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Virus-Induced Activation of Self-Specific TCR$\alpha\beta$ CD8$\alpha\alpha$ Intraepithelial Lymphocytes Does Not Abolish Their Self-Tolerance in the Intestine[1]

Leslie Saurer,[2]* Inge Seibold,* Silvia Rihs,* Claudio Vallan,* Tilman Dumrese,† and Christoph Mueller[3]*

TCR$\alpha\beta$ CD8$\alpha\alpha$ intestinal intraepithelial lymphocytes (IEL) represent an enigmatic subset of T cells, particularly, in regard to their potential functions and the apparent persistence of cells expressing self-specific TCR. We have used mice that are transgenic for the TCR$\alpha\beta$ specific for the lymphocytic choriomeningitis virus (LCMV)-derived peptide gp33, and TCR$\alpha\beta$-transgenic mice that coexpress the gp33 Ag ubiquitously, to analyze the functional properties of TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL in the presence, or absence, of their specific MHC-restricted Ag, and to assess the impact of molecular mimicry during a potent LCMV infection on potentially self-reactive TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL. In this study, we show that the presence of the specific self-Ag results in reduced expression of IL-2, IFN-γ, and IL-10 by resident TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL while expression of mRNA for TGFβ is not affected. We further demonstrate that despite their secluded location in the epithelium, TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL are activated after infection of the intestinal mucosa with LCMV. Importantly, LCMV-induced activation of self-specific TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL does not reverse their tolerance as no cytotoxic activity or up-regulated expression of proinflammatory cytokines is detected and no overt signs of autoimmunity are seen. Taken together, these results are in support of an immunoregulatory role for self-specific TCR$\alpha\beta$ CD8$\alpha\alpha$ in the intestinal mucosa and clearly speak against an involvement of this cell subset in inflammatory reactions and tissue destruction. ***The Journal of Immunology*, 2004, 172: 4176–4183.**

In the intestinal mucosa, the immune system faces a considerable challenge in its efforts to maintain local tissue homeostasis: It is confronted with a large array of Ags and has to mount protective immune responses against invading pathogens while preventing pathogenic responses to dietary Ags and commensal enteric bacteria. This multifarious task is mirrored by a diverse and specialized population of T cells located at the interphase of the intestinal mucosa and the bowel lumen: intraepithelial lymphocytes (IEL)[4] of the small murine intestine are comprised of three main subsets of $CD8^+$ T cells: Approximately one-half of the IEL express a $\gamma\delta$ TCR and a CD8$\alpha\alpha$ coreceptor; the rest, which expresses an $\alpha\beta$ TCR, can be subdivided into equal parts according to the expression of the heterodimeric (CD8$\alpha\beta$) or homodimeric (CD8$\alpha\alpha$) form of the coreceptor (1). TCR$\alpha\beta$ CD8$\alpha\beta$ IEL are subject to thymic selection (2) and can exert Ag-specific cytotoxicity against various pathogens (3–6). Thus, they closely resemble their $CD8^+$ counterparts in spleen and lymph nodes and may be derived from peripheral T cells that have entered the IEL compartment upon activation (7, 8). In contrast, TCR$\alpha\beta$ CD8$\alpha\alpha$ cells are restricted to the intestinal mucosa and bear features that are clearly distinct from peripheral T cells. Although it is now hypothesized that the thymus is involved in the selection process (9–11), at least some of the CD8$\alpha\alpha$ IEL may develop extrathymically (reviewed in Refs. 12 and 13), and although all TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL require β_2-microglobulin expression, they are not exclusively restricted by the classical MHC class I molecules K and D (14, 15). Moreover, antigenic signals received by CD8$\alpha\alpha$ IEL are likely to be different from those received by peripheral T cells, since TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL use different signaling molecules (16), and the CD8$\alpha\alpha$ coreceptor can modulate TCR transmitted signals by its semiautonomous interaction with the thymus leukemia (TL) Ag (17). Because of the unknown Ag specificity of CD8$\alpha\alpha$ IEL in wild-type mice, TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL were mainly studied in TCR-transgenic (tg) models. These models collectively presented the intriguing finding that TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL are not subject to negative selection as these cells persisted in TCR tg mice that coexpressed the specific MHC class I-restricted Ag (18–22). In normal mice, CD8$\alpha\alpha$ T cells are also enriched for cells expressing Vβ segments reactive for endogenous superantigens (23, 24). Furthermore, it was recently claimed that the selection of CD8$\alpha\alpha$ T cells appears to involve a true positive selection process as opposed to an escape of negative selection (10). Thus, it is tempting to speculate that TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL, which are specific for self-Ags, may fulfill particular functions in the intestinal mucosa. In contrast, these cells have a potential for autoreactivity and may cause chronic inflammation. With regard to the functional responses of self-specific TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL, however, so far rather discrepant results have been obtained. Although some studies suggested that these cells are functionally active (10, 18, 21), others observed a hyporesponsiveness to antigenic stimulation (19, 20, 24). To directly investigate the potential functions exerted by TCR$\alpha\beta$

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[4] Abbreviations used in this paper: IEL, intraepithelial lymphocyte; LCMV, lymphocytic choriomeningitis virus; TL, thymus leukemia; tg, transgenic.

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CD8$\alpha\alpha$ IEL in situ, we have used mice tg for the TCR$\alpha\beta$ specific for the lymphocytic choriomeningitis virus (LCMV) (3)-derived immunodominant epitope gp33 and TCR$\alpha\beta$ tg mice coexpressing the gp33 Ag. The gp33 transgene is under the control of a MHC class I promoter, resulting in a strong expression of gp33 also on intestinal epithelial cells. This model not only allowed us to reassess the effect of an ubiquitously expressed MHC class I-restricted Ag on selection and function of the TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL subset, but also to look at the impact of a potent LCMV infection on potentially self-reactive TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL.

Materials and Methods

Mice

C57BL/6 mice tg for the LCMV gp33-specific TCR$\alpha\beta$ (line 318) (25) and C57BL/6 mice tg for the LCMV gp33 (line H8) (26) were provided by H. Hengartner and R. M. Zinkernagel (University Hospital Zurich, Zurich, Switzerland). The tg mice were backcrossed to RAG2$^{-/-}$ mice, originally obtained from E. Wagner (Basel Institute for Immunology, Basel, Switzerland). 318 $\times$ RAG2$^{-/-}$ and H8 $\times$ RAG2$^{-/-}$ mice were then intercrossed to obtain double tg 318 $\times$ H8 $\times$ RAG2$^{-/-}$ mice. All mice were kept under specific pathogen-free conditions in the central animal facility of the Medical School, University of Bern (Bern, Switzerland). Experiments were performed in compliance with the regulations approved by the local committee on animal experimentation.

LCMV infections

A stock of LCMV strain WE was originally provided by S. Oehen (Zurich, Switzerland). Briefly, 2×10^4 PFU of LCMV-WE per mouse in a volume of 100 μl of MEM and 2% FCS were injected i.p.

