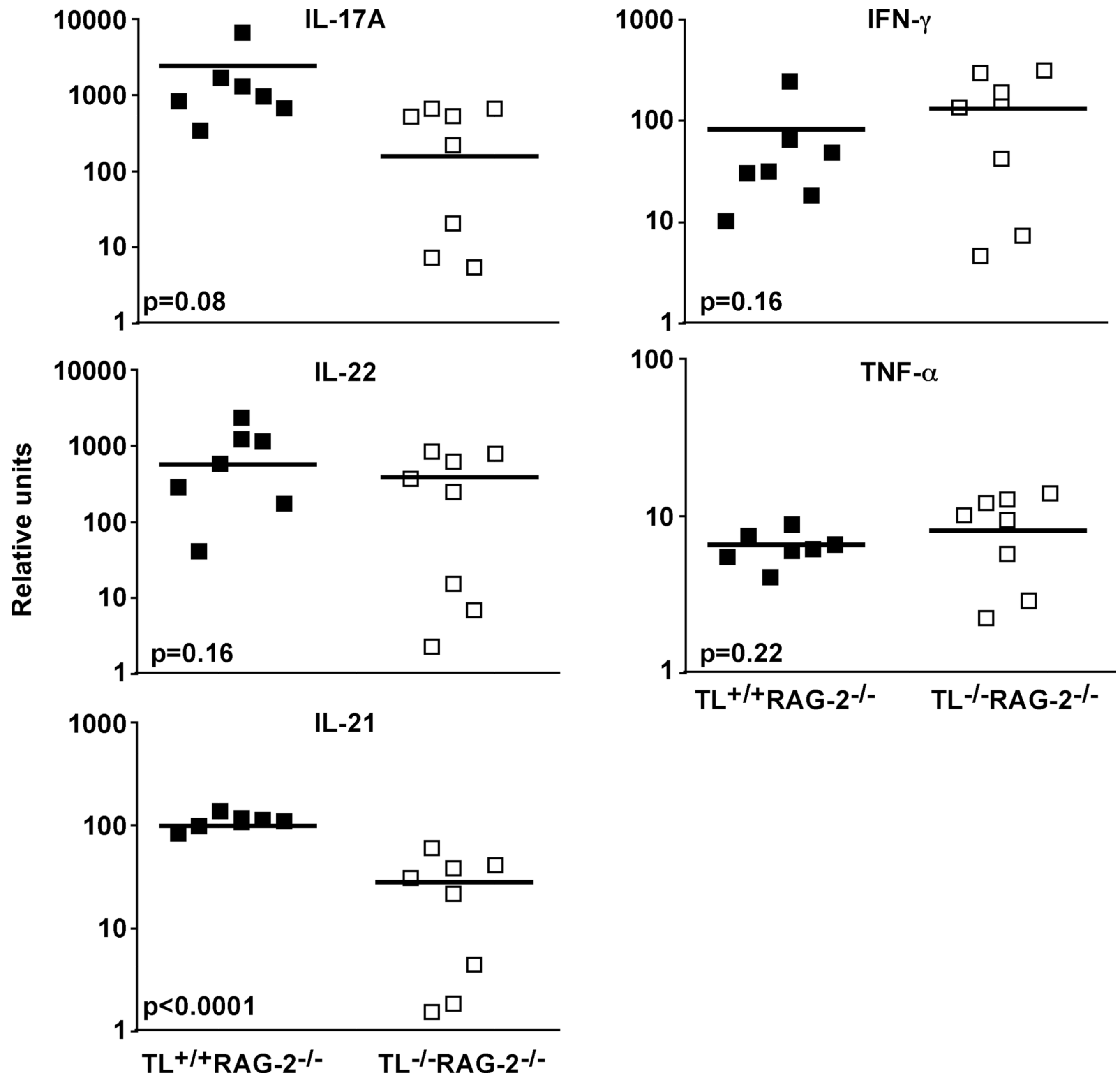

Figure 2. Cytokine mRNA expression in the colons of $TL^{-/-}RAG\text{-}2^{-/-}$ and $TL^{+/+}RAG\text{-}2^{-/-}$ recipient mice

Two weeks after adoptive transfer as in Fig. 1, mRNA was isolated from the colon and amplified by real-time PCR. Values are compared to untreated controls.

