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Mucosal memory $CD8^+$ T cells are selected in the periphery by an MHC class I molecule

Yujun Huang[1,7], Yunji Park[1,7], Yiran Wang-Zhu[1], Alexandre Larange[1], Ramon Arens[1], Iván Bernardo[1,6], Danyvid Olivares-Villagómez[2], Dietmar Herndler-Brandstetter[3], Ninan Abraham[4], Beatrix Grubeck-Loebenstein[3], Stephen P Schoenberger[1], Luc Van Kaer[2], Mitchell Kronenberg[1], Michael A Teitell[5] & Hilde Cheroutre[1]

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The presence of immune memory at pathogen-entry sites is a prerequisite for protection. Nevertheless, the mechanisms that warrant immunity at peripheral interfaces are not understood. Here we show that the nonclassical major histocompatibility complex (MHC) class I molecule thymus leukemia antigen (TL), induced on dendritic cells interacting with CD8αα on activated $CD8\alpha\beta^+$ T cells, mediated affinity-based selection of memory precursor cells. Furthermore, constitutive expression of TL on epithelial cells led to continued selection of mature $CD8\alpha\beta^+$ memory T cells. The memory process driven by TL and CD8αα was essential for the generation of $CD8\alpha\beta^+$ memory T cells in the intestine and the accumulation of highly antigen-sensitive $CD8\alpha\beta^+$ memory T cells that form the first line of defense at the largest entry port for pathogens.

A hallmark of immune memory is that repeated infections are met with accelerated and enhanced protective immunity[1]. Furthermore, unlike naive T lymphocytes or central memory T cells, which reside in lymphoid tissues, some antigen-experienced T cells gain the ability to persist long term as effector memory T cells (T_{EM} cells) in nonlymphoid tissues such as the intestine[2–5]. Central memory T cells, which respond with robust clonal expansion, are effective at protecting against infections by pathogens that replicate systemically[6], but they are probably inadequate to prevent the transmission of viruses (including human immunodeficiency virus (HIV)) or intracellular bacteria that penetrate the mucosal epithelia[3,7]. Effective resistance to the transmission of such pathogens requires the presence of local, antigen-specific T_{EM} cells before rechallenge[7]. Therefore, strategies aimed at inducing a powerful protective immune response that also warrants the formation of preexisting mucosal antigen-specific T_{EM} cells are considered an essential goal of successful vaccination.

Listeria monocytogenes, a Gram-positive intracellular pathogen of human and other mammals, including mice, is a food-borne pathogen; after ingestion and uptake by phagocytic cells, such as monocytes and dendritic cells (DCs), *L. monocytogenes* disseminate from the intestine into the bloodstream and spread to various systemic tissues such as the liver[8]. In humans, ingested *L. monocytogenes* may cause listeriosis because of its ability to also infect nonphagocytic cells, such as intestinal epithelial cells, through interaction of internalin (expressed by *L. monocytogenes*) and human E-cadherin (expressed on the basolateral pole of the enterocytes)[9]. In contrast, mice infected orally with *L. monocytogenes* do not develop listeriosis because of the inability of internalin to interact with mouse E-cadherin. Instead, mice clear the ingested bacteria via an effective CD8-dependent protective immune response, although bacteria that cross the mucosal barrier can spread to the liver and other organs via the blood, as can happen in humans[8]. Such observations have important implications for immunization strategies and indicate that the presence of local preexisting mucosal immunity might be key for the induction of effective protective immunity to ingested pathogens. Despite that, however, most knowledge of immune memory has been gained from model systems that use systemic immunization routes and memory generation in lymphoid tissues. Such preexisting immunity might be highly effective in combating systemic infection but is probably inadequate for controlling pathogens that invade the body via the oral route. Because of the protective mucosal CD8-mediated anti–*L. monocytogenes* response in mice and because of the emergence of genetically manipulated avirulent, attenuated strains of wild-type and recombinant *L. monocytogenes* deficient in ActA protein (which is essential for the motility and spreading of *L. monocytogenes*; $ActA^-$ *L. monocytogenes*) as important vectors for vaccination[8,10,11], immunization of mice with *L. monocytogenes* introduced via the oral route represents an optimal approach with which to examine the mechanisms and conditions that lead to the generation of preexisting mucosal immune memory.

Homodimers of CD8 (CD8αα) induced on activated $CD8\alpha\beta^+$ T cells, which maintain expression of CD8αβ, mark those primary effector cells that have a propensity to differentiate into memory cells[12]. In mice, the epithelium of the intestine is greatly enriched for memory $CD8\alpha\beta^+$ T cells

[1]Division of Developmental Immunology, La Jolla Institute for Allergy & Immunology, La Jolla, California, USA. [2]Department of Microbiology & Immunology, Vanderbilt University, School of Medicine, Nashville, Tennessee, USA. [3]Division of Immunology, Institute for Biomedical Aging Research, Austrian Academy of Sciences, Innsbruck, Austria. [4]Department of Microbiology and Immunology, Life Sciences Institute, Vancouver, Canada. [5]Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at University of California Los Angeles, Los Angeles, California, USA. [6]Present address: Clinical Laboratory. Histocompatibility Section, San Pedro Hospital, Logroño, Spain. [7]These authors contributed equally to this work. Correspondence should be addressed to H.C. (e-mail: hilde@liai.org).