Preparation of IEL

IEL were isolated from the small intestine as described previously (27). In brief, the entire small intestine was placed in Ca^{2+}- and Mg^{2+}-free HBSS, 10 mM HEPES, and 2% horse serum (HBSS-2). After longitudinally opening the gut, the tissue was cut into pieces of 1–2 cm each. Enterocytes and IEL were detached from the basement membrane by incubating pieces in HBSS-2 containing 2 mM DTT and 0.5 mM EDTA at 37°C with stirring. Isolated cells were sequentially passed through 70- and 40-μm pore size nylon mesh strainers (BD Biosciences, San Jose, CA). The IEL were then purified from enterocytes by 44/68% discontinuous Percoll (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation (15 min, 1000 $\times$ *g*, room temperature).

mAbs and gp33 tetramers

Fluorescence- or biotin-conjugated mAbs used in this study were either purchased from BD PharMingen (San Diego, CA; CD8α (53-6.7), CD8β (53-5.8), CD69 (H1.2F3), Vα2 (B20.1), Vβ8 (MR5-2)) or purified by protein G columns from supernatants of hybridomas originally obtained from American Type Culture Collection (Manassas, VA) and subsequently labeled according to standard protocols (CD3 (145-2C11); TCR$\gamma\delta$ (GL3)). Soluble H-2D^b tetramers complexed with biotinylated β_2-microglobulin and gp33 peptide were prepared as described elsewhere (28, 29) and were kindly provided by R. M. Zinkernagel (Zurich, Switzerland).

FACS analysis

Cells were taken up in PBS-buffered 4% paraformaldehyde and acquired on a FACScan (BD Biosciences) by gating on the lymphocyte population in the forward and side angle scatter. Stainings were analyzed using CellQuest software (BD Biosciences).

Cell sorting

For cell sorting, cells were stained with mAb against Vα2, CD8α, and CD8β and subsequently separated on a FACSVantage (BD Biosciences) into V$\alpha2^+$CD8α^+CD8β^- and V$\alpha2^+$CD8α^+CD8β^+ fractions (318 $\times$ RAG2$^{-/-}$ mice) and V$\alpha2^+$CD8α^+CD8β^- and V$\alpha2^+$CD8$^-$ fractions, respectively (318 $\times$ H8 $\times$ RAG2$^{-/-}$ mice). When cells were used for in vitro assays, staining for Vα2 was omitted and cells were sorted according to the expression of CD8α and CD8β as described above.

Proliferation assay

Briefly, 5×10^4 sorted cells were stimulated in triplicates in 96-well round-bottom microtiter plates (Costar, Cambridge, MA) with plate-bound anti-CD3 (10 μg/ml) or with 3×10^5 irradiated H8 $\times$ RAG2$^{-/-}$ spleen cells in a total volume of 200 μl of IMDM supplemented with 10% FCS. In some experiments, 50 U/ml IL-2 (Proleukin; Roche, Basel, Switzerland) was added. Cells were cultured for 48 h, pulsed with 1 μCi/well [^{3}H]thymidine, and harvested 18 h later.

Analysis of cytokine expression

Briefly, 1×10^5 sorted cells were stimulated with plate-bound anti-CD3 (10 μg/ml) in 200 μl of IMDM/10% FCS. For the IL-2 ELISA, supernatants were harvested from triplicate wells after 48 h, and IL-2 was detected using the anti-IL-2 mAb JES6-1A12 as a coating Ab and the mAb JES6-5H4 as a detecting Ab (BD PharMingen). The standard curve was constructed using recombinant mouse IL-2 (PeproTech, London, U.K.). For analysis of IL-2 mRNA expression, cells were harvested after 6 h and resuspended in 1 ml of Tri-Reagent (Molecular Research Center, Cincinnati, OH). For analysis of ex vivo mRNA expression, cells (1–2 $\times$ 10^5) were immediately taken up in 1 ml of Tri-Reagent after cell sorting.

RNA isolation and cDNA synthesis

RNA was isolated by phenol-chloroform extraction and Tri-Reagent according to the manufacturer's (Molecular Research Center) directions. RNA was DNase digested and reverse transcribed with random primers using a commercial cDNA kit (Promega, Madison, WI) and following the manufacturer's suggested conditions.

Real-time PCR

Serial dilutions of cDNA were made to determine the linear range for amplification. Real-time PCR was performed with appropriate dilutions of cDNA using the Applied Biosystems PRISM 7700 sequence detector and either Assays-on-Demand (IFN-γ, TNF-α, IL-10, and TGFβ) or the dsDNA binding dye SYBR Green (IL-2 and IL-4; Applied Biosystems, Foster City, CA). PCR amplifications were performed in a total volume of 25 μl according to the manufacturer's instructions (Assays-on-Demand) or using SYBR Green PCR Master Mix and 300 nM of the appropriate primers (IL-2: 5′- TGGAGCAGCTGTTGATGGACCTAC-3′, 5′-AGATGATGCTTTGACAGAAGGCTATC-3′; and IL-4: 5′-AAACGTCCTCACAGCAACGA-3′, 5′-GGCTTTCCAGGAAGTCTTTCAG-3′).

The real-time PCR was evaluated using the Sequence Detection System software version 1.7 (Applied Biosystems). Expression of specific cytokine genes was normalized based on levels of mRNA for 18S rRNA.

Cytotoxicity assay

The cytotoxicity assay was performed as described previously (30). Briefly, sorted effector cells were placed in a V-bottom microtiter plate (Costar) and serially diluted. A total of 3×10^4 RMA target cells, labeled with 75 μCi Na^{51}Cr and pulsed with 1 μg/ml gp33 or adn5, was added per well of the assay plate. After incubating the plate for 5 h at 37°C in 5% CO_2, 40 μl of supernatant from each well was harvested and measured for released ^{51}Cr on a Top Count liquid scintillation counter (Canberra Packard, Meriden, CT). Specific lysis was calculated as follows: (experimental counts − spontaneous counts)/(maximum counts − spontaneous counts) $\times$ 100

Results

Selection of TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL in TCR tg mice expressing the specific Ag

Previous studies of H-Y and 2C TCR tg mouse models (18–21) have demonstrated that in contrast to the CD8$\alpha\beta$ IEL subset, TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL do not appear to be negatively selected in the presence of their specific autoantigen. As shown in Fig. 1, this also applies to mice tg for the LCMV-derived immunodominant peptide gp33 and tg for the TCR$\alpha\beta$ specific for the gp33. In this model, TCR tg cells cannot only be identified by the mAbs Vα2 and Vβ8 that recognize the tg TCR, but also by gp33-loaded MHC class I tetramers that bind to gp33-specific T cells. No gp33 tetramer-positive cells could be detected in the TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL populations of C57BL/6 wild-type mice and of mice tg for the gp33 (H8), respectively (Fig. 1). However, in mice expressing the tg TCR$\alpha\beta$ (318), 3% of the TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL stained positive for the gp33 tetramers and, in 318 $\times$ H8 mice that express both the gp33 Ag and the specific TCR$\alpha\beta$, even 10% of the TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL were gp33 tetramer positive (Fig. 1). Thus, in the