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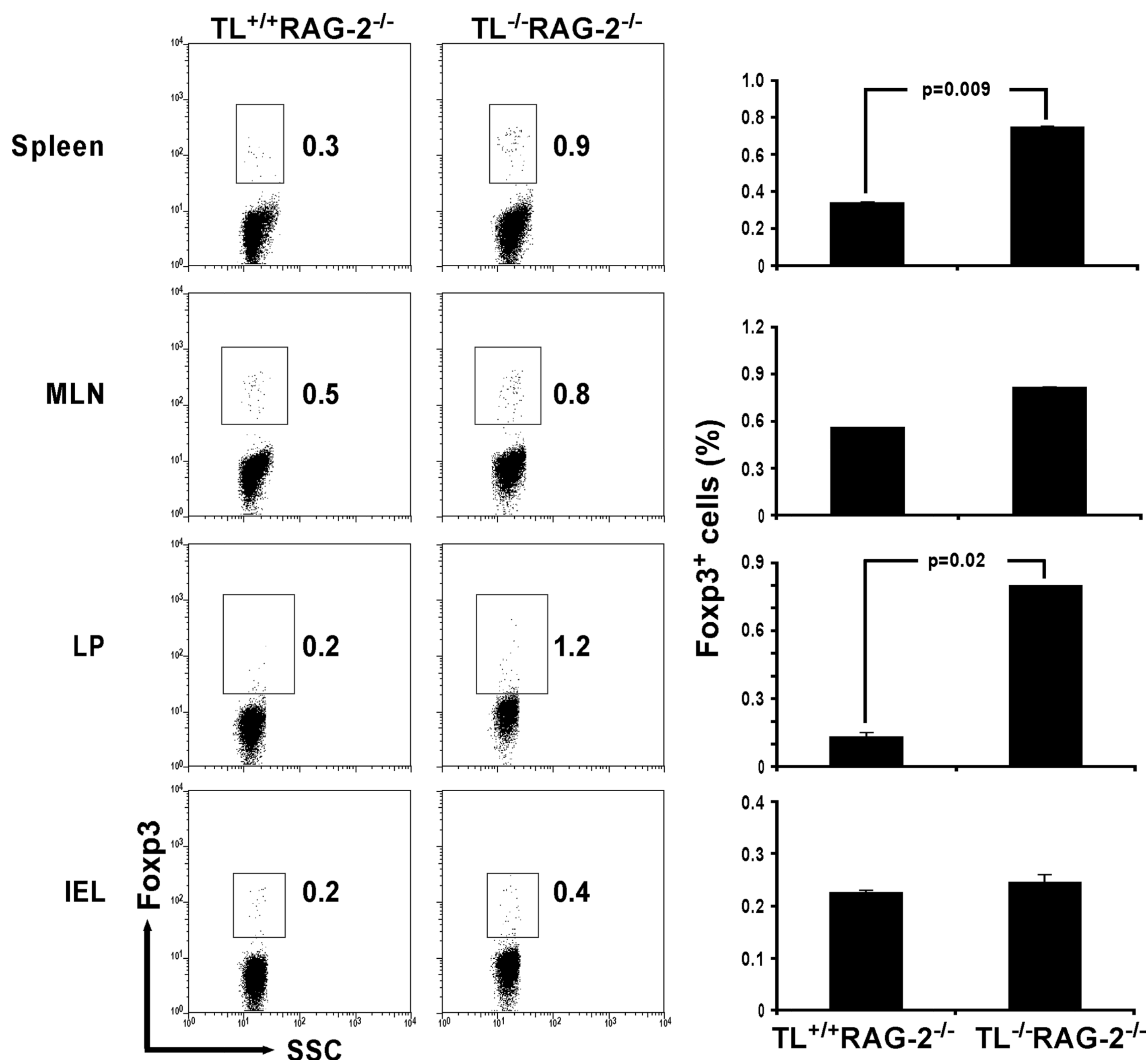