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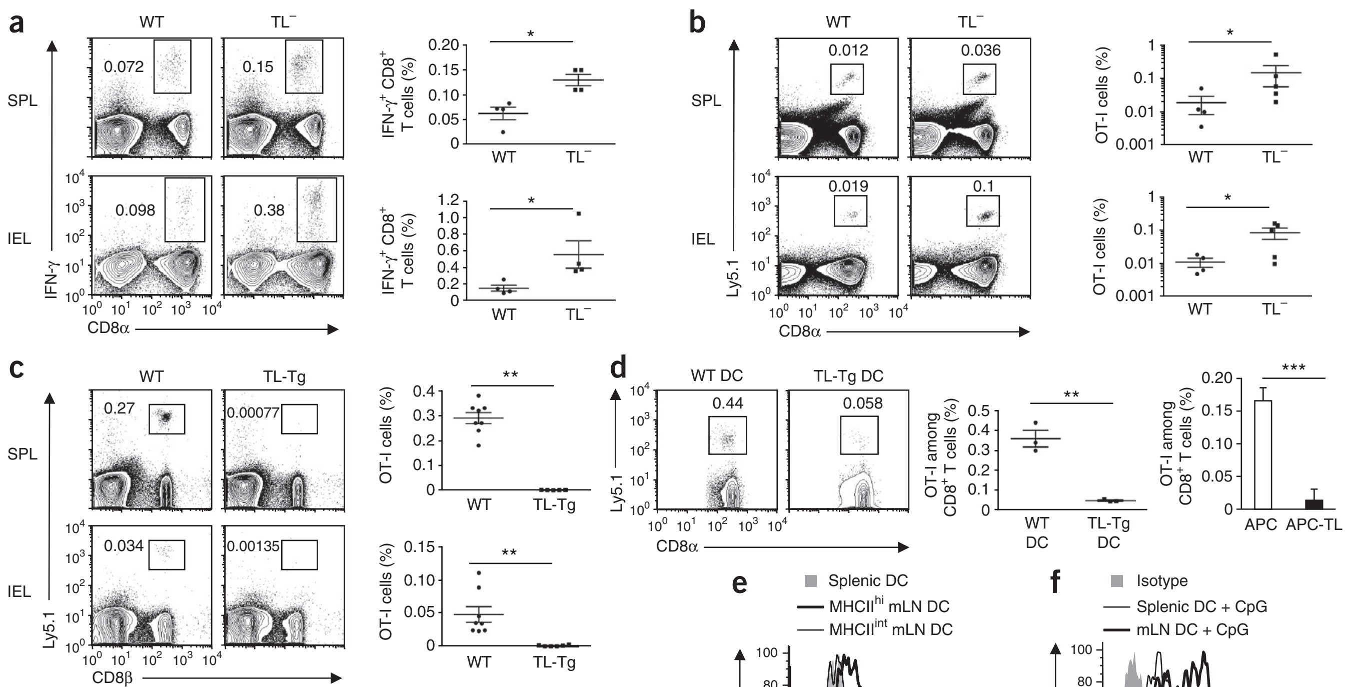

Figure 1 TL negatively affects the generation of memory cells from CD8αβ⁺ T cells. (**a**) Intracellular staining of interferon-γ (IFN-γ) and cell surface staining of CD8α (left) in or on splenocytes (SPL) and IELs isolated from wild-type (WT) or TL⁻ mice 30 d after oral infection with 1×10^9 ActA⁻ LM-OVA, then restimulated *ex vivo* with SIINFEKL. Right, pooled data. (**b**) Tracking of donor (Ly5.1⁺) OT-I cells among splenocytes and IELs (left) from Ly5.2⁺ wild-type or TL⁻ recipients given adoptive transfer of 5×10^4 naive Ly5.1⁺ CD8⁺ OT-I cells, then orally infected with 1×10^9 ActA⁻ LM-OVA 1 d later; cells were obtained 2 months after infection. Graph, pooled data. (**c**) Tracking of donor OT-I cells among splenocytes and IELs (left) from wild-type or TL-transgenic (TL-Tg) recipients given 1×10^6 naive Ly5.1⁺ OT-I cells, then infected and assessed as in **b**. (**d**) Flow cytometry of memory OT-I cells from the spleens of B6 mice given 5×10^4 Ly5.1⁺ naive OT-I cells, then immunized intravenously with 5×10^5 SIINFEKL-loaded DCs generated from wild-type or TL-transgenic bone marrow cells (left; middle, pooled data), or given OT-I cells primed *in vitro* with APCs that do not express TL (APC) or APCs transfected to express TL (APC-TL; far right); cells were analyzed 2 months after immunization with DCs or transfer of *in vitro*–activated OT-I cells. (**e**) Flow cytometry analysis of TL expression by splenic or mLN DCs sorted on the basis of their expression of CD11c and MHC class II (MHCII). (**f**) TL expression by freshly isolated splenic or mLN DCs activated for 1 d with CpG. Isotype, isotype-matched control antibody. Numbers adjacent to outlined areas (**a**–**d**) indicate percent cells in each throughout. Each symbol represents an individual mouse (**a**–**d**); small horizontal lines indicate the mean (and s.e.m.). $*P < 0.05$, $**P < 0.001$ and $***P < 0.01$ (unpaired *t*-test). Data are representative of three independent experiments.

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that coexpress CD8αα[13]. Thymus leukemia antigen (TL) is a high-affinity ligand for CD8αα[14]. TL is a nonclassical, nonpolymorphic major histocompatibility complex (MHC) class I molecule encoded on chromosome 17 in the *H2-T* region. This locus has undergone genetic rearrangements to produce at least two functional alleles, *H2-T3* (in H-2^b mouse strains such as C57BL/6 (B6)) and *H2-T18* (in H-2^d mouse strains such as BALB/c). Despite its name, TL is constitutively expressed on intestinal epithelial cells that are adjacent to the CD8αα⁺ T cells[15,16]. Such findings suggest a role for TL in the accumulation of mucosal CD8αα⁺CD8αβ⁺ memory T cells; however, the mechanisms that drive the CD8αα-dependent generation of mucosal immune memory remain unknown.