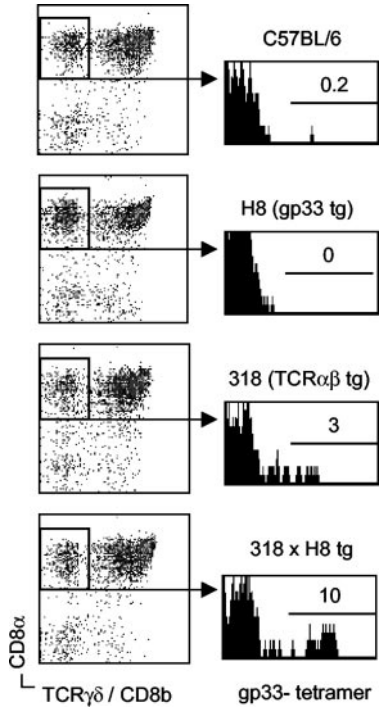

FIGURE 1. LCMV gp33-specific TCRαβ CD8αα IEL are enriched in TCRαβ (318) and LCMV gp33 (H8) double tg mice. IEL isolated from C57BL/6 wild-type mice, 318 tg mice, H8 tg mice, and 318 × H8 tg mice were stained with gp33 tetramers and mAbs against TCRγδ, CD8β, and CD8α, and analyzed by FACS. TCRαβ CD8αα IEL were gated by excluding TCRαβ CD8αβ and TCRγδ CD8αα IEL, and the percentage of gp33 tetramer-positive cells was assessed in the TCRαβ CD8αα IEL population. Experiment was performed twice with comparable results.

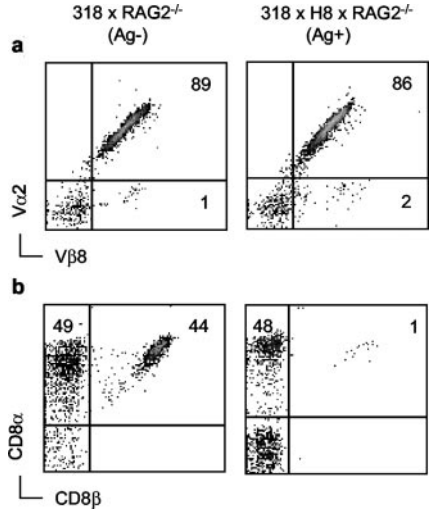

FIGURE 2. Expression of the specific Ag in TCRαβ tg × RAG2$^{-/-}$ mice results in deletion of CD8αβ, but not of CD8αα IEL. *a*, IEL were isolated from 318 × RAG2$^{-/-}$ (Ag$^-$) and 318 × H8 × RAG2$^{-/-}$ (Ag$^+$) mice and stained with the mAbs Vα2 and Vβ8 for expression of the tg TCR. *b*, CD8 coreceptor expression was assessed by staining IEL with the CD8α, CD8β, and Vα2 mAbs and gating on Vα2$^+$ IEL. Numbers indicate the percentage of cells positive for the tg TCR in the lymphocyte population (*a*) and of cells positive for the CD8αβ and CD8αα coreceptor in the TCR tg IEL population, respectively (*b*). Representative data from >10 experiments are shown.

presence of their cognate Ag, TCRαβ CD8αα IEL with self-specific TCR are indeed not deleted, but persistent and may even be enriched.

Generation of a monospecific TCRαβ CD8αα IEL population

For a better characterization of these potentially self-reactive TCRαβ CD8αα IEL, 318 × H8 mice were crossed onto a RAG2$^{-/-}$ background to thus obtain a monospecific TCRαβ CD8αα IEL population and increased total TCRαβ CD8αα cell numbers in the absence of TCRγδ and TCRαβ CD8αβ T cells. Analysis of 318 × H8 × RAG2$^{-/-}$ mice showed that all IEL now only expressed the tg TCRαβ, as determined by a staining with the mAbs Vα2 and Vβ8 (Fig. 2*a*). The relative frequency of TCR tg IEL in the IEL compartment of 318 × H8 × RAG2$^{-/-}$ (Ag$^+$) mice was the same as in 318 × RAG2$^{-/-}$ mice (Ag$^-$) that did not express the specific Ag (Fig. 2*a*), and there was no difference in actual numbers of IEL recovered from Ag$^+$ and Ag$^-$ mice, respectively (data not shown). However, whereas Ag$^-$ mice had equal numbers of TCRαβ CD8αα and TCRαβ CD8αβ IEL, the latter population was almost completely absent from the Ag$^+$ mice (Fig. 2*b*). In addition to the TCRαβ CD8αα IEL, which remained present in the 318 × H8 × RAG2$^{-/-}$ mice, a major population of TCRαβ CD8$^-$ IEL was present among the TCR tg cells in the IEL compartment of these mice (Fig. 2*b*). The appearance of CD8$^-$ IEL has also been reported in other TCR tg mouse models in which the specific Ag was coexpressed (20, 21, 31). Although this may suggest that these cells arise from TCRαβ CD8αβ or CD8αα IEL that have down-regulated the CD8 coreceptor in response to constant antigenic stimulation, we found no evidence for intermediate forms of CD8 expression or down-modulation of the coreceptor upon in vitro stimulation of TCRαβ CD8αα IEL (data not shown).

Response of TCRαβ CD8αα IEL from Ag$^+$ and Ag$^-$ mice to stimulation in vitro

In 318 × H8 × RAG2$^{-/-}$ (Ag$^+$) mice, the tg expression of the gp33 Ag is under the control of a MHC class I promoter. Hence, gp33 is expressed ubiquitously in the gut by a variety of cell types, including intestinal epithelial cells, and is thus likely to directly influence the functional behavior of resident TCRαβ CD8αα IEL. As shown in Fig. 3*a*, sorted TCRαβ CD8αα IEL from Ag$^+$ mice indeed failed to proliferate in response to plate-bound anti-CD3 and Ag-bearing irradiated stimulator cells, as compared with TCRαβ CD8αα IEL from Ag$^-$ (318 × RAG2$^{-/-}$) mice. To determine whether the absence of a proliferative response could be attributed to an impaired IL-2 production, IL-2 mRNA expression was assessed in anti-CD3-stimulated TCRαβ CD8αα IEL from Ag$^+$ and Ag$^-$ mice, respectively, by means of real-time RT-PCR. Short-term stimulation (6 h) of TCRαβ CD8αα IEL from Ag$^-$ mice resulted in a considerable induction of IL-2 mRNA expression, whereas TCRαβ CD8αα IEL from Ag$^+$ mice expressed only basal levels of mRNA for IL-2, even after stimulation with plate-bound anti-CD3 (Fig. 3*b*). Very similar results were obtained on a protein level with almost no IL-2 detectable in supernatants of stimulated TCRαβ CD8αα IEL from Ag$^+$ mice while IL-2 was readily detected in the supernatants of TCRαβ CD8αα IEL from Ag$^-$ mice (Fig. 3*c*). Addition of exogenous IL-2 to cultures, however, only partially rescued proliferation of TCRαβ CD8αα IEL from Ag$^+$ mice (data not shown).