Figure 3. Decreased levels of donor-derived Foxp3$^+$ cells in TL-expressing mucosa

Two weeks after adoptive transfer as in Fig. 1, donor cells were analyzed for Foxp3 expression in the indicated organs. Plots are gated on CD4$^+$TCRβ$^+$ cells. Data is representative of two independent experiments, with n = 3 to 4 mice per experimental group.

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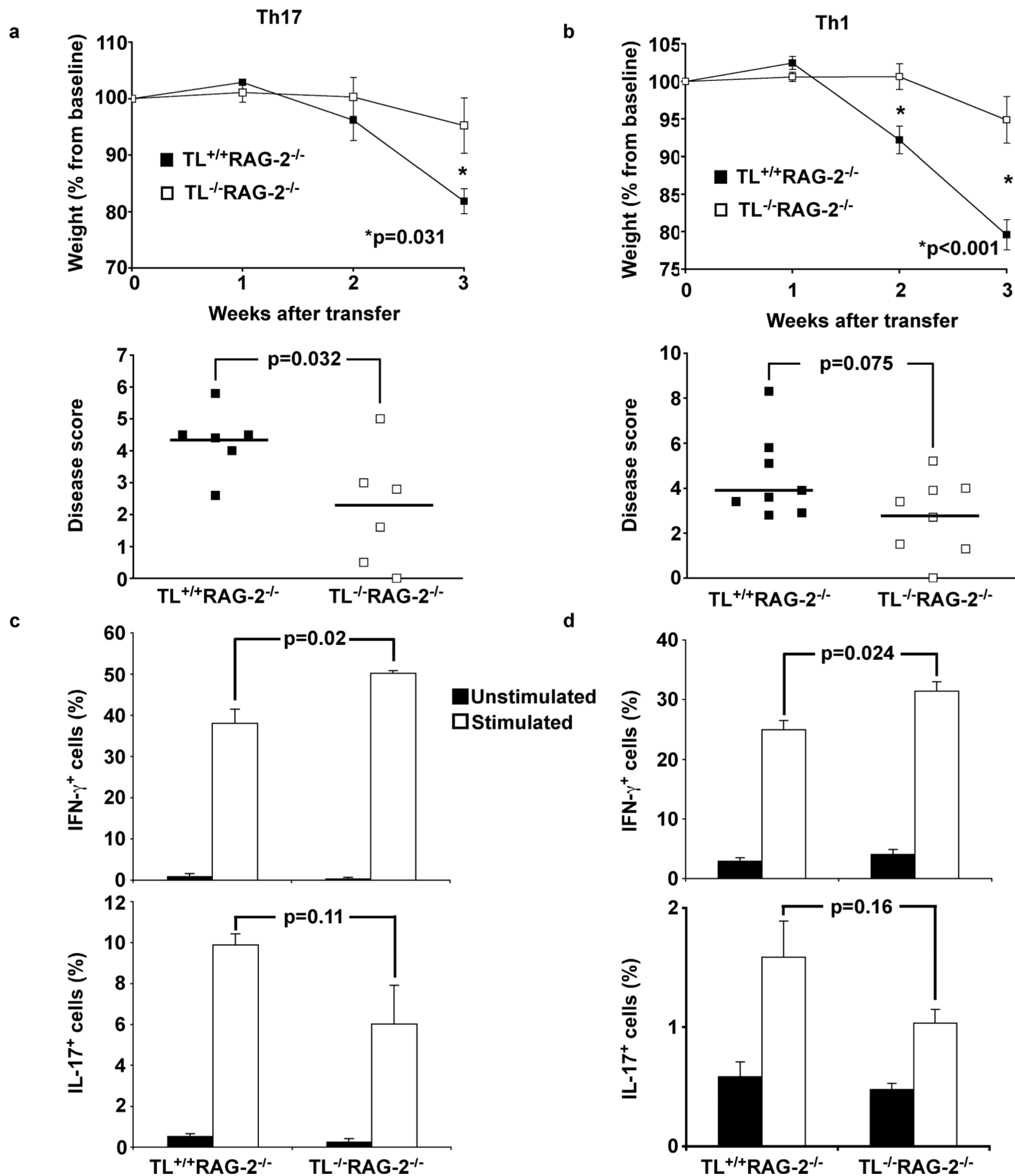