Through the use of a model of oral infection with *L. monocytogenes* to elicit a CD8-driven protective immune response initiated at the mucosal entry site, we define here an affinity-based selection mechanism controlled by TL expression. This was induced on antigen-presenting cells (APCs) and led to the survival and differentiation of high-affinity, CD8αα⁺CD8αβ⁺ memory precursor cells. Furthermore, constitutive expression of TL on the epithelium of the intestine continued to impose selection pressure, which contributed to the affinity maturation of the resident mucosal CD8αβ⁺ T_{EM} cells.

RESULTS

TL is not required for memory CD8αβ⁺ T cells

Among the MHC class I molecules encoded by the mouse genome, TL is distinguished because it has a particularly high affinity for CD8αα mediated by three unique amino acid alterations in the highly conserved membrane-proximal α3 domain of its MHC class I heavy chain[17]. To assess if TL, the most likely physiological ligand for CD8αα *in vivo*[12], also has a role in the generation of CD8αβ⁺ effector memory cells, we analyzed the differentiation of CD8αβ⁺ memory T cells in mice with deletion of the gene encoding TL (called 'TL⁻ mice' here)[18]. We assessed the generation of ovalbumin (OVA)-specific CD8αβ⁺ memory T cells in the spleens of TL⁻ mice infected orally with *L. monocytogenes* that express OVA antigen (LM-OVA) and found that the absence of TL did not impair but instead enhanced the generation of these cells (**Fig. 1a**). We observed a similar effect in the epithelium of the intestine of these mice when we analyzed intraepithelial lymphocytes (IELs; **Fig. 1a**). Likewise, the transfer of naive OT-I T cells transgenic for the expression of monoclonal T cell antigen receptor (TCR) specific for the OVA peptide SIINFEKL (amino acids 257–264) and H-$2K^b$ into wild-type or TL⁻ recipient

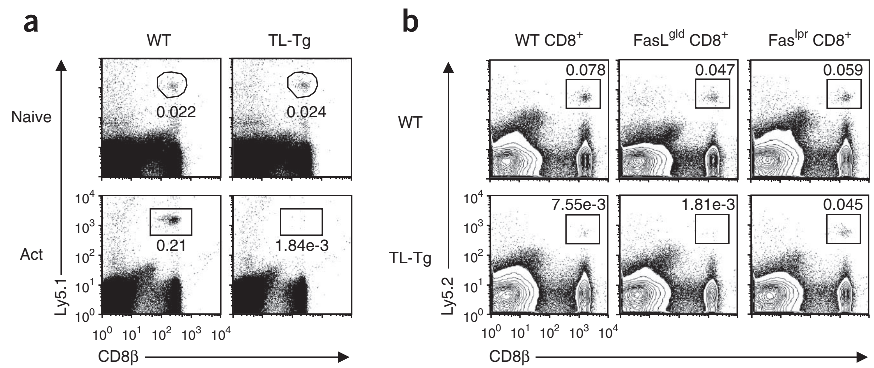

Figure 2 TL mediates the death of activated CD8αβ+ T cells. (**a**) Tracking of donor cells in the spleens of wild-type or TL-transgenic recipients of 1×10^6 naive Ly5.1+ OT-I cells (top; $n = 5$ mice per group) or 1×10^6 *in vitro*–activated Ly5.1+ OT-I cells (bottom (Act); $n = 8$ mice per group), assessed 1 month after transfer. (**b**) Tracking of Ly5.2+ donor in the spleens of Ly5.1+ wild-type or TL-transgenic recipients ($n = 4$ mice per group) of CD8+ T cells (0.5×10^6) sorted from wild-type mice or mice deficient in Fas ligand ($FasL^{gld}$) or Fas (Fas^{lpr}) and preactivated (before transfer) for 3 d *in vitro* with anti-CD3 and anti-CD28 beads, assessed 1 month after transfer. Data are representative of two independent experiments.

mice subsequently orally infected with LM-OVA also resulted in the generation of more OT-I TCR–transgenic memory T cells in the absence of TL expression (**Fig. 1b**). Those observations were consistent with published data of mice with transgenic expression of single-chain MHC class I on a β_2-microglobulin-deficient background also indicating that in the absence of TL, normal or slightly enhanced memory forms in response to viral infection[19]. Together the data show that whereas CD8αα promotes the memory differentiation of CD8αβ+ effector T cells[12], its high-affinity ligand TL seems to inhibit this process.

TL expressed on APCs kills primed CD8αβ+ T cells

TL has a restricted pattern of expression[20] that includes induction on APCs, such as DCs, in addition to expression on epithelial cells[12]. The greater abundance of memory CD8αβ+ T cells seen in the absence of TL in TL− mice suggested that under normal conditions, TL expression on subsets of priming DCs might negatively influence the survival or differentiation of CD8αβ+ memory precursor cells. To assess this possibility, we analyzed the effect of constitutive TL expression during priming through the use of TL-transgenic mice that express an allelic form of the gene encoding TL under control of the promoter of the gene encoding the MHC class I molecule H-2D. In contrast to the outcome in TL− hosts, transferred OT-I T cells primed *in vivo* in TL-transgenic recipient mice orally infected with LM-OVA failed to generate or sustain immune memory either locally, in the intestine, or systemically, in the spleen or liver (**Fig. 1c** and **Supplementary Fig. 1**). Moreover, OT-I T cells primed systemically *in vivo* with TL-transgenic bone marrow DCs loaded with SIINFEKL transferred failed to generate memory cells in the spleens of wild-type hosts (**Fig. 1d**). Similarly, OT-I cells initially primed *in vitro* by APCs transfected to express TL did not generate memory cells after adoptive transfer (**Fig. 1d**). These data indicated that TL expression on APCs interfered with the survival and memory programming of primary CD8αβ+ effector cells.