Cytokine gene expression profile of ex vivo isolated TCRαβ CD8αα IEL from Ag$^+$ and Ag$^-$ mice

To further assess the impact of the specific self-Ag on the functional behavior of TCRαβ CD8αα IEL, we compared the expression of additional proinflammatory and immunomodulatory cytokine genes in TCRαβ CD8αα IEL upon isolation from Ag$^+$ and

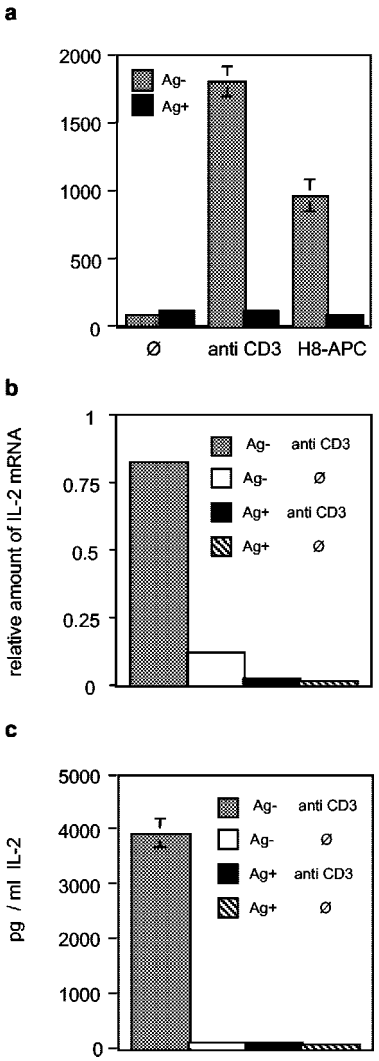

FIGURE 3. TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL from Ag^+ mice exhibit a defective proliferative response and a failure in activation-induced IL-2 expression. *a,* Sorted TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL (5×10^4 cells/well) isolated from 318 $\times$ RAG2$^{-/-}$ (Ag^-) and 318 $\times$ H8 $\times$ RAG2$^{-/-}$ (Ag^+) mice, respectively, were stimulated with plate-bound anti-CD3 (10 μg/ml) or with irradiated spleen cells obtained from H8 $\times$ RAG2$^{-/-}$ mice (3×10^5 cells/well). Cells were cultured for 48 h, pulsed with [^{3}H]thymidine, and harvested 18 h later. *b* and *c,* 10^5 sorted TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL were stimulated with plate-bound anti-CD3 (10 μg/ml). *b,* After 6 h, cells were harvested for extraction of RNA and expression of IL-2 mRNA was assessed by real-time RT-PCR. *c,* Supernatants from triplicate wells were harvested after 48 h and analyzed for the presence of IL-2 by ELISA. One of two similar experiments is shown using pooled cells from three mice for each experiment. Error bars indicate the SEM of triplicate cultures.

Ag^- mice, respectively. In the absence of their specific Ag (318 $\times$ RAG2$^{-/-}$ mice), TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL constitutively expressed comparable amounts of mRNA for IFN-γ, IL-10, and TGFβ (Fig. 4). The presence of the specific self-Ag in the 318 $\times$ H8 $\times$ RAG2$^{-/-}$ mice substantially reduced the expression of mRNA for IFN-γ and IL-10 by TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL, whereas expression of mRNA for TGFβ remained unaffected and even was slightly increased (Fig. 4). In both Ag^+ and Ag^- mice, little or no expression of mRNA for TNF-α or IL-4, respectively, could be detected. Thus, tolerance induction in self-specific TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL not only affected proliferation and IL-2 production but also involved reduced expression of certain immunomodulatory cytokine genes.

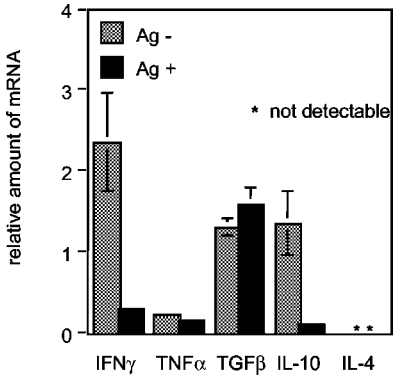

FIGURE 4. Distinct ex vivo cytokine gene expression profiles for TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL from Ag^+ and Ag^- mice. RNA was extracted from sorted TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL isolated ex vivo from five 318 $\times$ RAG2$^{-/-}$ and six 318 $\times$ H8 $\times$ RAG2$^{-/-}$ mice, respectively, and analyzed for mRNA for IFN-γ, TNF-α, TGFβ, IL-10, and IL-4 by real-time RT-PCR. Error bars indicate the SEM.

Effects of a systemic LCMV infection on TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL in Ag^+ mice

It has repeatedly been shown that viral infections can interfere with or even break tolerance of T cells to self-Ags (26, 32–34). LCMV not only produces the target self-Ag of our tg model, but when administered systemically also infects $CD11c^+$ dendritic cells in the intestinal mucosa (6). Hence, systemic infection of 318 $\times$ H8 $\times$ RAG2$^{-/-}$ (Ag^+) mice with LCMV seemed an ideal model system to determine whether virus-induced activation of self-specific TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL leads to a breakdown in tolerance and results in the generation of autoaggressive effector T cells and subsequent intestinal pathologies.

FACS analysis of IEL isolated from Ag^+ mice on different time points after LCMV infection (days 4, 6, and 8) indicated that despite their secluded location TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL indeed responded to the ongoing virus infection. Thus, although no increased expression of CD25 was noted, there was a very distinct up-regulation of the early activation marker CD69 on day 6 after infection, concomitant with an apparent infection of the intestinal mucosa at this time point (Fig. 5*a*). LCMV-induced activation of self-specific TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL, however, did not provoke increased expression of mRNA for the proinflammatory cytokines IFN-γ or TNF-α (Fig. 6) while mean expression levels of TGFβ mRNA increased ~2-fold following LCMV infection. No cytotoxic activity could be detected in TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL on day 8 after infection, i.e., at the time when maximum cytotoxic activity is observed for the CD8$\alpha\beta$ IEL subset, isolated as a positive control from IEL-reconstituted and LCMV-infected RAG2$^{-/-}$ mice (Fig. 7). Most importantly, analysis of H&E-stained tissue sections revealed no signs of obvious histopathological alterations in the intestinal mucosa such as epithelial erosions or increased proliferative activity of enterocytes (data not shown), and all of the LCMV-infected 318 $\times$ H8 $\times$ RAG2$^{-/-}$ mice remained healthy. These results indicate that tolerance of self-specific TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL is not readily abolished and that in consequence these cells are unlikely to initiate and mediate inflammatory reactions in the intestine.