Figure 4. Colitis severity, but not weight loss, is more pronounced for Th17 cells than Th1 cells introduced into an environment where IEC express TL

Differentiated Th17 cells (a, c) or Th1 cells (b, d) were adoptively transferred into $TL^{+/+}RAG\text{-}2^{-/-}$ or $TL^{-/-}RAG\text{-}2^{-/-}$ mice (0.25×10^6 cells/recipient). Hosts were followed for weight change (a and b, top) and disease scores were analyzed 3 weeks after transfer (a and b, bottom). 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 is from one of two independent experiments of 3–4 mice per group.

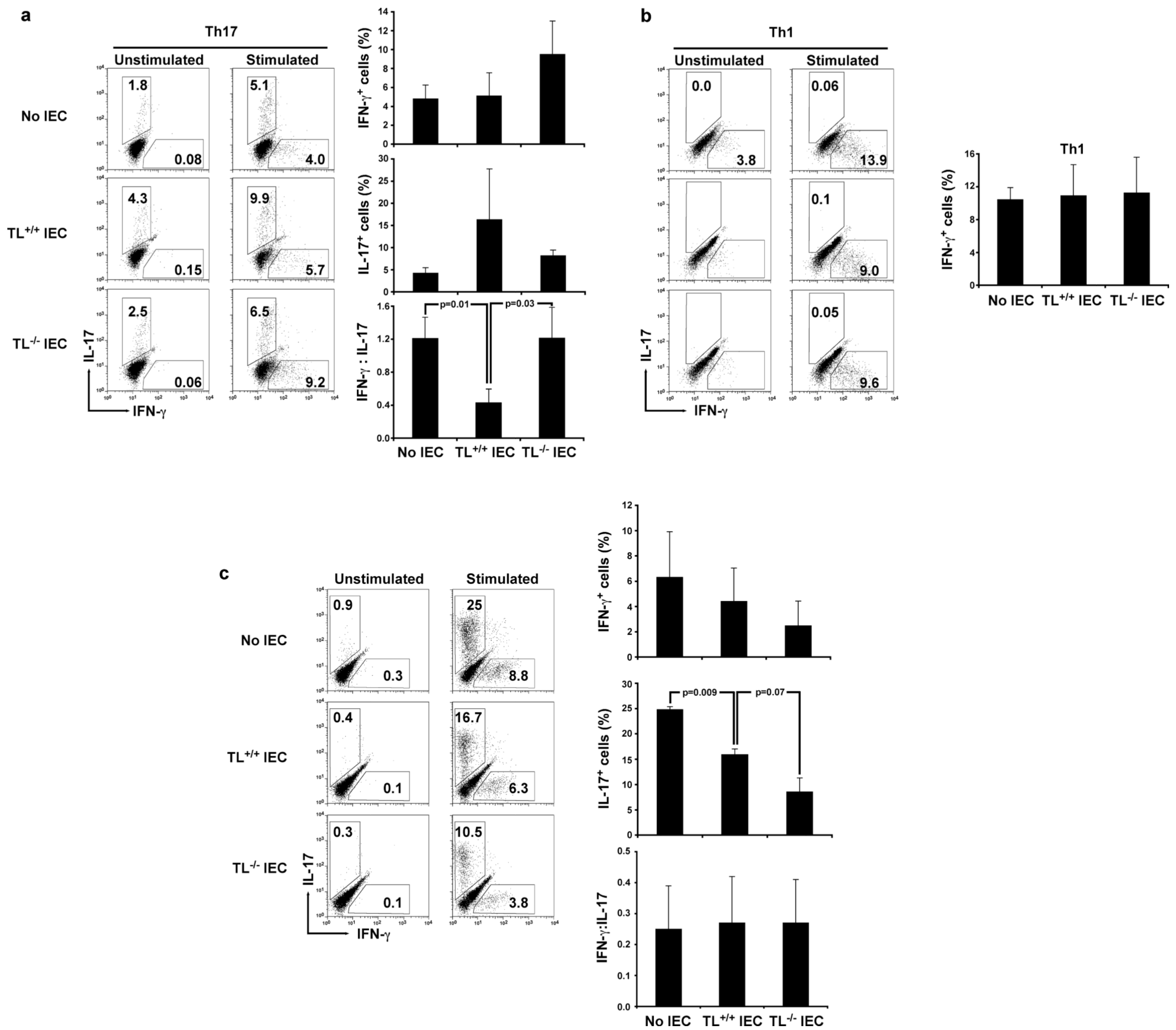

Figure 5. TL+IEC promote IL-17 production in differentiated Th17 cells

(a) Differentiated Th17 cells were cultured in the absence (top dot plots) or in the presence of highly purified IEC (1:3 ratio) from TL-competent (middle dot plots) or TL-deficient (bottom dot plots) donors. Cells were activated with plate-bound anti-CD3. Four days after co-culture, cells were analyzed for intracellular cytokine production by incubation in the presence (stimulated) or absence (unstimulated) of PMA/ionomycin. Bar graphs 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 $TCR\beta^+CD4^+$ cells. All data is representative of at least two independent experiments.