Under steady-state conditions, resting splenic DCs normally do not have detectable expression of TL surface protein, although some induce it after activation[12]. However, analysis of various DC subsets indicated that in contrast to splenic DCs, a subset of mesenteric lymph node (mLN) DCs had constitutively low expression of TL. This TL+ subset had the phenotype of mature migratory DCs (high expression of MHC class II, CD11c+ and CD103+CCR7+; **Supplementary Fig. 2a**) also typical of those DCs that directed retinoic acid–based induction of gut-homing receptors on the T cells they primed[21] (**Fig. 1e** and **Supplementary Fig. 2b**). The expression of TL on these mucosal DCs was further upregulated during priming and was considerably enhanced in response to innate immune stimuli such as CpG oligodeoxynucleotides (**Fig. 1f**). These observations indicated that naive T cells responding *in vivo* to gut-derived antigens were primed in the context of TL expressed by the migratory DCs and upregulated considerably under inflammatory conditions.

TL kills T cells via the receptor Fas

Although TL has structural features characteristic of MHC class I molecules, it does not function as a typical antigen-presenting molecule. The narrow distance between the α-helices that form the boundaries of the antigen-binding groove do not permit peptide binding and presentation by TL[22], and therefore TL fails to engage with the αβ TCR. Nevertheless, the high degree of conserved sequence in the α3 domain allows TL to interact with the coreceptor CD8αβ despite the exclusion of TL from the TCR activation complex[14]. A similar interaction of soluble HLA class I molecules with the CD8αβ TCR coreceptor, separately from TCR ligation, has been shown to lead to cell death induced by the tumor necrosis factor receptor superfamily member 6 Fas (CD95) and its ligand[23–26]. To determine if the interaction of TL with CD8αβ on activated T cells might also lead to cell death, we monitored the survival of naive and activated CD8αβ+ cells in the presence of constitutive TL expression in TL-transgenic hosts. Whereas naive donor cells survived similarly in wild-type or TL-transgenic mice, activated CD8αβ+ OT-I cells survived only in wild-type hosts but not in TL-transgenic hosts (**Fig. 2a**), which supported the proposal that TL-induced cell death targeted activated CD8αβ+ T cells. However, activated Fas-deficient ($Fas^{lpr/lpr}$) CD8αβ+ donor T cells were not deleted from TL-transgenic recipient mice (**Fig. 2b**), which provided evidence that similar to the reported cell death mediated by soluble HLA-G[23,24], TL-induced cell death also involved the death pathway mediated by Fas and its ligand.

Activation-induced CD8αα 'rescues' CD8αβ+ effector T cells

In contrast to CD8αβ, CD8αα does not function as a TCR coreceptor and, similar to TL, CD8αα also does not participate directly in the TCR activation complex[27,28]. However, whereas TL induces the death of activated CD8αβ+ T cells, CD8αα, which has higher affinity for TL than does CD8αβ[14], promotes the survival of CD8αβ+ effector cells[12], which suggests that activation-induced CD8αα might interfere with TL-induced cell death. To assess this, we compared the survival and memory differentiation of CD8αβ+ effector T cells in the presence or absence of CD8αα. Because of deletion of the enhancer region I in the promoter of the gene encoding CD8α[29], OT-I CD8αβ+ donor cells on the $E8_I$-deficient background (called '$\Delta E8_I$ OT-I CD8αβ+ T cells' here) do not induce detectable amounts of activation-dependent CD8αα[12]. *In vitro*, $\Delta E8_I$ OT-I CD8αβ+ T cells primed by SIINFEKL-loaded mLN DCs, which expressed TL (**Fig. 1e**), underwent more activation-induced death than did their counterparts primed by splenic DCs (**Fig. 3a**). However, similar enhanced death of $\Delta E8_I$ OT-I CD8αβ+

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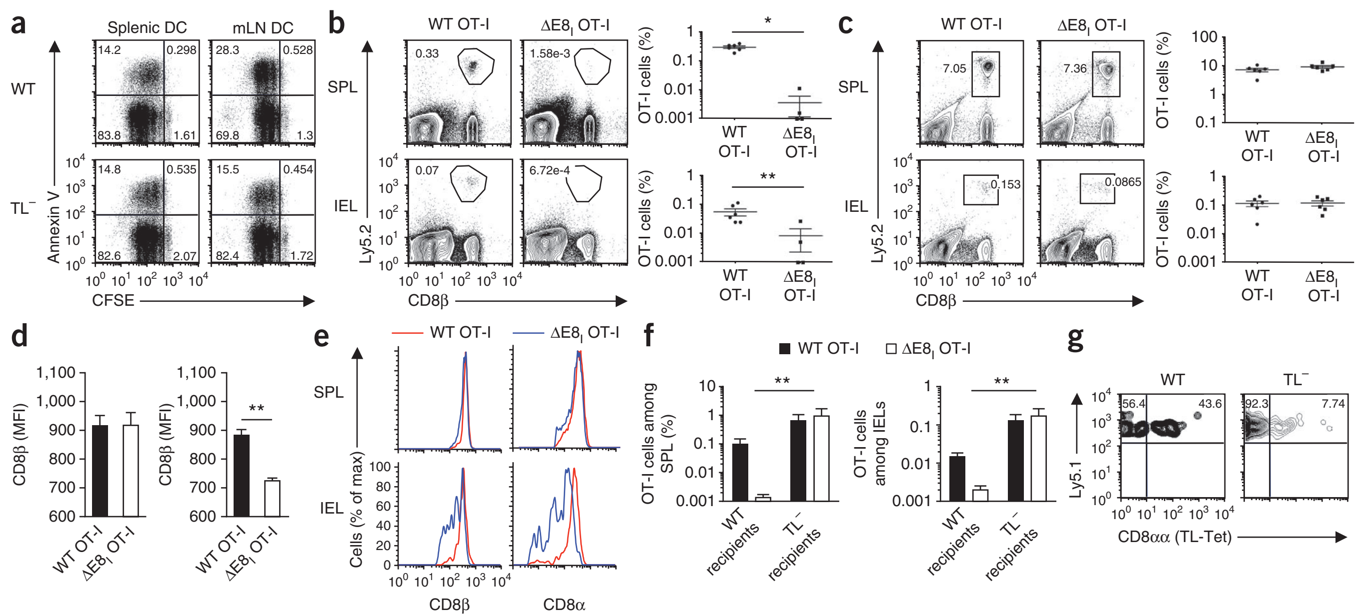