Discussion

Clonal deletion in the thymus represents a major mechanism for the elimination of self-reactive T cells and the establishment of tolerance to self-peptides (reviewed in Refs. 35 and 36). Intriguingly, however, this process does not seem to extend to a subset of

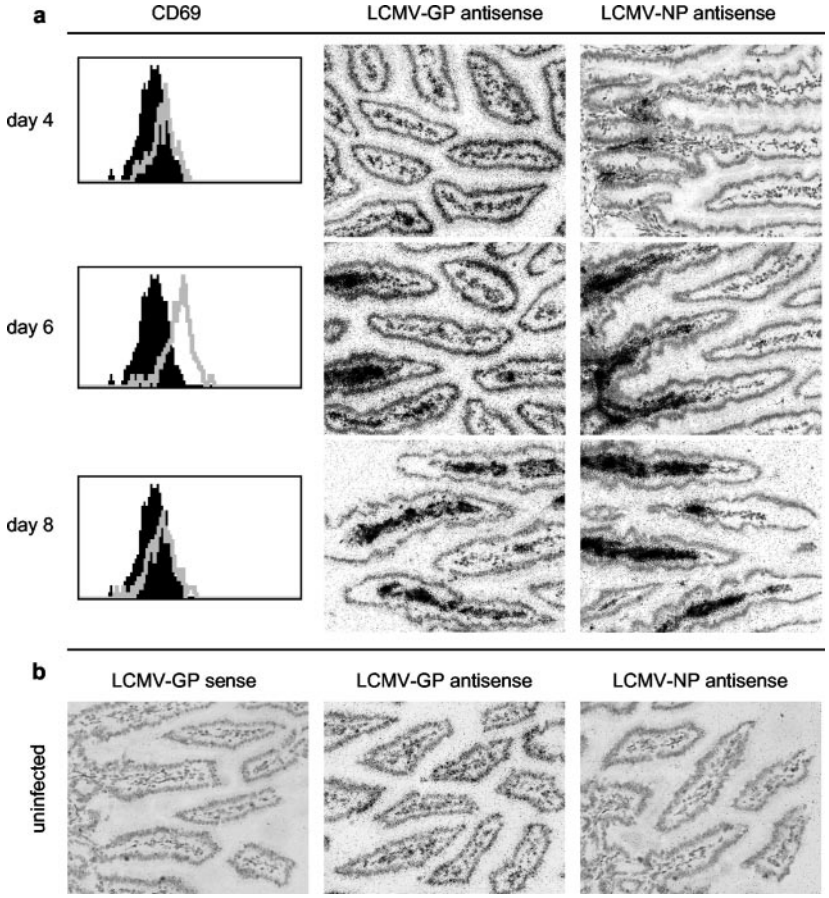

FIGURE 5. Infection of the intestinal mucosa with LCMV leads to activation of TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL. Expression of the early activation marker CD69 was analyzed on CD8α^+CD8β^- IEL isolated from uninfected (filled histogram) and LCMV-infected (lines) 318 × H8 × RAG2$^{-/-}$ mice at the indicated times after LCMV infection. In situ hybridization using LCMV-NP and LCMV-GP antisense ^{35}S-labeled RNA probes allowed for the localization of LCMV-infected cells and of LCMV-infected cells along with cells expressing the gp33 transgene, respectively, on tissue sections from LCMV-infected (*a*) and uninfected 318 × H8 × RAG2$^{-/-}$ mice (*b*). Magnification, ×80. *b,* As a negative control, a tissue section from a 318 × H8 × RAG2$^{-/-}$ mouse was also hybridized with a LCMV-GP sense ^{35}S-labeled RNA probe. For each postinfection interval, three mice were analyzed with comparable results.

intestinal IEL as several studies of TCR tg models have reported the persistence of TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL expressing self-specific TCR (18–22). This finding also holds true for the TCR$\alpha\beta$ 318 tg RAG2$^{-/-}$ mice which, in the presence of the ubiquitously expressed LCMV-gp33 transgene, almost completely delete the TCR$\alpha\beta$ CD8$\alpha\beta$ T cells while TCR$\alpha\beta$ CD8$\alpha\alpha$ T cells remain present in the IEL compartment.

Induction of functional unresponsiveness or anergy has been described as a mechanism to maintain peripheral tolerance in potentially autoreactive T cells that have escaped thymic negative selection (reviewed in Ref. 37). The lack of a proliferative response and the defect in activation-induced IL-2 expression observed in TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL from 318 × H8 × RAG2$^{-/-}$ (Ag$^+$) mice apparently supports a role for such a mechanism in our model, in particular, since we also found no evidence for ex vivo gp33-specific cytotoxic activity mediated by self-specific TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL in a short-term ^{51}Cr release assay (data not shown).

It indeed makes sense that tolerance induction should extend to potentially autoreactive CD8$\alpha\alpha$ T cells in the IEL compartment

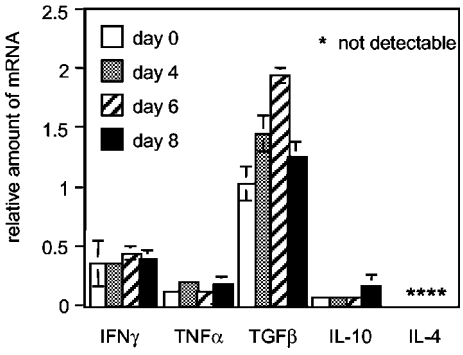

FIGURE 6. Cytokine gene expression by TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL during systemic LCMV infection. TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL were sorted from uninfected and LCMV-infected (days 4, 6, and 8 after infection) 318 × H8 × RAG2$^{-/-}$ mice. RNA was extracted, reverse transcribed, and analyzed for the cytokines IFN-γ, TNF-α, TGFβ, IL-10, and IL-4 by Real-time RT-PCR. Error bars indicate the range between two experiments. At each interval, three mice were analyzed.

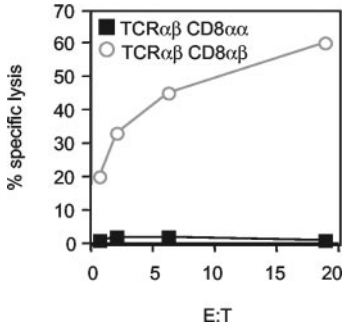

FIGURE 7. TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL isolated from LCMV-infected Ag$^+$ mice do not exert gp33-specific ex vivo cytotoxic activity. TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL were purified from two LCMV-infected 318 × H8 × RAG2$^{-/-}$ mice on day 8 after infection and tested for in vitro LCMV-gp33-specific cytotoxic activity in a 5-h ^{51}Cr release assay (■). For a positive control, cytotoxic activity was assessed in CD8$\alpha\beta$ IEL purified from a RAG2$^{-/-}$ mouse that had been reconstituted with total IEL from a C57BL/6 mouse for 5 wk and had subsequently been infected with LCMV for 8 days (○). Unspecific cytotoxicity was measured using target cells pulsed with the irrelevant adenovirus-derived peptide adn5 instead of gp33 was <5% (data not shown).

since uncontrolled proliferation or cytotoxic activity would be particularly deleterious in the intestinal mucosa. In fact, despite the high precursor frequency of potentially self-reactive TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL, we observed no signs of histopathological alterations in the intestinal mucosa of 318 × H8 × RAG2$^{-/-}$ mice. Why should, however, the murine intestine preserve self-specific T cells in the limited niches of the IEL compartment, if these cells are to be unfunctional? Guehler et al. (20) suggested that in the 2C TCR tg model, TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL from Ag$^+$ mice had undergone an immune deviation and differentiation into a T_H2-like phenotype capable of mediating humoral immune responses, as these cells expressed mRNA for IL-4 upon in vitro restimulation. That T cells with an anergic phenotype need not necessarily be unfunctional is further illustrated by the CD4$^+$CD25$^+$ subset of T cells which proliferates very poorly upon TCR stimulation in vitro, yet has the potent capacity of suppressing immune responses in vivo by release of immunomodulatory cytokines (reviewed in Ref. 38).

