# Thymus leukemia antigen controls intraepithelial lymphocyte function and inflammatory bowel disease

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**Intestinal intraepithelial lymphocytes (IEL) bear a partially activated phenotype that permits them to rapidly respond to antigenic insults. However, this phenotype also implies that IEL must be highly controlled to prevent misdirected immune reactions. It has been suggested that IEL are regulated through the interaction of the CD8$\alpha\alpha$ homodimer with the thymus leukemia (TL) antigen expressed by intestinal epithelial cells. We have generated and characterized mice genetically-deficient in TL expression. Our findings show that TL expression has a critical role in maintaining IEL effector functions. Also, TL deficiency accelerated colitis in a genetic model of inflammatory bowel disease. These findings reveal an important regulatory role of TL in controlling IEL function and intestinal inflammation.**

CD8$\alpha\alpha$ | mucosal immunity | colitis | nonclassical MHC | immunoregulation

The intestinal mucosa represents one of the major entry points for antigens into the body, and thus requires a refined immunological system that can prevent the invasion and dissemination of both commensal and pathogenic microorganisms. One of the main immunological compartments involved in regulating mucosal immune responses is comprised by the intraepithelial lymphocytes (IEL). IEL are a population of lymphocytes that reside within the intestinal epithelium, and constitute one of the largest populations of lymphocytes in the body. In mice, 3 main subpopulations of IEL have been identified: TCR$\gamma\delta^+$ T cells expressing mostly the CD8$\alpha\alpha$ homodimer, TCR$\alpha\beta^+$ T cells expressing either CD4 or CD8$\alpha\beta$ and sometimes coexpressing CD8$\alpha\alpha$, and TCR$\alpha\beta^+$ T cells expressing CD8$\alpha\alpha$ (1). Many reports indicate that these populations function in the recognition of stress signals (2), are involved in the recovery from tissue damage (3, 4), function as conventional memory cells (1), or have natural autoreactivity, suggesting a regulatory role (5, 6). Despite their varied function and phenotype, the great majority of IEL are characterized by a "partial activation" state (7). This phenotype suggests that IEL are capable of rapidly responding to stimuli, and therefore, must be held in tight check to prevent unwanted reactions. Thus, an intriguing aspect is the regulation of the effector functions of IEL, which remains incompletely understood.

The thymus leukemia (TL) antigen is a nonclassical MHC class I molecule encoded by a locus within the MHC complex (8). TL expression is confined to the surface of intestinal epithelial cells (IEC) (9, 10) and it does not appear to bind an antigenic moiety (11, 12). Recently, it has been demonstrated that TL binds preferentially to the CD8$\alpha\alpha$ homodimer (11–17), and it has been suggested that this interaction, at least in vitro, modulates IEL responses.

Considering that CD8$\alpha\alpha$ is a prevalent surface marker on IEL, and that these cells reside in close proximity to TL-expressing IEC, we hypothesized that TL has a key role in regulating IEL effector functions. In the present study, we have analyzed mice deficient in the expression of TL and report that these animals exhibit alterations in the proliferation and function of IEL. Also, we show that TL serves as a regulatory element that delays the progression of chronic colitis in a genetic model of inflammatory bowel disease (IBD).

## Results

**Generation of TL-Deficient Mice.** We generated TL-deficient animals by using embryonic stem (ES) cells derived from C57BL/6 mice by conventional gene targeting techniques (Fig. 1*A*). C57BL/6 mice have 1 functional TL gene (*T3*) and carry a natural deletion of the *T18* gene (18). This strategy allowed us to obtain TL-deficient mice directly in a homogeneous C57BL/6 background. Mutant mice lack TL expression in IEC, as indicated by staining with a TL-specific antibody (Fig. 1*B*). Also, RT-PCR analysis showed that expression of TL in both the small and large intestine is lost in TL-deficient animals (Fig. 1*C*).

**TL Deficiency Does Not Alter the Numbers or Proportion of Different IEL Subsets.** The constitutive expression of TL on IEC could imply an important role for this molecule in mediating homing and/or maintenance of IEL. Therefore, we carefully analyzed the IEL populations in these mice. We observed that the total number of small intestine IEL was similar among $TL^{-/-}$ and $TL^{+/+}$ mice [$TL^{-/-}$, $2.68 \times 10^6 \pm 0.25 \times 10^6$ ($n = 56$); $TL^{+/+}$, $2.30 \times 10^6 \pm 0.18 \times 10^6$ ($n = 52$)]. We also analyzed the proportion and total cell number of the different IEL populations and found that CD8$\alpha\alpha^+$ (defined by TL-tetramer staining), TCR$\beta^+$, TCR$\gamma\delta^+$, and CD4$^+$ cells, among others, were similar between $TL^{-/-}$ and $TL^{+/+}$ mice (Fig. 1 *D* and *E Left*). Also, there was no significant difference in the percentages of the different colonic IEL populations derived from $TL^{-/-}$ and $TL^{+/+}$ mice (Fig. 1*E Right*). Analysis of the lymphocyte populations in other organs did not reveal alterations in either T or B cells (data not shown).

**TL Modulates Colonic IEL Homeostatic Proliferation and IEL Function.** To determine the role of TL in homeostatic proliferation of IEL in vivo, we analyzed the incorporation of BrdU in IEL derived from $TL^{-/-}$ and $TL^{+/+}$ mice. As shown in Fig. 2*A Left*, the proportion of BrdU$^+$ cells from the small intestine was similar among TL-deficient and TL-competent mice, both for TCR$\gamma\delta^+$ and TCR$\alpha\beta^+$ IEL. However, analysis of colonic IEL showed that TCR$\beta^+$CD8$\alpha\alpha^+$ and TCR$\gamma\delta^+$CD8$\alpha\alpha^+$ cells consistently incorporated more BrdU than their counterparts isolated from $TL^{+/+}$ mice. The levels of BrdU$^+$ TCR$\beta^+$CD8$\alpha\beta^+$ cells were the same in IEL isolated from $TL^{-/-}$ and $TL^{+/+}$ mice (Fig. 2*A Right*). It is possible that the high antigenic load present in the large intestine triggers