Figure 3 Activation-induced CD8αα rescues CD8αβ⁺ primary effector T cells from TL-induced cell death. (**a**) Death of $\Delta E8_I$ OT-I cells (2×10^5) labeled with the cytosolic dye CFSE and cultured for 2 d with 4×10^4 SIINFEKL-pulsed DCs from the spleen or mLNs of wild-type or TL⁻ mice, analyzed by annexin V staining. Numbers in quadrants indicate percent cells in each throughout. (**b**) Tracking of donor OT-I cells among splenocytes and IELs of Ly5.1⁺ recipient mice given adoptive transfer of 5×10^4 naive Ly5.2⁺ wild-type or Ly5.2⁺ $\Delta E8_I$ OT-I cells, then orally infected with 1×10^9 ActA⁻ LM-OVA 1 d after transfer, and assessed 2 months after infection. Right, pooled data (as in **Fig. 1a**–**d**). (**c**) Tracking of donor OT-I cells among splenocytes and IELs of Ly5.1⁺ recipient mice given adoptive transfer of 5×10^4 naive Ly5.2⁺ wild-type or Ly5.2⁺ $\Delta E8_I$ OT-I cells, then intravenously infected with 2.5×10^5 ActA⁻ LM-OVA 1 d after transfer and assessed 2 months after infection. Right, pooled data (as in **Fig. 1a**–**d**). (**d**) Expression of CD8β on effector wild-type OT-I or $\Delta E8_I$ OT-I cells from recipients treated as in **b** (left) or **c** (right), assessed 7 d after infection. MFI, mean fluorescence intensity. (**e**) Expression of CD8β on memory wild-type OT-I or $\Delta E8_I$ OT-I cells among splenocytes and IELs of recipients treated as in **c**, assessed 2 months after infection. (**f**) Tracking of memory OT-I cells among splenocytes and IELs of Ly5.1⁺ wild-type or Ly5.1⁺ TL⁻ mice ($n = 5$ per group) given 5×10^4 naive Ly5.1⁺Ly5.2⁺ wild-type OT-I cells and 5×10^4 naive Ly5.2⁺ $\Delta E8_I$ OT-I cells (transferred together) and then infected orally with 1×10^9 ActA⁻ LM-OVA 1 d after transfer, assessed 2 months after infection (pooled data). (**g**) CD8αα expression, assessed by staining with TL tetramers (TL-Tet), on gated memory wild-type OT-I cells among IELs of wild-type or TL⁻ recipient mice as in **f**, asssessed 2 months after infection. $*P < 0.001$ and $**P < 0.01$ (unpaired *t*-test). Data are representative of three (**a**–**c**,**e**–**g**) or two (**d**) independent experiments (mean and s.e.m. in **d**,**f**).

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T cells primed by SIINFEKL-loaded mLN DCs isolated from TL⁻ mice was not induced by those mucosal APCs (**Fig. 3a**).

Furthermore, similar to published results showing impaired splenic memory generation by $\Delta E8_I$ CD8αβ⁺ T cells in response to intraperitoneal infection with lymphocytic choriomeningitis virus (LCMV)[12], $\Delta E8_I$ OT-I CD8αβ⁺ donor cells primed *in vivo* with LM-OVA via the oral route also failed to generate detectable memory cells in the spleens or intestines of wild-type recipient mice (**Fig. 3b**). In contrast, another published study using the same systemic LCMV–immunization approach has shown that $\Delta E8_I$ CD8αβ⁺ T cells do generate systemic memory[30]. However, there was substantial downregulation of CD8αβ during the initial priming phase in that study[30], which was not seen on either wild-type CD8αβ⁺ T cells or the LCMV-primed $\Delta E8_I$ CD8αβ⁺ T cells in the other study noted above[12]. We considered the possibility that a difference in the strength of the LCMV viral stock might have caused downregulation of CD8αβ and effects on survival similar to those mediated by activation-induced CD8αα on normal wild-type CD8αβ⁺ T cells. To investigate this, we assessed whether, in the model used here, the memory response of $\Delta E8_I$ OT-I CD8αβ⁺ T cells was altered in mice primed systemically with LM-OVA introduced intravenously, a route that induces a much more potent response than does the oral route[8]. In contrast to oral immunization, systemic priming of the $\Delta E8_I$ OT-I CD8αβ⁺ T cells with LM-OVA resulted in a distinct memory response in the spleen and intestine similar to that of wild-type OT-I cells (**Fig. 3c**). Furthermore, similar to the study discussed above[30], CD8αβ was downregulated substantially during the potent priming of $\Delta E8_I$ OT-I CD8αβ⁺ T cells activated via the intravenous route, whereas such downregulation did not occur in $\Delta E8_I$ OT-I CD8αβ⁺ T cells primed via the oral route (**Fig. 3d**). Although activation-induced downregulation of CD8αβ could explain the survival of $\Delta E8_I$ OT-I CD8αβ⁺ T cells during systemic priming, it does not explain the long-term survival of these cells in the intestine, where TL is constitutively expressed on intestinal epithelial cells. To further investigate this, we analyzed the CD8αβ expression of $\Delta E8_I$ OT-I CD8αβ⁺ memory T cell subsets in the spleen and the intestine. Notably, whereas $\Delta E8_I$ OT-I CD8αβ⁺ memory cells in the periphery expressed normal amounts of CD8αβ, memory $\Delta E8_I$ OT-I CD8αβ⁺ IELs that persisted in the presence of TL expressed much less CD8αβ (**Fig. 3e**). These results indicated that the mechanism of TL-induced cell death continuously and selectively shapes the repertoire of the memory cells that accumulate at the mucosal site of the intestine. In support of that hypothesis, in the absence of TL expression in the intestine of TL⁻ hosts, as with systemic priming, there was no difference in the efficiency of memory formation in the spleen or intestine when wild-type OT-I or $\Delta E8_I$ OT-I CD8αβ⁺ T cells were primed via the oral route (**Fig. 3f**). Furthermore, there was also no selective accumulation of CD8αα-expressing wild-type OT-I CD8αβ IELs in TL⁻ hosts (**Fig. 3g**), which indicated that TL on the epithelial cells in the intestine imposed selective pressure to promote the local accumulation