In support of a possible regulatory role for TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL, we found that these cells express moderate levels of mRNA for IFN-γ, TGFβ, and IL-10 in the complete absence of their specific MHC-restricted Ag, indicating that TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL may constitutively express mRNA for immunoregulatory cytokines as part of their genetic program or that other than TCR-mediated signals may induce and sustain cytokine gene expression. In fact, it has recently been shown for in vitro-stimulated CD8$^+$ IEL that the interaction of the TL ligand with the CD8$\alpha\alpha$ homodimer enhances IL-2 and IFN-γ production independently of the TCR MHC specificity (17). Although it was beyond the scope of the present study to also assess the relative role of TL-CD8$\alpha\alpha$ and additional non-MHC-restricted cell-cell interactions for modulating the effector functions of TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL, our results clearly demonstrate that Ag-specific, TCR-mediated signaling profoundly influences cytokine gene expression since TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL from Ag$^+$ mice consistently exhibit reduced levels of mRNA for IFN-γ and IL-10, but similar or even increased levels of mRNA for TGFβ.

The consistent expression of TGFβ by TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL is intriguing. TGFβ is the major switch factor for IgA-producing plasma cells in the intestinal mucosa and appears to play a critical protective role in experimental models of intestinal inflammation (39, 40), possibly by inhibiting T cell proliferation and APC functions or by preventing the production of proinflammatory cytokines and chemokines by enterocytes (39, 41, 42). With their preferential expression of this important immunomodulatory cytokine and their reduced proliferative capacity, self-specific TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL seem well adapted to an environment in which space for clonal expansion is lacking and preservation of tissue integrity is vital. Based on the results of Leishman et al. (10), which suggest a true positive selection process of self-specific TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL as opposed to an escape of negative selection (10), it is therefore tempting to speculate that the presence of self-specific TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL may indeed be of relevance to the maintenance of intestinal tissue homeostasis.

Evidence for a cytokine-dependent regulatory role of self-specific TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL has very recently been provided by a study that demonstrated an IL-10-mediated protective capacity of adoptively transferred TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL in an experimental model of colitis (43). This may be in apparent contrast to our finding that IL-10 mRNA expression was consistently reduced in TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL from 318 × H8 × RAG2$^{-/-}$ mice in favor of the expression of TGFβ. Moreover, our tg system also failed to confirm a role for the expression of IL-4 by self-specific TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL as previously observed for the 2C TCR tg model (20). These discrepancies between different models may be attributed to different methods used for the analysis of cytokine expression. A conclusive analysis of cytokine expression on a protein level is indeed difficult to accomplish for IEL as these cells seem to depend on survival signals provided by the epithelial cells and tend to rapidly undergo apoptosis in ex vivo culture (44). In fact, similar to Poussier et al. (43) who were unable to detect secretion of IL-10 in disease-preventing TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL (43), we were not successful in detecting TGFβ in supernatants of in vitro-stimulated TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL from 318 × H8 × RAG2$^{-/-}$ mice (data not shown). Given these circumstances, we believe that by assessing the effect of the presence or absence of the specific self-Ag on the expression of immunomodulatory cytokine genes directly in ex vivo isolated cells, thereby circumventing the problems associated with an in vitro culture of IEL, we have chosen an approach that is most likely to reflect the actual in vivo situation.

The seemingly conflicting results obtained so far for immunomodulatory cytokines preferentially expressed by self-specific TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL may be best reconciled by a concept that also considers the interrelated roles of these factors in immunoregulation. With the general role of IL-4 in the intestinal mucosa remaining controversial, this may be particularly true for TGFβ and IL-10, both of which have been ascribed vital roles in the prevention of intestinal pathologies. In this respect, it has recently been suggested that TGFβ may serve as a primary mechanism for the counterregulation of Th1 T cell-mediated mucosal inflammation while IL-10 is necessary as a secondary factor that facilitates TGFβ production (45).

Although TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL specific for self-Ags may be involved in the maintenance of intestinal tissue homeostasis, it is important to bear in mind that these cells have a potential for autoreactivity and could themselves initiate and contribute to intestinal inflammation. This in particular has to be considered with the constant and high exposure of the intestine to infectious and proinflammatory agents and the fact that also antigenically unrelated infections can prime for autoaggressiveness (26, 46). Although it has previously been shown in vitro that cytolytic responses of self-specific TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL can be retrieved in the presence of increasing levels of exogenous self-peptide (47), no investigation has so far addressed the important issue of whether autoaggressive TCR$\alpha\beta$ CD8$\alpha\alpha$ effector T cells can be generated in vivo. In this study, we have been able to directly assess the consequences of molecular mimicry on self-specific TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL in situ by the administration of a potent LCMV infection to the 318 × H8 × RAG2$^{-/-}$ (Ag$^+$) mice.

It may be argued that due to their secluded location in the intestinal epithelium, TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL may not be in contact with LCMV-infected, professional APCs. However, dendritic cells have been shown to penetrate the gut epithelium with their dendrites (48, 49), and the observed up-regulation of the early activation marker CD69 concomitant with an apparent infection of the intestinal mucosa on day 6 after infection clearly argues for a virus-induced activation of the TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL. Activation appears to be Ag specific, since after LCMV infection of OT-I × RAG2$^{-/-}$ mice, which are TCR tg for an OVA-derived peptide, no up-regulation of CD69 could be detected on TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL (data not shown).

Thus, we for the first time have been able to demonstrate an Ag-specific response of TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL in situ, while previous attempts to detect an immune reaction by these cells after an in vivo priming have been hampered by unknown Ag specificities or low precursor frequencies (30). However, this potent activation of TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL in situ does not lead to the generation of autoaggressive T cells. This is in clear contrast to CD8$\alpha\beta$ T cells, where tolerance against the gp33 Ag expressed in the pancreatic β cells was readily abolished following systemic LCMV infection,

resulting in CD8 T cell-mediated β cell destruction and the onset of overt diabetes (32, 50). Hence, it is intriguing that in our system all LCMV-infected mice remained healthy and no signs of intestinal pathologies such as epithelial erosion or crypt hyperplasia were observed. The absence of any overt signs of autoimmunity is supported by our observation that LCMV infection does not induce cytotoxic activity or even provoke the expression of proinflammatory cytokines in self-specific TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL. Although these findings may be accounted for by the consistent and even increased expression of TGFβ by self-specific IEL throughout the course of LCMV infection, the exact molecular mechanism underlying the attenuation of immune effector functions in this IEL subset are still elusive.