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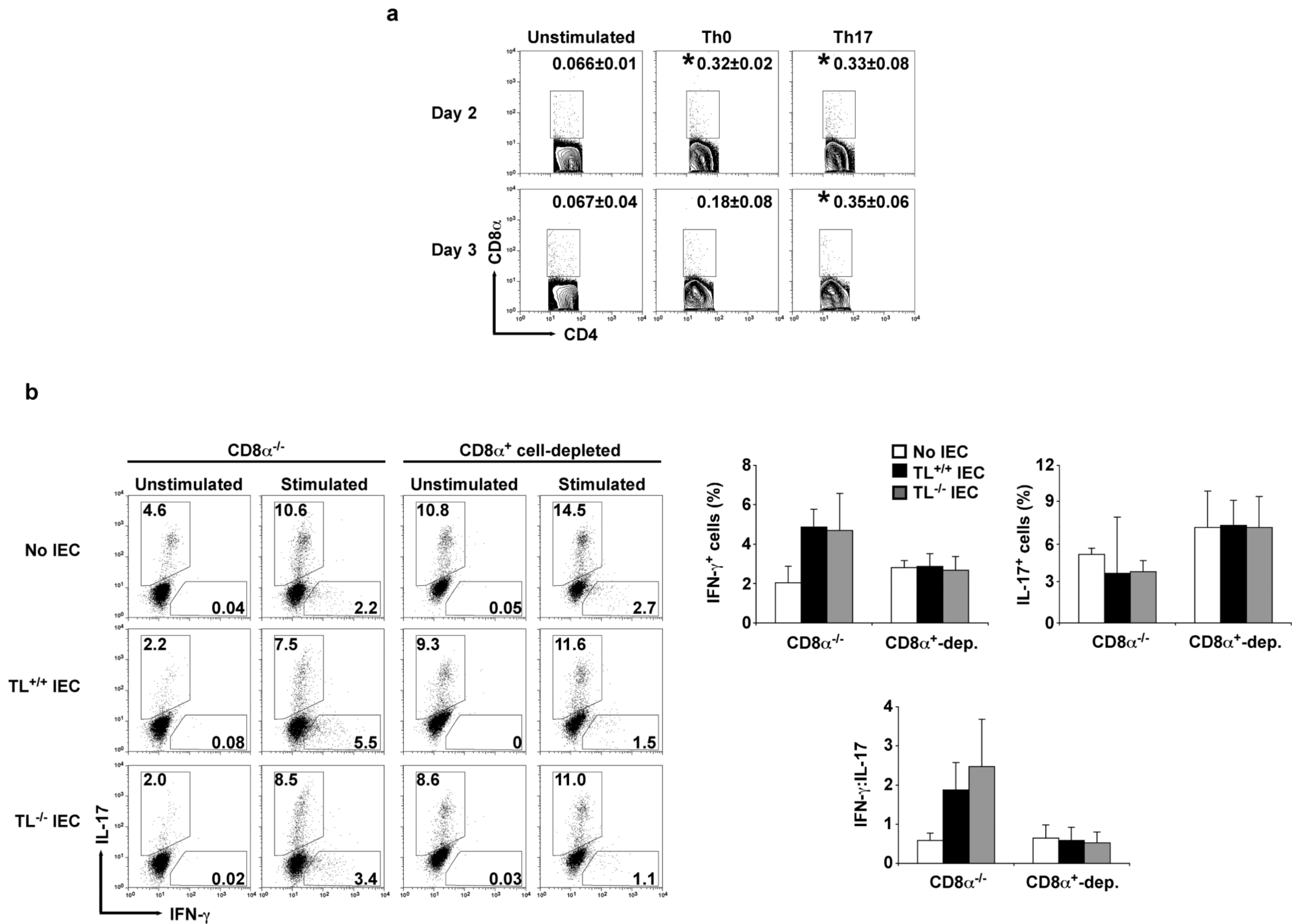

Figure 6. The TL/CD8αα interaction is critical for modulation of $CD4^+$ T cell responses by IEC

(a) Activated $CD4^+$ T cells transiently express CD8αα. In order to avoid background staining from conventional $CD8^+$ T cells, we used total splenocytes from β_2-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 days after activation, cells were stained for surface markers. Results are shown for cells gated on $CD8\beta^-$ cells as an irrelevant marker for noise reduction. Similar results were obtained when splenocytes from WT mice were used. * indicates $p>0.03$, comparing CD8α staining of Th0 or Th17 versus unstimulated cells. Data is representative of two independent experiments using 2–3 mice per condition. (b) Purified, fully differentiated Th17 cells from CD8α-deficient mice or Th17 cells from WT mice depleted of the transient $CD4^+CD8\alpha^+$ T cell population (see Material and Method section for procedure and Fig. S3b for purification efficiency) were co-cultured in the absence (left, top panels) or in the presence of purified IEC from TL-competent (left, middle panels) or -deficient (left, bottom panels) donor animals. Four days after co-culture, cells were analyzed for intracellular cytokine production by incubation in the presence (stimulated) or absence (unstimulated) of PMA/ionomycin. Bar graphs 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 represents results from 2 independent experiments.