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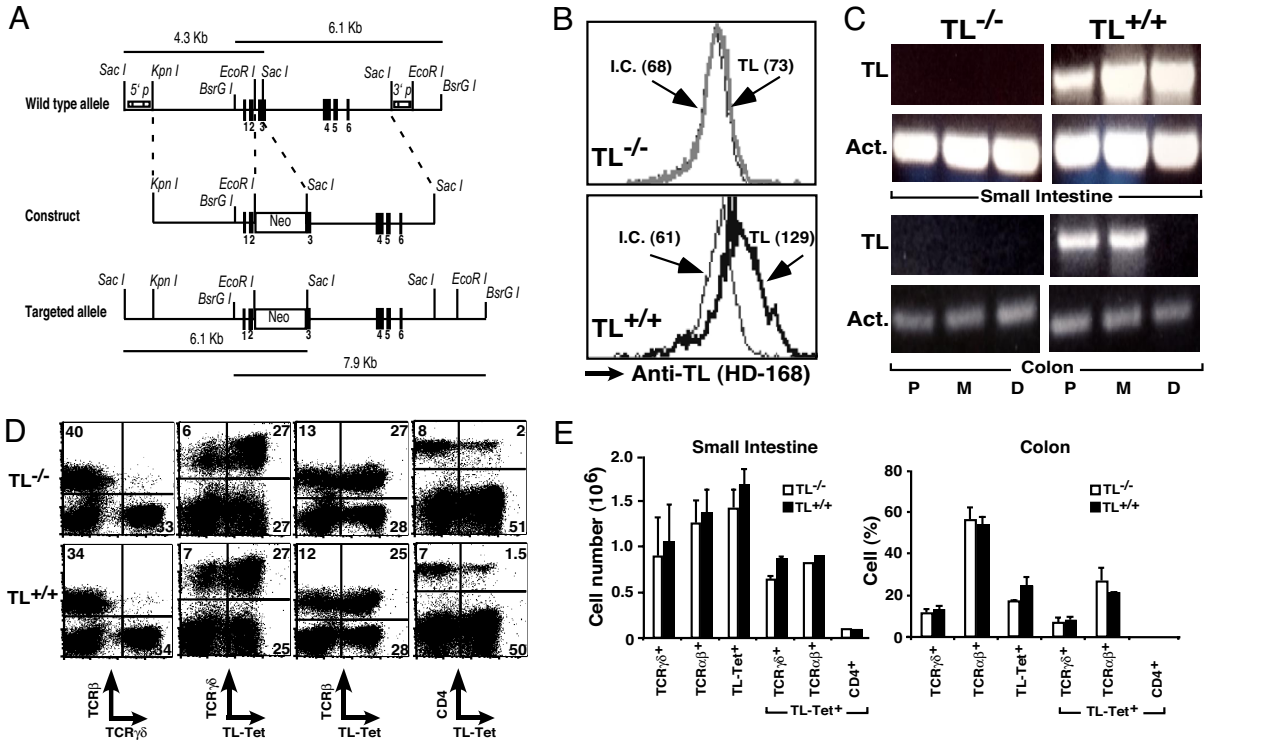


**Fig. 1.** Generation of TL deficient mice. (*A*) *T3* targeting strategy in C57BL/6 ES cells resulting in partial deletion of exon 3; 5′p and 3′p indicate probes used for hybridization. (*B*) TL expression on IEC recovered from IEL preparations were analyzed for anti-TL (HD-168) or isotype control (I.C.) staining. Parentheses indicate mean fluorescence intensity. IEC were gated based on their forward and side scatter properties. (*C*) TL RT-PCR amplification of RNA samples isolated from small intestine or colon. P, proximal; M, middle; D, distal. (*D*) FACS analysis of IEL populations derived from the small intestine of $TL^{-/-}$ ($n = 5$) or $TL^{+/+}$ ($n = 5$) mice. (*E*) Representation of the total cell number of distinct IEL populations. Due to the low and inconsistent number of recovered cells, colon IEL were pooled for analysis. Data are representative of at least 3 independent experiments.

more IEL proliferation in the absence of TL. Noteworthy, $TL^{-/-}$ mice do not develop any type of colonic inflammation suggesting that other mechanisms exist to keep IEL in check.

Previous reports have indicated that the presence of IEC, which constitutively express TL, prevents IEL proliferation in response to anti-CD3 stimulation in vitro (19–21). To test whether TL deletion modulates IEL proliferation, we stimulated crude preparations of IEL derived from $TL^{-/-}$ and $TL^{+/+}$ mice with graded doses of plate-bound anti-CD3 antibody in vitro. As shown in Fig. 2*B*, anti-CD3-stimulated IEL from $TL^{-/-}$ mice proliferated more extensively than IEL from $TL^{+/+}$ mice. Differences were significant at relatively low concentrations of anti-CD3 antibody ($P = 0.03$ at 1 $\mu$g/mL of anti-CD3), suggesting that TL inhibits proliferation below a specific threshold of TCR activation. To test whether the enhanced proliferation of IEL isolated from $TL^{-/-}$ mice is intrinsic to IEL or is a consequence of the absence of TL expression on IEC in the cultures, we cultured anti-CD3-stimulated IEL from wild-type and TL mutant mice in the presence of TL-transfected RMA tumor cells. Results showed that TL-expressing RMA cells reduced proliferation of IEL from TL mutant mice to a level similar to that of IEL from wild-type mice ($P = 0.001$) (Fig. 2*C*), indicating that control of IEL proliferation might be regulated by TL expressed on the surrounding IECs in vivo. To further examine the suppressive role of TL in proliferation, we performed blocking experiments by using a TL-specific monoclonal antibody. As expected, blocking TL had a greater effect on IEL derived from $TL^{+/+}$ mice than from $TL^{-/-}$ animals (Fig. 2*D*). Interestingly, total $CD8\alpha^+$ (which includes $CD8\beta^+$ cells) and $CD8\alpha^+\beta^+$ IEL from $TL^{+/+}$ mice showed significantly more desuppression than cells from $TL^{-/-}$ mice (Fig. 2*D Upper* and *Lower*, respectively). Of significance, $CD8\alpha\alpha^+$ cells (excluding $CD8\beta^+$ cells) did not produce measurable IL-2 in our experimental conditions (data not shown).

To determine the proliferative potential of $TCR\gamma\delta^+$ and $TCR\alpha\beta^+$ (the 2 main IEL populations) in the absence of TL, we depleted IEL preparations of $TCR\gamma\delta^+$ or $TCR\alpha\beta^+$ cells but maintained the IEC. We found that IEL preparations in which the great majority of IEL expressed $TCR\alpha\beta$ proliferated more when isolated from $TL^{-/-}$ mice compared with preparations from $TL^{+/+}$ mice (Fig. 2*E Upper*). Interestingly, IEL preparations enriched in $TCR\gamma\delta^+$ cells proliferated similarly regardless of whether the IEL were isolated from $TL^{-/-}$ or $TL^{+/+}$ mice (Fig. 2*E Lower*).