The clearance from LCMV in a virus-infected immunocompetent host critically depends on the presence of cytotoxic TCR$\alpha\beta$ CD8$\alpha\beta$ T cells (51). Hence, in Ag^+ mice where TCR$\alpha\beta$ tg CD8$\alpha\beta$ T cells are centrally deleted, the absence of cytotoxic activity in TCR$\alpha\beta$ CD8$\alpha\alpha$ T cells may contribute to the observed persistence of virus beyond day 8, i.e., at a time point where virus is generally cleared in an immunocompetent mouse. Because of these differences in the distribution, frequency, and persistence of virus-infected cells in Ag^+ and Ag^- mice, however, a conclusive comparative analysis of TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL in the presence or absence of the autoantigen was not possible.

In summary, our results emphasize the unique nature of TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL and imply that this cell subset is unlikely to initiate autoreactive responses or contribute to chronic inflammatory reactions in the intestine. Moreover, along with our finding that in the presence of their specific self-Ag TCR$\alpha\beta$ CD8$\alpha\alpha$ IEL preferentially express mRNA for TGFβ, these findings further underline the potential contribution of self-specific T cells in modulating local immune responses and in maintaining local tissue homeostasis.

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References

1. Camerini, V., C. Panwala, and M. Kronenberg. 1993. Regional specialization of the mucosal immune system. Intraepithelial lymphocytes of the large intestine have a different phenotype and function than those of the small intestine. *J. Immunol. 151:1765*.
2. Guy Grand, D., N. Cerf Bensussan, B. Malissen, M. Malassis Seris, C. Briottet, and P. Vassalli. 1991. Two gut intraepithelial $CD8^+$ lymphocyte populations with different T cell receptors: a role for the gut epithelium in T cell differentiation. *J. Exp. Med. 173:471*.
3. Offit, P. A., and K. I. Dudzik. 1989. Rotavirus-specific cytotoxic T lymphocytes appear at the intestinal mucosal surface after rotavirus infection. *J. Virol. 63:3507*.
4. Chardes, T., D. Buzoni Gatel, A. Lepage, F. Bernard, and D. Bout. 1994. *Toxoplasma gondii* oral infection induces specific cytotoxic CD8α/β^+Thy-1^+ gut intraepithelial lymphocytes, lytic for parasite- infected enterocytes. *J. Immunol. 153:4596*.
5. Sydora, B. C., B. D. Jamieson, R. Ahmed, and M. Kronenberg. 1996. Intestinal intraepithelial lymphocytes respond to systemic lymphocytic choriomeningitis virus infection. *Cell. Immunol. 167:161*.
6. Muller, S., M. Buhler-Jungo, and C. Mueller. 2000. Intestinal intraepithelial lymphocytes exert potent protective cytotoxic activity during an acute virus infection. *J. Immunol. 164:1986*.
7. Kim, S. K., D. S. Reed, W. R. Heath, F. Carbone, and L. Lefrancois. 1997. Activation and migration of CD8 T cells in the intestinal mucosa. *J. Immunol. 159:4295*.
8. Camerini, V., B. C. Sydora, R. Aranda, C. Nguyen, C. MacLean, W. H. McBride, and M. Kronenberg. 1998. Generation of intestinal mucosal lymphocytes in SCID mice reconstituted with mature, thymus-derived T cells. *J. Immunol. 160:2608*.
9. Guy-Grand, D., N. Pardigon, S. Darche, O. Lantz, P. Kourilsky, and P. Vassalli. 2001. Contribution of double-negative thymic precursors to CD8$\alpha\alpha^+$ intraepithelial lymphocytes of the gut in mice bearing TCR transgenes. *Eur. J. Immunol. 31:2593*.
10. Leishman, A. J., L. Gapin, M. Capone, E. Palmer, H. R. MacDonald, M. Kronenberg, and H. Cheroutre. 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*.
11. Guy-Grand, D., O. Azogui, S. Celli, S. Darche, M. C. Nussenzweig, P. Kourilsky, and P. Vassalli. 2003. Extrathymic T cell lymphopoiesis: ontogeny and contribution to gut intraepithelial lymphocytes in athymic and euthymic mice. *J. Exp. Med. 197:333*.
12. Lefrancois, L., and L. Puddington. 1995. Extrathymic intestinal T-cell development: virtual reality? *Immunol. Today 16:16*.
13. Guy-Grand, D., and P. Vassalli. 2002. Gut intraepithelial lymphocyte development. *Curr. Opin. Immunol. 14:255*.
14. Gapin, L., H. Cheroutre, and M. Kronenberg. 1999. Cutting edge: TCR$\alpha\beta^+$ CD8$\alpha\alpha^+$ T cells are found in intestinal intraepithelial lymphocytes of mice that lack classical MHC class I molecules. *J. Immunol. 163:4100*.
15. Park, S. H., D. Guy-Grand, F. A. Lemonnier, C. R. Wang, A. Bendelac, and B. Jabri. 1999. Selection and expansion of CD8α/α1 T cell receptor α/β1 intestinal intraepithelial lymphocytes in the absence of both classical major histocompatibility complex class I and nonclassical CD1 molecules. *J. Exp. Med. 190:885*.
16. Guy Grand, D., B. Rocha, P. Mintz, M. Malassis Seris, F. Selz, B. Malissen, and P. Vassalli. 1994. Different use of T cell receptor transducing modules in two populations of gut intraepithelial lymphocytes are related to distinct pathways of T cell differentiation. *J. Exp. Med. 180:673*.
17. Leishman, A. J., O. V. Naidenko, A. Attinger, F. Koning, C. J. Lena, Y. Xiong, H. C. Chang, E. Reinherz, M. Kronenberg, and H. Cheroutre. 2001. T cell responses modulated through interaction between CD8$\alpha\alpha$ and the nonclassical MHC class I molecule, TL. Science *294:1936*.
18. Rocha, B., H. von Boehmer, and D. Guy-Grand. 1992. Selection of intraepithelial lymphocytes with CD8α/α co-receptors by self-antigen in the murine gut. *Proc. Natl. Acad. Sci. USA 89:5336*.
19. Poussier, P., H. S. Teh, and M. Julius. 1993. Thymus-independent positive and negative selection of T cells expressing a major histocompatibility complex class I restricted transgenic T cell receptor α/β in the intestinal epithelium. *J. Exp. Med. 178:1947*.
20. Guehler, S. R., J. A. Bluestone, and T. A. Barrett. 1996. Immune deviation of 2C transgenic intraepithelial lymphocytes in antigen-bearing hosts. *J. Exp. Med. 184:493*.
21. Cruz, D., B. C. Sydora, K. Hetzel, G. Yakoub, M. Kronenberg, and H. Cheroutre. 1998. An opposite pattern of selection of a single T cell antigen receptor in the thymus and among intraepithelial lymphocytes. *J. Exp. Med. 188:255*.
22. Levelt, C. N., Y. P. de Jong, E. Mizoguchi, C. O'Farrelly, A. K. Bhan, S. Tonegawa, C. Terhorst, and S. J. Simpson. 1999. High- and low-affinity single-peptide/MHC ligands have distinct effects on the development of mucosal CD8$\alpha\alpha$ and CD8$\alpha\beta$ T lymphocytes. *Proc. Natl. Acad. Sci. USA 96:5628*.
23. Rocha, B., P. Vassalli, and D. Guy Grand. 1991. The Vβ repertoire of mouse gut homodimeric α $CD8^+$ intraepithelial T cell receptor α/β^+ lymphocytes reveals a major extrathymic pathway of T cell differentiation. *J. Exp. Med. 173:483*.
24. Poussier, P., P. Edouard, C. Lee, M. Binnie, and M. Julius. 1992. Thymus-independent development and negative selection of T cells expressing T cell receptor α/β in the intestinal epithelium: evidence for distinct circulation patterns of gut- and thymus-derived T lymphocytes. *J. Exp. Med. 176:187*.
25. Pircher, H., T. W. Mak, R. Lang, W. Ballhausen, E. Ruedi, H. Hengartner, R. M. Zinkernagel, and K. Burki. 1989. T cell tolerance to Mlsa encoded antigens in T cell receptor Vβ8.1 chain transgenic mice. *EMBO J. 8:719*.
26. Ehl, S., J. Hombach, P. Aichele, T. Rulicke, B. Odermatt, H. Hengartner, R. Zinkernagel, and H. Pircher. 1998. Viral and bacterial infections interfere with peripheral tolerance induction and activate $CD8^+$ T cells to cause immunopathology. *J. Exp. Med. 187:763*.
27. Lefrançois, L. 1993. Isolation of mouse small intestinal intraepithelial lymphocytes. In *Current Protocols in Immunology*. J. E. Coligan, A. M. Kruisbeek, D. H. Marguelies, E. M. Shevach, and W. Strober, eds. Wiley, New York, p. 5.3.2.
28. Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. Science *274:94*.
29. Gallimore, A., A. Glithero, A. Godkin, A. C. Tissot, A. Pluckthun, T. Elliott, H. Hengartner, and R. Zinkernagel. 1998. Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. *J. Exp. Med. 187:1383*.
30. Corazza, N., S. Muller, T. Brunner, D. Kagi, and C. Mueller. 2000. Differential contribution of Fas- and perforin-mediated mechanisms to the cell-mediated cytotoxic activity of naive and in vivo-primed intestinal intraepithelial lymphocytes. *J. Immunol. 164:398*.
31. Podd, B. S., C. Aberg, K. L. Kudla, L. Keene, E. Tobias, and V. Camerini. 2001. MHC class I allele dosage alters CD8 expression by intestinal intraepithelial lymphocytes. *J. Immunol. 167:2561*.
32. Ohashi, P. S., S. Oehen, K. Buerki, H. Pircher, C. T. Ohashi, B. Odermatt, B. Malissen, R. M. Zinkernagel, and H. Hengartner. 1991. Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell 65:305*.
33. Rocken, M., J. F. Urban, and E. M. Shevach. 1992. Infection breaks T-cell tolerance. *Nature 359:79*.
34. Vezys, V., S. Olson, and L. Lefrancois. 2000. Expression of intestine-specific antigen reveals novel pathways of CD8 T cell tolerance induction. *Immunity 12:505*.
35. Sprent, J., and S. R. Webb. 1995. Intrathymic and extrathymic clonal deletion of T cells. *Curr. Opin. Immunol. 7:196*.