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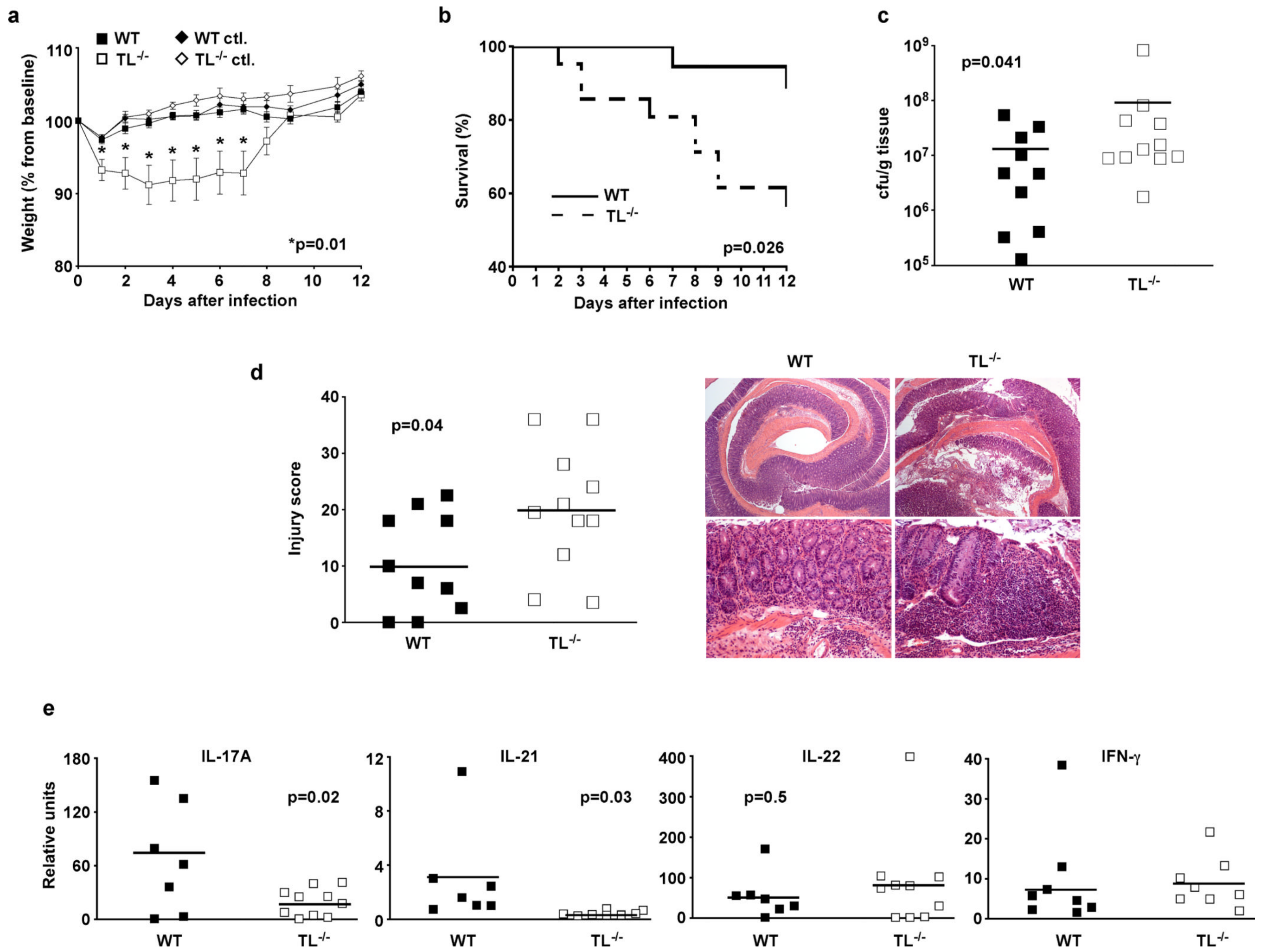

Figure 7. TL expression by IEC protects against *Citrobacter rodentium* infection

WT and $TL^{-/-}$ mice were infected orally with 1×10^8 CFU of *C. rodentium*. (a) Weight and (b) survival were monitored for 12 days. 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 days post-infection. Representative data is shown. (d) Left, injury scores of colons at 12 days after infection. See Materials and Methods section for a full description of injury score. Right, representative H&E staining from dissected colons (4× and 20× magnification). (e) Real time PCR analysis from colons isolated at 6 days after infection.