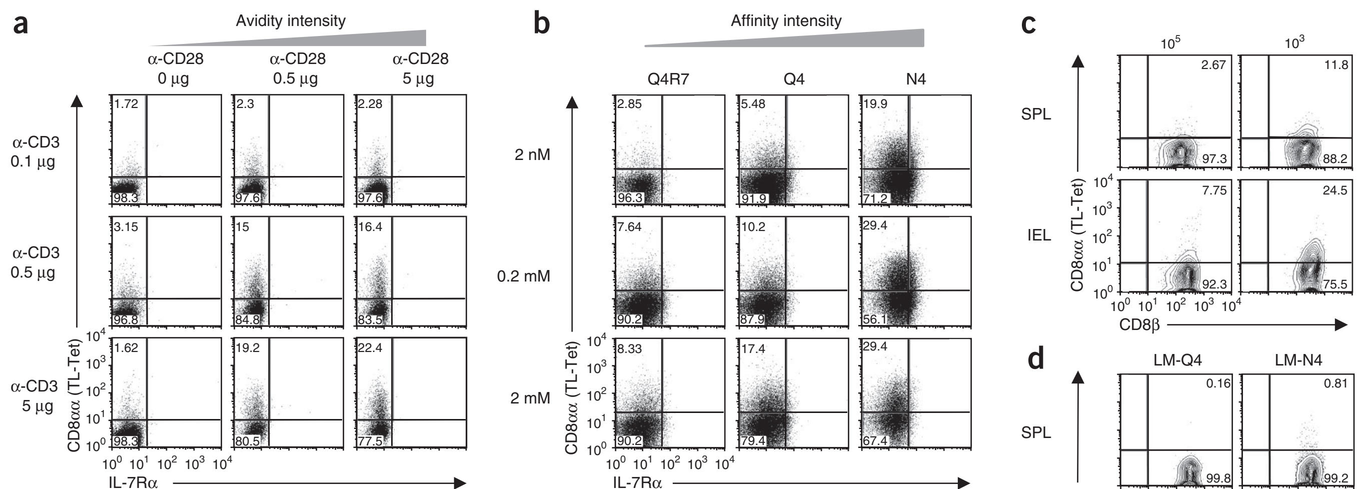

Figure 4 CD8αα expression correlates with the intensity of TCR activation. (**a**) Expression of CD8αα (assessed as in **Fig. 3g** throughout) and IL-7Rα on splenocytes cultured for 3 d *in vitro* in the presence of graded concentrations (wedge) of soluble anti-CD3 (α-CD3) and anti-CD28 (α-CD28), gated on CD8$^+$ T cells. (**b**) Expression of CD8αα and IL-7Rα on OT-I cells cultured for 2 d *in vitro* with artificial APCs (MEC.B7 adherent fibroblasts, which express the costimulatory molecule B7.1) in the presence of a graded concentration (wedge) of SIINFEKL (with asparagine (N) at position 4 (N4)) or the altered peptide ligands Q4R7 and Q4. (**c**) Expression of CD8αα and CD8β on Ly5.1$^+$ CD8$^+$ OT-I cells among splenocytes and IELs from B6 recipient mice ($n = 4$ per group) given 1×10^5 or 1×10^3 sorted naive Ly5.1$^+$ CD8$^+$ OT-I cells, then infected orally with 1×10^9 ActA$^-$ LM-OVA 1 d after transfer and assessed 7 d after infection. (**d**) Expression of CD8αα and CD8β on donor OT-I cells among splenocytes and IELs from wild-type recipient mice ($n = 5$ per group) given 5×10^4 naive Ly5.1$^+$ CD8$^+$ OT-I cells, then infected orally with 2×10^8 wild-type LM-Q4 or LM-N4 1 d after transfer, assessed 7 d after infection. Data are representative of three (**a**,**b**) or two (**c**,**d**) independent experiments.

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of CD8αα-expressing effector memory T cells. These data are consistent with a role for activation-induced CD8αα in sequestering TL away from the coreceptor CD8αβ, which thereby prevents TL-induced death of CD8αβ$^+$ primary effector T cells.