36. Stockinger, B. 1999. T lymphocyte tolerance: from thymic deletion to peripheral control mechanisms. *Adv. Immunol. 71:229*.
37. Walker, L. S., and A. K. Abbas. 2002. The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat. Rev. Immunol. 2:11*.
38. Maloy, K. J., and F. Powrie. 2001. Regulatory T cells in the control of immune pathology. *Nat. Immunol. 2:816*.
39. Buzoni-Gatel, D., A. C. Lepage, I. H. Dimier-Poisson, D. T. Bout, and L. H. Kasper. 1997. Adoptive transfer of gut intraepithelial lymphocytes protects against murine infection with *Toxoplasma gondii*. *J. Immunol. 158:5883*.
40. Powrie, F., J. Carlino, M. W. Leach, S. Mauze, and R. L. Coffman. 1996. A critical role for transforming growth factor-β but not interleukin 4 in the suppression of T helper type 1-mediated colitis by $CD45RB^{low}CD4^{+}$ T cells. *J. Exp. Med. 183:2669*.
41. Gorelik, L., and R. A. Flavell. 2000. Abrogation of TGFβ signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity 12:171*.
42. Takeuchi, M., P. Alard, and J. W. Streilein. 1998. TGF-β promotes immune deviation by altering accessory signals of antigen-presenting cells. *J. Immunol. 160:1589*.
43. Poussier, P., T. Ning, D. Banerjee, and M. Julius. 2002. A unique subset of self-specific intraintestinal T cells maintains gut integrity. *J. Exp. Med. 195:1491*.
44. Brunner, T., D. Arnold, C. Wasem, S. Herren, and C. Frutschi. 2001. Regulation of cell death and survival in intestinal intraepithelial lymphocytes. *Cell Death Differ. 8:706*.
45. Fuss, I. J., M. Boirivant, B. Lacy, and W. Strober. 2002. The interrelated roles of TGF-β and IL-10 in the regulation of experimental colitis. *J. Immunol. 168:900*.
46. Vezys, V., and L. Lefrancois. 2002. Cutting edge: inflammatory signals drive organ-specific autoimmunity to normally cross-tolerizing endogenous antigen. *J. Immunol. 169:6677*.
47. Guehler, S. R., R. J. Finch, J. A. Bluestone, and T. A. Barrett. 1998. Increased threshold for TCR-mediated signaling controls self-reactivity. *J. Immunol. 160:5341*.
48. Maric, I., P. G. Holt, M. H. Perdue, and J. Bienenstock. 1996. Class II MHC antigen (Ia)-bearing dendritic cells in the epithelium of the rat intestine. *J. Immunol. 156:1408*.
49. Rescigno, M., M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonasio, F. Granucci, J. P. Kraehenbuhl, and P. Ricciardi-Castagnoli. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol. 2:361*.
50. Oldstone, M. B., M. Nerenberg, P. Southern, J. Price, and H. Lewicki. 1991. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. *Cell 65:319*.
51. Kagi, D., B. Ledermann, K. Burki, P. Seiler, B. Odermatt, K. J. Olsen, E. R. Podack, R. M. Zinkernagel, and H. Hengartner. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature 369:31*.