To determine the intrinsic proliferative response of IEL, we depleted IEC from IEL preparations (>90% pure) isolated from $TL^{-/-}$ and $TL^{+/+}$ mice and stimulated them with anti-CD3. As shown in Fig. 2*F*, there was no detectable difference in IEL proliferation between $TL^{-/-}$ and $TL^{+/+}$ mice. This finding suggests that the intrinsic proliferative capacity of IEL is not affected by the absence of TL.

The current model for TL proposes a role for this molecule as a regulator of IEL function (15), decreasing IEL cytotoxicity while enhancing cytokine production. Although not statistically significant, we observed that IEL from $TL^{-/-}$ mice were more cytotoxic than cells from $TL^{+/+}$ mice (Fig. 2*G*), but in contrast to the model, we found higher IFN-$\gamma$ secretion in IEL from $TL^{-/-}$ mice on anti-CD3 stimulation (Fig. 2*H*).

**TL Deficiency Accelerates the Onset and Increases the Severity of Disease in a Spontaneous Model of T Cell-Dependent Colitis.** We reasoned that TL expression on IECs might contribute to maintain immune quiescence of IEL under normal conditions and that the absence of TL might render $TL^{-/-}$ mice susceptible to IBD. To test this hypothesis, we used $TCR\alpha^{-/-}$ mice, which spontaneously develop IBD similar to ulcerative colitis (UC) in humans (22). Disease in these animals is mediated by a population of IL-4-

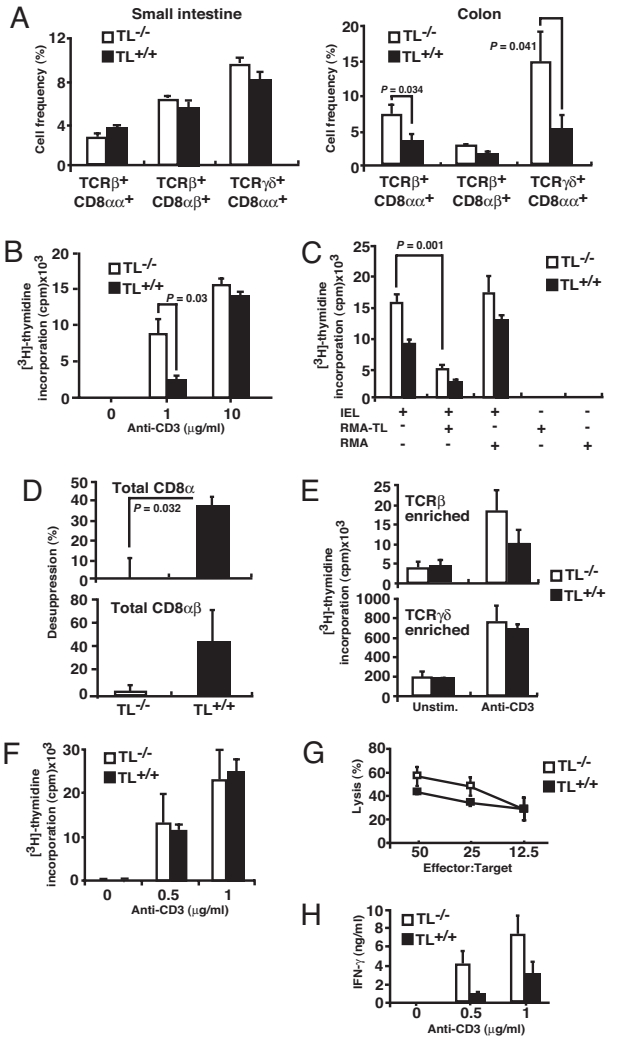


**Fig. 2.** IEL from TL-deficient mice have altered effector functions. (*A*) BrdU incorporation of IEL. Due to the low number of colon isolated, colon IEL were pooled for analysis. Combined data of 2 or 3 experiments are shown. (*B*) IEL isolated from $TL^{-/-}$ ($n = 3$) and $TL^{+/+}$ ($n = 3$) mice were stimulated for 60 h with graded doses of plate bound anti-CD3 antibody and pulsed with [$^3$H]thymidine. (*C*) IEL preparations from $TL^{-/-}$ ($n = 2$) and $TL^{+/+}$ ($n = 3$) mice were cultured in the presence of RMA or RMA cells transfected with TL and stimulated with 1 $\mu$g/mL of plate bound anti-CD3 antibody; 60 h later, cells were pulsed as in *B*. (*D*) IEL from $TL^{-/-}$ or $TL^{+/+}$ mice were stimulated for 24 h with plate-bound anti-CD3 (1 $\mu$g/mL) in the presence or absence of anti-TL antibody (HD168, 50 $\mu$g/mL). Cells were then stained for intracellular IL-2. After subtracting background (isotype control staining), percentage of desuppression was calculated as follows: [(stimulated-unstimulated)/stimulated] $\times$ 100. (*E*) IEL enriched for TCR$\beta^+$ or TCR$\gamma\delta^+$ cells were stimulated for 60 h with or without plate-bound anti-CD3 (0.5 to 1 $\mu$g/mL). Cells were pulsed as described above. (*B–E*) Representative data of 2 or 3 experiments. (*F*) IEL were purified by depleting epithelial cells by using a combination of anti-g8.8 monoclonal antibody followed by anti-rat-PE-labeled antibody. Cells were then separated by a magnetic column. Cells were stimulated with anti-CD3 as described above. Results shown are a combination of 2 independent experiments. (*G*) IEL from $TL^{-/-}$ or $TL^{+/+}$ mice were pooled (4 to 5 mice) and cultured at different ratios with $^{51}$Cr-labeled P815 cells ($5 \times 10^3$ cells) in the presence of anti-CD3 antibody (2 $\mu$g/mL); 8 h later $^{51}$Cr-release was measured. Combined data of 3 experiments are shown. (*H*) Supernatants from IEL stimulated with graded doses of anti-CD3 as in *B* were collected 60 h after culture and analyzed for IFN-$\gamma$ levels by ELISA. Results are representative of at least 2 independent experiments.

producing CD4$^+$ T cells that express TCR$\beta\beta$ homodimers (23–26). We crossed $TL^{-/-}$ mice with $TCR\alpha^{-/-}$ mice to generate $TL^{-/-}TCR\alpha^{-/-}$ compound mice. We followed a cohort of $TL^{+/+}TCR\alpha^{-/-}$ and $TL^{-/-}TCR\alpha^{-/-}$ mice for a period of >30 weeks and recorded the emergence of clinical signs of colitis (diarrhea, scruffiness, rectal prolapse, and rectal bleeding). In our colony, $TL^{+/+}TCR\alpha^{-/-}$ mice had a disease incidence of 14% at 30 weeks of age. Strikingly, at 30 weeks of age 83% of $TL^{-/-}TCR\alpha^{-/-}$ mice presented with signs of colitis (Table 1). Also, whereas the $TL^{+/+}TCR\alpha^{-/-}$ mice developed the first signs of IBD $\approx$20 weeks of age (Fig. 3*A*), consistent with published reports (22), $\approx$20% of the double knockout mice showed clear signs of disease by 15 weeks of age (Fig. 3*A*). Also, the overall mortality due to IBD in $TL^{+/+}TCR\alpha^{-/-}$ mice was 8.3%, compared with 35% in double mutant mice.