CD8αα marks the intensity of TCR activation

Because of the relatively high-affinity interaction of CD8αα with TL, activation-induced expression of CD8αα can be distinguished from CD8αβ expression through the use of TL tetramers[12]. Activated CD8αβ$^+$ T cells do not all induce CD8αα to the same extent, and a variegated expression pattern of high expression (CD8αα^{hi}) and low expression (CD8αα^{lo-neg}) is typically observed through the use of TL tetramers. Together with the fact that the initial induction of CD8αα requires TCR stimulation, this suggests that there might be a close link between the intensity of TCR activation and the degree of CD8αα induction. In support of that, TL tetramer staining of polyclonal CD8αβ$^+$ wild-type T lymphocytes activated *in vitro* with variable concentrations of soluble antibody to CD3 (anti-CD3) and anti-CD28 showed a graded increase in CD8αα expression with higher concentrations of anti-CD3 plus anti-CD28 (**Fig. 4a**). Furthermore, OT-I T cells stimulated with various altered peptide ligands that bind as well to the H-2K^b MHC class I molecule as does the original OT-I TCR ligand SIINFEKL but have different antigenic potencies[31] also demonstrated tight association between the extent of CD8αα induction and the degree of TCR activation. Thus, the original high-affinity ligand SIINFEKL induced higher CD8αα expression than did SIINFEKL variants with lower affinity (**Fig. 4b**). We obtained results *in vivo* that also supported the idea of affinity-based induction of CD8αα when we analyzed mice infected orally with LM-OVA after they received either a high or low number of naive donor OT-I cells, which because of antigenic competition will result in either a low or high antigen dose, respectively (**Fig. 4c**). Additionally, we analyzed two groups of recipient mice given adoptive transfer of an equal number of OT-I precursor cells but orally infected with *L. monocytogenes* expressing the low-affinity peptide variant SIIQFEKL (LM-Q4) or the high-affinity SIINFEKL peptide (LM-N4). Mice infected with LM-N4 generated more CD8αα-expressing CD8αβ$^+$ T cells than did those infected with LM-Q4 (**Fig. 4d**). In each of these approaches, the results consistently indicated that the extent of CD8αα induction represents a sensitive measurement of the intensity of the signal strength received through the activated TCR.

Similar to activation-induced expression of CD8αα, the expression of IL-7 receptor α-chain (IL-7Rα) also has been proposed as a marker for memory precursor cells[32]. However, in contrast to CD8αα expression, IL-7R expression did not correlate with TCR activation; instead, IL-7R was constitutively expressed on naive cells and was initially downregulated during activation at the time when CD8αα was first upregulated (**Supplementary Fig. 3a**). The reciprocal expression of CD8αα and IL-7R suggested different roles for these molecules in memory programming and/or survival. Consistent with that hypothesis, a mutation in the sequence encoding the cytoplasmic IL-7Rα Y449XXM motif (where '449' indicates the position of the tyrosine residue and 'X' indicates any amino acid) known to impair the long-term survival of IL-7R-dependent CD8$^+$ memory T cells[33] did not interfere with the induction of CD8αα or the survival and generation of OT-I memory T cells with this mutation in response to oral immunization with LM-OVA (**Supplementary Fig. 3b**). These data indicated that the affinity-based selective programming of memory precursor cells does not depend on IL-7R signals. Overall, the data indicate that in addition to being a memory precursor marker[12], CD8αα expression also 'reports on' the affinity or avidity of the antigen signal received by activated CD8αβ$^+$ primary effector cells. The affinity-based induction of CD8αα, together with the ability of CD8αα to sequester TL and prevent TL-induced cell death, therefore represents a mechanism by