IBD was also scored by examination of colons, based both on macroscopic and microscopic parameters, as described (27). On macroscopic analysis, colons from $TL^{-/-}TCR\alpha^{-/-}$ mice appeared partially or fully thickened and were accompanied by loose stool (Fig. 3*B Upper*). Histological analysis of H&E stained colon sections showed higher incidence of pathological lesions in $TL^{-/-}TCR\alpha^{-/-}$ than in $TL^{+/+}TCR\alpha^{-/-}$ mice, including loss of goblet cells, elongation of crypts, and cellular infiltration (Fig. 3*B Lower*). This analysis clearly showed that $TL^{-/-}TCR\alpha^{-/-}$ mice presented with significantly ($P = 0.034$) more severe IBD as compared with $TL^{+/+}TCR\alpha^{-/-}$ mice (Fig. 3*C*).

Next, we examined IEL populations from $TL^{-/-}TCR\alpha^{-/-}$ and $TL^{+/+}TCR\alpha^{-/-}$ mice. We found that the numbers of TCR$\beta\beta$ and TCR$\gamma\delta$ cells in the colon were increased even in disease free $TL^{-/-}TCR\alpha^{-/-}$ mice (in a representative experiment: TCR$\beta\beta$, $0.1 \times 10^4$ for $TL^{-/-}TCR\alpha^{-/-}$ and $0.04 \times 10^4$ for $TL^{+/+}TCR\alpha^{-/-}$; TCR$\gamma\delta$, $2.2 \times 10^4$ for $TL^{-/-}TCR\alpha^{-/-}$ and $1.2 \times 10^4$ for $TL^{+/+}TCR\alpha^{-/-}$). Although the colon is the primary organ affected in $TCR\alpha^{-/-}$ mice, a hallmark of these mice is the presence of hyperplastic mesenteric lymph nodes (MLNs) due to a predominant expansion of B cells and TCR$\gamma\delta$ T cells that is more prevalent in diseased animals (24). $TL^{-/-}TCR\alpha^{-/-}$ mice also harbored hyperplastic MLN, and their cellularity was consistently higher than that of $TL^{+/+}TCR\alpha^{-/-}$ mice, regardless of IBD status (Fig. 3*D Left*). Even young (6 to 9 weeks old), disease-free $TL^{-/-}TCR\alpha^{-/-}$ mice presented a higher cellularity in the MLN than $TL^{+/+}TCR\alpha^{-/-}$ mice (data not shown), indicating that the factors causing disease are present at a relative young age in the double mutant mice. However, despite the increase in cell numbers, lymphocyte subset proportions among the 2 types of mice were indistinguishable (Fig. 3*D Right*).

Besides an expanded B cell population, $TCR\alpha^{-/-}$ mice also have elevated titers of IgG antibodies, including Th1-induced (IgG2a) and Th2-induced (IgG1) isotypes, which have been reported to increase in diseased mice (24). We found that both types of mice had similar increased levels of total Ig, IgG1, and IgG2a as compared with $TCR\alpha^+$ mice (data not shown). Also, we observed no difference in the proportion of B cells (defined as B220$^+$) in MLN ($TL^{-/-}TCR\alpha^{-/-}$ mice, 78.0 $\pm$ 4.5; $TL^{+/+}TCR\alpha^{-/-}$ mice, 73.3 $\pm$ 2.2). Because the predominant cytokine involved in the pathogenesis of IBD in $TCR\alpha^{-/-}$ mice is IL-4 (23, 26) we investigated its levels in $TL^{-/-}TCR\alpha^{-/-}$ mice. MLN are the primary lymphoid organs that harbor emigrant activated IEL, and in $TCR\alpha^{-/-}$ mice their lymphocyte populations resemble those present in colonic IEL (24). We enriched MLN for TCR$\gamma\delta^+$ or TCR$\beta\beta^+$ cells and measured IL-4 production after PMA/ionomycin or anti-CD3 stimulation. As shown in Fig. 3*E Left*, enriched TCR$\gamma\delta^+$ and TCR$\beta\beta^+$ cells from $TL^{+/+}TCR\alpha^{-/-}$ and $TL^{-/-}TCR\alpha^{-/-}$ animals proliferated similarly independent of the stimulus. However, consistent with previous reports (23–26), TCR$\beta\beta^+$ cells were the main population of cells responsible for IL-4 production, which was increased in $TL^{-/-}TCR\alpha^{-/-}$ mice (Fig. 3*E Right*). Our results suggest that lack of TL results in increased IL-4 secretion by pathogenic TCR$\beta\beta$ T cells, which in turn affects the development and severity of IBD in the $TCR\alpha^{-/-}$ mice.

Spleen, inguinal lymph nodes, and Peyer's patches of

**Table 1. IBD incidence in TL-deficient and TL-competent TCR$\alpha^{-/-}$ mice**

| | *n* | Mice with signs of IBD | Incidence, % | Moribund mice | Mortality, % |
|---|---|---|---|---|---|
| TL$^{-/-}$TCR$\alpha^{-/-}$ | 29 | 24 | 82.8 | 10 | 34.5 |
| TL$^{+/+}$TCR$\alpha^{-/-}$ | 36 | 5 | 13.9 | 3 | 8.3 |
| TL$^{-/-}$TCR$\alpha^{+}$ | 6 | 0 | 0 | 0 | 0 |

Mice were followed for a period of 33 weeks after birth and monitored for signs of disease, including diarrhea, rectal prolapse, rectal bleeding, and scruffiness.

TL$^{+/+}$TCR$\alpha^{-/-}$ and TL$^{-/-}$TCR$\alpha^{-/-}$ animals exhibited similar cellularity and proliferative and cytokine responses (data not shown), indicating that the alterations in cellularity and T cell function imposed by TL deficiency are unique to the IEL and MLN compartments.

**TL Expression Is Not Required for Efficient CD8 T Cell Memory Differentiation.** In addition to its abundant expression on IEL, CD8$\alpha\alpha$ is transiently induced on primary effector CD8$^+$ T cells that gain the capacity to differentiate to memory T cells (28). It is possible that interaction of CD8$\alpha\alpha$ expressed by the activated CD8$\alpha\beta$ T cells with TL expressed by activated APCs is required for survival and/or differentiation of memory precursor cells. To investigate this possibility, we examined primary and memory CD8$^+$ T cell responses in TL-deficient mice in response to an infection with lymphocytic choriomeningitis virus (LCMV). TL$^{+/+}$ and TL$^{-/-}$ mice generated CD8$^+$IFN-$\gamma^+$ cells and virus-specific effector and memory cells in similar proportion (Fig. 4), indicating that interaction of CD8$\alpha\alpha$ with its ligand TL is not required for efficient effector and memory differentiation of LCMV-specific CD8 T cells. These findings are consistent with another study showing that mice expressing a single MHC class I molecule in the absence of TL expression generated normal numbers of memory CD8 T cells (29). Although these results indicate that the role of CD8$\alpha\alpha$ in memory differentiation does not depend on its interaction with TL, they do not rule out a role for CD8$\alpha\alpha$ in the differentiation and/or survival of CD8 memory precursor cells.

## Discussion

We report the initial characterization of mice with defective expression of TL, a molecule implicated as a modulator of the function of CD8$\alpha\alpha$-expressing T cells (13, 15). Our findings offer strong support for the hypothesis that TL modulates IEL proliferation, cytotoxicity, and cytokine secretion in the intestine. Also, we provide evidence that TL delays development of T cell mediated IBD, indicating that it is an important modulator of inflammatory T cell responses.

One unusual feature of IEL is that, regardless of the activation stimulus, these cells have a limited capacity to proliferate (20, 30, 31). Yamamoto *et al.* (21) reported that highly purified IEL preparations devoid of IEC responded more strongly to anti-CD3 stimulation than IEL cocultured in the presence of IEC. Reduced

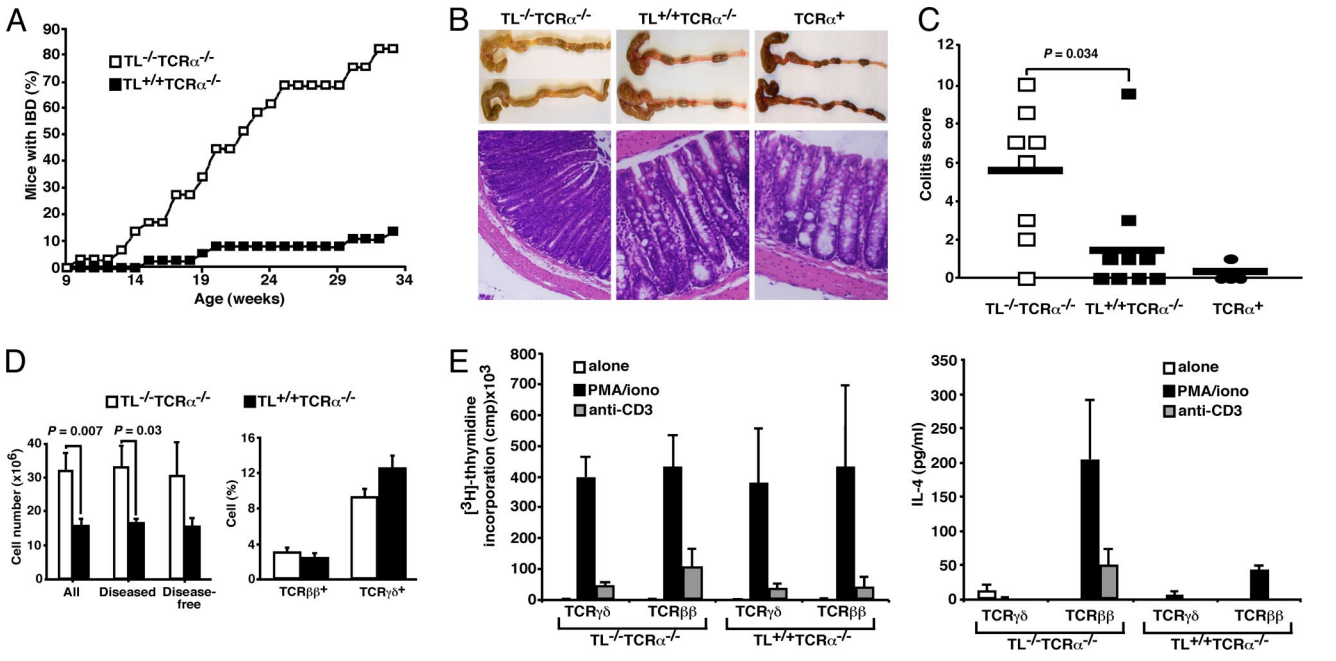


**Fig. 3.** TL deficiency results in higher IBD incidence in TCR$\alpha^{-/-}$ mice. (*A*) A cohort of 29 TL$^{-/-}$TCR$\alpha^{-/-}$, 36 TL$^{+/+}$TCR$\alpha^{-/-}$, and 6 TL$^{-/-}$TCR$\alpha^{+/+}$ mice were monitored for 33 weeks after birth for signs of IBD, including diarrhea, rectal prolapse, rectal bleeding, and scruffiness. Mice were considered to have IBD when one of these signs was present. (*B*) Representative colons from the different groups of mice analyzed. Colons were scored for macroscopic morphological alterations (*Upper*). H&E tissue sections from representative colons (*Lower*). (*C*) Macroscopic and microscopic combined score of 15- to 19-week old TL$^{-/-}$TCR$\alpha^{-/-}$ ($n = 8$), TL$^{+/+}$TCR$\alpha^{-/-}$ ($n = 9$), and TCR$\alpha^{+/+}$ ($n = 4$) mice. (*D*) Total cell numbers in MLNs of 15- to 30-week old TL$^{-/-}$TCR$\alpha^{-/-}$ ($n = 21$; 13 diseased) and TL$^{+/+}$TCR$\alpha^{-/-}$ ($n = 25$; 5 diseased) mice (*Left*); cell proportions in MLN (*Right*). (*E*) Pooled MLN from TL$^{-/-}$TCR$\alpha^{-/-}$ ($n = 4$; 10 weeks old) and TL$^{+/+}$TCR$\alpha^{-/-}$ ($n = 5$; 11 to 14 weeks old) mice were enriched for TCR$\gamma\delta$ or TCR$\beta\beta$ cells and incubated in the presence of media alone, PMA plus ionomycin, or 5 $\mu$g/mL of plate bound anti-CD3 antibody. Proliferation (*Left*) was measured by [$^3$H]thymidine incorporation and supernatants were analyzed for IL-4 production (*Right*) by ELISA. Results are representative of at least 3 independent experiments.

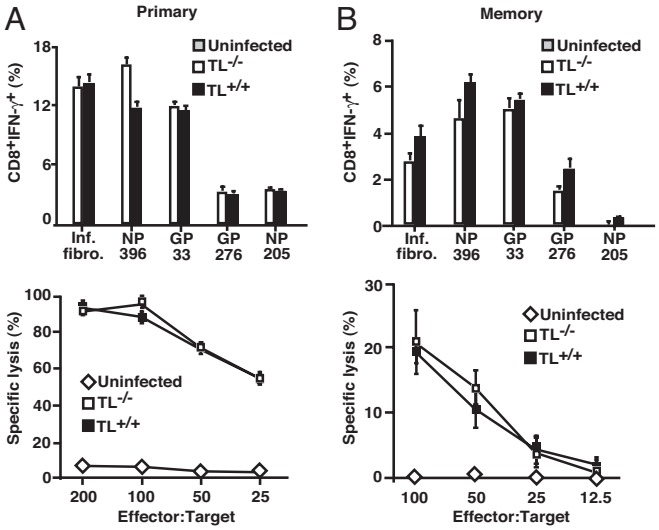


**Fig. 4.** TL-deficient mice generate effective memory responses against LCMV. Splenocytes from mice infected with LCMV for 8 days (*A*) or 5 days after rechallenge with virus (*B*) were tested for IFN-$\gamma$ secretion on stimulation with LCMV-infected fibroblasts or LCMV-derived peptides (*Upper*) and cytotoxicity against LCMV-infected C57SV fibroblasts (*Lower*). Data are representative of 3 independent experiments. Each experiment was performed with at least 5 mice per group.

IEL proliferation was restored when purified IEC membranes (but not soluble factors) were added to the culture. Interestingly, this effect could not be blocked by adding antibodies against TGF$\beta$, CD1d, E-cadherin, class I, or class II molecules (21). Our finding that IEL from TL$^{-/-}$ mice exhibited enhanced proliferative responses in the presence of TL$^{-/-}$ IEC together with the anti-TL blocking experiments (Fig. 2*B*), is consistent with a role of TL expression on IEC in suppressing IEL proliferation in vivo.

It is interesting to note that TL primarily diminished IEL proliferation at low antigenic stimulation (0.5–1.0 $\mu$g/mL plate-bound anti-CD3), indicating that TL may not be able to prevent proliferation on full activation of IEL. Thus, it is likely that TL serves to prevent aberrant proliferation of IEL in the antigen-rich environment of the intestine without interfering with protective immune responses. This scenario is supported by our finding that IEL from TL$^{-/-}$ mice have higher levels of in vivo homeostatic proliferation (based on BrdU incorporation) than IEL isolated from TL$^{+/+}$ mice (Fig. 2*A*). Surprisingly, we only observed this difference when colonic IEL were analyzed. One possibility is that the high turnover of IEL in the colon is due to the presence of a higher antigenic load in this organ compared with the small intestine.

We observed that the regulatory properties of TL are different for the diverse IEL populations. For example, TL control of a full activation through TCR stimulation (anti-CD3) was more evident in TCR$\beta^+$ cells, mainly CD8$\alpha^+\beta^+$ (which are known to up-regulate CD8$\alpha\alpha$ in the mucosa), whereas TL appeared to regulate homeostatic proliferation of colonic TCR$\beta^+$CD8$\alpha\alpha^+$ and TCR$\gamma\delta^+$CD8$\alpha\alpha^+$ IEL. Although the reasons for this differential regulation are unknown, we suggest that TL prevents a full activation of effector TCR$\beta^+$CD8$\alpha\beta^+$ IEL directed toward nonspecific or nonpathogenic epitopes in the antigen-rich environment of the intestine. In a similar fashion, TCR$\beta^+$CD8$\alpha\alpha^+$ in the colon, which are thought to have a regulatory role, are constantly exposed to high loads of both commensal and pathogenic bacteria. Therefore, in the colon, TL might regulate proliferation of these cells to ensure a proper balance between T cell responses that can be effective in clearing pathogenic bacteria and tolerant responses to commensal bacteria.

Although IEL from TL$^{-/-}$ mice showed greater proliferation capacity and enhanced cytotoxic activity, these animals did not develop signs of spontaneous bowel inflammation or IBD (Table 1 and data not shown), implying that regulatory factors other than TL (e.g., T$_{regs}$) prevent IEL from disrupting the epithelium and causing unwanted immune responses. However, the absence of TL had a significant impact on the development and severity of spontaneously induced colitis in the TCR$\alpha^{-/-}$ mouse model. The significantly increased severity of disease was also consistent with a higher production of pathogenic IL-4 by the mucosal T cells of TL$^{-/-}$TCR$\alpha^{-/-}$ mice. These findings suggest that TL has a role in regulating IL-4 cytokine production of pathogenic CD4$^+$TCR$\beta\beta$ T cells, which may account, at least in part, for the increased susceptibility of TL-deficient TCR$\alpha^{-/-}$ mice to T cell dependent inflammatory colitis. It is important to note that we did not detect expression of CD8$\alpha\alpha$ on CD4$^+$TCR$\beta\beta^+$ T cells from the MLN and IEL compartments (data not shown). Nevertheless, it is possible that CD4$^+$TCR$\beta\beta^+$ T cells transiently express CD8$\alpha\alpha$ on entering the intestinal epithelium. However, a fraction of TCR$\gamma\delta$ cells present in the IEL compartment expressed CD8$\alpha\alpha$ (data not shown), and their function may be affected by the absence of TL in TL$^{-/-}$TCR$\alpha^{-/-}$ mice. However, the specific function(s) of these cells in the TCR$\alpha^{-/-}$ colitis model is not completely understood. TCR$\gamma\delta$ T cells may be involved in tissue recovery and, if the absence of TL affects this role, a more severe disease might be observed.

Although TL deficiency profoundly affected spontaneous T cell dependent colitis in the TCR$\alpha^{-/-}$ model, it did not influence disease in chemically induced IBD models such as DSS or TNBS (data not shown). Each of these chemicals (or the vehicle used) disrupts the integrity of the mucosal epithelium, thus potentially altering the levels of TL present in IEC. Under these circumstances, the effect of TL absence on IECs may not be critical. Another established protocol for the induction of IBD is transferring pathogenic CD4$^+$ T cells into immunodeficient animals, such as RAG-deficient mice. We crossed TL$^{-/-}$ mice with RAG-2$^{-/-}$ animals to serve as recipients in adoptive transfer experiments. Interestingly, untreated TL$^{-/-}$RAG-2$^{-/-}$ mice develop a severe wasting disease as young adults. Because this disease interfered with the adoptive transfer experiments we could not establish the role of TL in this IBD model.

In summary, our findings indicate that TL has a critical role in the regulation of IEL function and serves as an important checkpoint in the process of inflammation and some forms of IBD development. These findings provide important insight into the mechanisms that regulate IEL function and should prove useful for developing prophylactic and/or therapeutic strategies against inflammatory conditions of the gastrointestinal tract.

## Materials and Methods

**Generation of TL-Deficient Mice.** To generate TL-deficient mice, the *T3$^b$* gene was isolated from a genomic C57BL/6 library by probing with a 188 bp fragment from exon 3. To disrupt *T3$^b$*, exon 3 was interrupted by introducing a neomycin resistance cassette (Fig. 1*A*). This construct was transfected into BL/6-III ES cells derived from C57BL/6 mice (32). ES cells carrying a targeted TL allele were microinjected into BALB/c blastocysts. The resulting chimeric male mice were bred with C57BL/6 females and offspring with a black coat color were tested for germ-line transmission. Heterozygous mutant animals were intercrossed to obtain homozygous mutants in a pure C57BL/6 background. To confirm the lack of expression of TL, tail DNA was amplified with the following set of primers: *T3* forward, 5′-TGGGCGAGAGAGACAGAGAT-3′; *T3* reverse, 5′-CCAACCAAACAAGCAAACAA-3′; and *T3*-Neo, 5′-CCAGAAAGCGAAGGAACAAA-3′. To analyze expression of TL in the bowel, RT-PCR was performed by using the following primers: forward, 5′-AGAGATCGTCACAACCAATGCA-3′ and reverse 5′-GTCACGTGTGTTTTTGGAGGAT-3′.

**Mice.** C57BL/6 (B6) and TCR$\alpha$ chain deficient mice (33) were purchased from The Jackson Laboratory.

**Isolation of Lymphocytes from Lymph Nodes, Spleen, and Intestinal Epithelium.** Lymphocytes from spleen and lymph nodes were isolated following conventional procedures. IEL were isolated following an established protocol (34). IEL purity was >70% with mostly IEC contaminants. Lymphocyte enrichment was performed by using anti-PE MACS beads (Miltenyi Biotec) as indicated by the manufacturer's instructions.

**Reagents and Flow Cytometry.** Anti-TL (HD-168) hybridoma was obtained from the American Type Culture Collection. Anti-TCR$\gamma\delta$, -TCR$\beta$, -CD3, -CD8$\alpha$, -CD8$\beta$, -CD4 (clone TH11–18H10), -IL-2, -IFN-$\gamma$, -g8.8, and -rat Ig monoclonal antibodies were purchased from BD Biosciences. Anti-BrdU antibody and isotype control were purchased from eBiosciences. TL-tetramer was generated as previously described (15). Single-cell suspensions of the lymphocytes from different compartments were stained with fluorescently labeled mAbs. To generate RMA-TL cells, the TL gene was cloned and expressed under the control of the CMV promoter (Clontech).

**Proliferation Assays.** Single cell suspensions were plated in flat-bottomed 96-well plates at 1 to $2 \times 10^5$ cells per well in RPMI supplemented with 10% FBS. Cells were stimulated with graded doses of plate-bound anti-CD3 antibody or PMA/Ionomycin (20 ng/mL and 1 $\mu$M, respectively); 48 h later, 1 $\mu$Ci ((1 Ci = 37 GBq)) of [$^3$H]thymidine (PerkinElmer, Life Sciences) was added to the wells; 12 h later, cells were harvested and uptake of radioactivity was measured. For measurement of cytokine secretion in vitro, supernatants were harvested after 60 h of culture, and cytokine levels were evaluated by ELISA. For TL blocking proliferation experiments, 50 $\mu$g/mL of anti-TL antibody (HD-168) were added to the cells and incubated for 45 min at 37 °C. Then, cells were transferred to an anti-CD3 coated plate and stimulated for 24 h.

**Redirected Cytotoxicity Assays.** We labeled $2 \times 10^6$ P815 cells with 300 $\mu$Ci of $^{51}$Cr (PerkinElmer) in complete RPMI for 1 h at 37 °C. Anti-CD3$\varepsilon$ (clone 145–2C11; BD Biosciences) antibody was then added at a final concentration of 2 $\mu$g/mL. Target cells were plated in round-bottomed plates at a concentration of $5 \times 10^3$ cells per well in 100 $\mu$L of medium. IEL were cocultured with antibody-bound P815 target cells in 100 $\mu$L. Percentage lysis was calculated by using the following formula: [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100.

**In Vivo BrdU Treatment.** Mice were injected i.p. with 100 $\mu$g of 5-bromo-2-deoxyuridine (Sigma–Aldrich) once every day for 2 days. On the third day, mice received 2 injections 4 h apart; 24 h later, IEL were isolated and stained for surface molecules. Cells were stained with anti-BrdU antibody following conventional methodology.

**IBD Analysis.** TL$^{-/-}$TCR$\alpha^{-/-}$, TL$^{+/+}$TCR$\alpha^{-/-}$, TL$^{-/-}$, and TCR$\alpha^+$ mice were monitored biweekly in a blinded fashion for signs of colitis including diarrhea, rectal prolapse, rectal bleeding, and scruffiness. We considered mice to have IBD when one of these signs was present. Macroscopic examination of the colon was performed as reported (27). For microscopic examination, pathological IBD lesions were scored as previously described (22). Macroscopic and microscopic scores were combined as a final IBD score. A standard serum ELISA was performed to determine Ig serum levels.

**Infection with LCMV.** TL$^{-/-}$ and TL$^{+/+}$ littermates were infected with $5 \times 10^4$ PFU of LCMV i.p. for 8 days and splenocytes were harvested for IFN-$\gamma$ and cytotoxicity assays. To measure the memory response, the mice were infected i.p. with $5 \times 10^4$ PFU of LCMV, rechallenged 50 days later with the same dose of virus and killed 5 days after secondary challenge. Cytotoxic responses against the virus were measured by using C57SV (*H-2$^b$*) fibroblasts infected for 3 days at 0.1 MOI.

**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.

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1. Cheroutre H (2004) Starting at the beginning: New perspectives on the biology of mucosal T cells. *Annu Rev Immunol* 22:217–246.
2. Groh V, Steinle A, Bauer S, Spies T (1998) Recognition of stress-induced MHC molecules by intestinal epithelial $\gamma\delta$ T cells. *Science* 279:1737–1740.
3. Boismenu R, Havran WL (1994) Modulation of epithelial cell growth by intraepithelial $\gamma\delta$ T cells. *Science* 266:1253–1255.
4. Chen Y, Chou K, Fuchs E, Havran WL, Boismenu R (2002) Protection of the intestinal mucosa by intraepithelial $\gamma\delta$ T cells. *Proc Natl Acad Sci USA* 99:14338–14343.
5. Leishman AJ, *et al.* (2002) Precursors of functional MHC class I- or class II-restricted CD8$\alpha\alpha$+ T cells are positively selected in the thymus by agonist self-peptides. *Immunity* 16:355–364.
6. Yamagata T, Mathis D, Benoist C (2004) Self-reactivity in thymic double-positive cells commits cells to a CD8$\alpha\alpha$ lineage with characteristics of innate immune cells. *Nat Immunol* 5:597–605.
7. Montufar-Solis D, Garza T, Klein JR (2007) T-cell activation in the intestinal mucosa. *Immunol Rev* 215:189–201.
8. Boyse EA, Old LJ, Luell S (1964) Genetic Determination of the Tl (Thymus leukaemia) Antigen in the Mouse. *Nature* 201:779.
9. Wu M, Van Kaer L, Itohara S, Tonegawa S (1991) Highly restricted expression of the thymus leukemia antigens on intestinal epithelial cells. *J Exp Med* 174:213–218.
10. Hershberg R, *et al.* (1990) Expression of the thymus leukemia antigen in mouse intestinal epithelium. *Proc Natl Acad Sci USA* 87:9727–9731.
11. Liu Y, *et al.* (2003) The crystal structure of a TL/CD8$\alpha\alpha$ complex at 2.1 A resolution: Implications for modulation of T cell activation and memory. *Immunity* 18:205–215.
12. Weber DA, *et al.* (2002) Peptide-independent folding and CD8$\alpha\alpha$ binding by the nonclassical class I molecule, thymic leukemia antigen. *J Immunol* 169:5708–5714.
13. Attinger A, *et al.* (2005) Molecular basis for the high affinity interaction between the thymic leukemia antigen and the CD8$\alpha\alpha$ molecule. *J Immunol* 174:3501–3507.
14. Devine L, Rogozinski L, Naidenko OV, Cheroutre H, Kavathas PB (2002) The complementarity-determining region-like loops of CD8$\alpha$ interact differently with $\beta$2-microglobulin of the class I molecules H-2K$^b$ and thymic leukemia antigen, while similarly with their $\alpha$3 domains. *J Immunol* 168:3881–3886.
15. Leishman AJ, *et al.* (2001) T cell responses modulated through interaction between CD8$\alpha\alpha$ and the nonclassical MHC class I molecule, TL. *Science* 294:1936–1939.
16. Teitell M, Mescher MF, Olson CA, Littman DR, Kronenberg M (1991) The thymus leukemia antigen binds human and mouse CD8. *J Exp Med* 174:1131–1138.
17. Tsujimura K, *et al.* (2003) Thymus leukemia antigen (TL)-specific cytotoxic T lymphocytes recognize the $\alpha$1/$\alpha$2 domain of TL free from antigenic peptides. *Int Immunol* 15:1319–1326.
18. Weiss EH, *et al.* (1984) Organization and evolution of the class I gene family in the major histocompatibility complex of the C57BL/10 mouse. *Nature* 310:650–655.
19. Mosley RL, Whetsell M, Klein JR (1991) Proliferative properties of murine intestinal intraepithelial lymphocytes (IEL): IEL expressing TCR$\alpha\beta$ or TCR$\gamma\delta$ are largely unresponsive to proliferative signals mediated via conventional stimulation of the CD3-TCR complex. *Int Immunol* 3:563–569.
20. Sydora BC, *et al.* (1993) Intestinal intraepithelial lymphocytes are activated and cytolytic but do not proliferate as well as other T cells in response to mitogenic signals. *J Immunol* 150:2179–2191.
21. Yamamoto M, Fujihashi K, Kawabata K, McGhee JR, Kiyono H (1998) A mucosal intranet: Intestinal epithelial cells down-regulate intraepithelial, but not peripheral, T lymphocytes. *J Immunol* 160:2188–2196.
22. Mombaerts P, *et al.* (1993) Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice. *Cell* 75:274–282.
23. Mizoguchi A, Mizoguchi E, Bhan AK (1999) The critical role of interleukin 4 but not interferon $\gamma$ in the pathogenesis of colitis in T-cell receptor $\alpha$ mutant mice. *Gastroenterology* 116:320–326.
24. Mizoguchi A, *et al.* (1996) Cytokine imbalance and autoantibody production in T cell receptor-$\alpha$ mutant mice with inflammatory bowel disease. *J Exp Med* 183:847–856.
25. Takahashi I, Kiyono H, Hamada S (1997) CD4$^+$ T-cell population mediates development of inflammatory bowel disease in T-cell receptor $\alpha$ chain-deficient mice. *Gastroenterology* 112:1876–1886.
26. Iijima H, *et al.* (1999) Alteration of interleukin 4 production results in the inhibition of T helper type 2 cell-dominated inflammatory bowel disease in T cell receptor $\alpha$ chain-deficient mice. *J Exp Med* 190:607–615.
27. Mizoguchi A, Mizoguchi E, Smith RN, Preffer FI, Bhan AK (1997) Suppressive role of B cells in chronic colitis of T cell receptor $\alpha$ mutant mice. *J Exp Med* 186:1749–1756.
28. Madakamutil LT, *et al.* (2004) CD8$\alpha\alpha$-mediated survival and differentiation of CD8 memory T cell precursors. *Science* 304:590–593.
29. Williams MA, Bevan MJ (2005) Cutting edge: A single MHC class Ia is sufficient for CD8 memory T cell differentiation. *J Immunol* 175:2066–2069.
30. Dillon SB, MacDonald TT (1986) Functional characterization of Con A-responsive Lyt2-positive mouse small intestinal intraepithelial lymphocytes. *Immunology* 59:389–396.
31. Mowat AM, MacKenzie S, Baca ME, Felstein MV, Parrott DM (1986) Functional characteristics of intraepithelial lymphocytes from mouse small intestine. II. In vivo and in vitro responses of intraepithelial lymphocytes to mitogenic and allogeneic stimuli. *Immunology* 58:627–634.
32. Ledermann B, Burki K (1991) Establishment of a germ-line competent C57BL/6 embryonic stem cell line. *Exp Cell Res* 197:254–258.
33. Mombaerts P, *et al.* (1992) Mutations in T-cell antigen receptor genes $\alpha$ and $\beta$ block thymocyte development at different stages. *Nature* 360:225–231.
34. Oida T, *et al.* (2000) Role of gut cryptopatches in early extrathymic maturation of intestinal intraepithelial T cells. *J Immunol* 164:3616–3626.