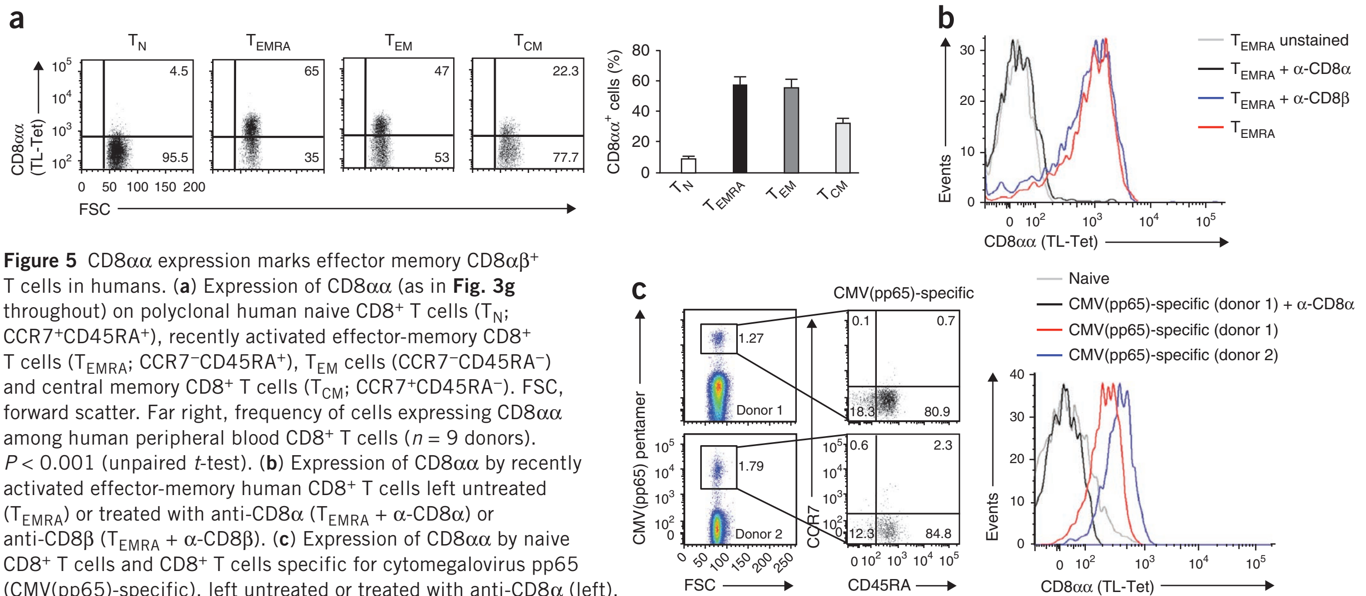

Figure 5 CD8αα expression marks effector memory CD8αβ+ T cells in humans. (**a**) Expression of CD8αα (as in **Fig. 3g** throughout) on polyclonal human naive CD8+ T cells (T_N; CCR7+CD45RA+), recently activated effector-memory CD8+ T cells (T_{EMRA}; CCR7−CD45RA+), T_{EM} cells (CCR7−CD45RA−) and central memory CD8+ T cells (T_{CM}; CCR7+CD45RA−). FSC, forward scatter. Far right, frequency of cells expressing CD8αα among human peripheral blood CD8+ T cells (n = 9 donors). P < 0.001 (unpaired t-test). (**b**) Expression of CD8αα by recently activated effector-memory human CD8+ T cells left untreated (T_{EMRA}) or treated with anti-CD8α (T_{EMRA} + α-CD8α) or anti-CD8β (T_{EMRA} + α-CD8β). (**c**) Expression of CD8αα by naive CD8+ T cells and CD8+ T cells specific for cytomegalovirus pp65 (CMV(pp65)-specific), left untreated or treated with anti-CD8α (left). Left and middle, staining of human CD8+ T cells with a pentamer of cytomegalovirus pp65 (CMV(pp65) pentamer; left), followed by gating on cells specific for cytomegalovirus pp65 as recently activated effector-memory CD8+ T cells (middle). Data are representative of three experiments with three donors each (**a**), two independent experiments (**b**) or two experiments with three donors each (**c**; two donors among six presented here).

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which the most avid CD8αβ+ primary effector cells are selectively preserved as part of the memory precursor pool.

CD8αα marks human high-affinity CD8αβ+ T cells

Published evidence has indicated that tetramers of TL, in addition to detecting mouse CD8αα, also detect expression of human CD8αα homodimers[12,17]. Consistent with activation-induced expression of CD8αα observed on mouse CD8 T cells, TL tetramers also stained human CD8αβ+ effector cells, whereas they did not stain naive T cells (**Fig. 5a**). Staining with the TL tetramer was blocked with an antibody specific for human CD8α but not by anti-CD8β (**Fig. 5b** and **Supplementary Fig. 4**), which confirmed the specificity of the TL tetramer for human CD8αα. Furthermore, a subset of high-affinity CD8αβ+ T cells specific for the dominant epitope derived from the internal matrix protein pp65 of cytomegalovirus[34], isolated from people seropositive for cytomegalovirus, stained almost exclusively with the TL tetramer, and this was blocked with anti-CD8α (**Fig. 5c**). These data indicate that CD8αα expression on human CD8αβ+ effector T cells also is associated with high-affinity effector cells.

Retinoic acid and transforming growth factor-β enhance CD8αα

The epithelium of the intestine is enriched for CD8αα-expressing CD8αβ+ T cells, which suggests that a selective process based on CD8αα expression might drive this localized accumulation. Consistent with that, after priming *in vitro*, CD8αα expression on the activated T cells was higher after priming by mLN DCs than after priming by splenic DCs (**Fig. 6a**,**b**). In the presence of exogenous retinoic acid, normally released by mLN DCs during priming[21], splenic DCs also mediated strong induction of CD8αα on the OT-I T cells they primed (**Fig. 6a**,**b**). Conversely, treatment with an inhibitor of the retinoic acid receptor resulted in lower CD8αα expression on T cells primed by mLN DCs (**Fig. 6a**). Likewise, the addition of transforming growth factor-β (TGF-β), known to be an important modulator of mucosal T cell differentiation, resulted in more CD8αα induction on activated CD8αβ+ primary effector cells primed by splenic DCs than on cells primed with splenic DCs alone (**Fig. 6c**). However, we observed that in each condition, the enhanced induction of CD8αα occurred only in the presence of substantial antigen activation (**Fig. 6b**,**c**), which suggested that they influenced CD8+ effector differentiation by further promoting the selective marking of high-affinity effector cells by CD8αα.

The expression of TL on mucosal migratory DCs known to release retinoic acid and TGF-β (**Fig. 1e**,**f**), together with the enhanced induction of CD8αα on high-affinity effectors in the presence of retinoic acid as well as TGF-β, indicated that the selective 'rescue' of high-affinity memory precursor cells was geared toward effector cells that home to the gut. In agreement with that, CD8αα-expressing OT-I cells were first detectable at mucosal induction sites, such as mLNs and Peyer's patches, early after oral exposure to antigen (**Fig. 6d**). They then gradually accumulated during the contraction and memory phases in the pool of T_{EM} cells positive for the integrin $\alpha_E\beta_7$ (CD103+) in the gut epithelium but not in the CD103− memory cells that persisted systemically (**Fig. 6e**).

TL on intestinal epithelium selects mature CD8αβ T_{EM} cells

The constitutive expression of TL on intestinal epithelial cells[15,16] suggests that TL might continuously shape the resident mucosal memory CD8αβ+ T cell population even after rechallenge. To investigate this possibility, we examined the fate of primary and secondary $CD8\alpha\alpha^{hi}CD8\alpha\beta^+$ or $CD8\alpha\alpha^{lo-neg}CD8\alpha\beta^+$ effector T cells *in vivo*. We initially primed OT-I cells *in vitro* in the absence of TL with APCs that do not naturally express TL, then sorted the cells into $CD8\alpha\alpha^{hi}$ and $CD8\alpha\alpha^{lo-neg}$ primary effector T cells and adoptively transferred them into wild-type recipient mice. Both subsets of primary effector cells showed a similar short-term homing ability (**Supplementary Fig. 5a**), and both effector T cell types responded the same way when tested *in vitro* for interferon-γ production (**Supplementary Fig. 5b**) or *in vivo* for cytotoxicity (**Supplementary Fig. 5c**). However, in response to oral rechallenge with LM-OVA, primary memory OT-I T cell populations derived from the $CD8\alpha\alpha^{hi}$ precursor cells

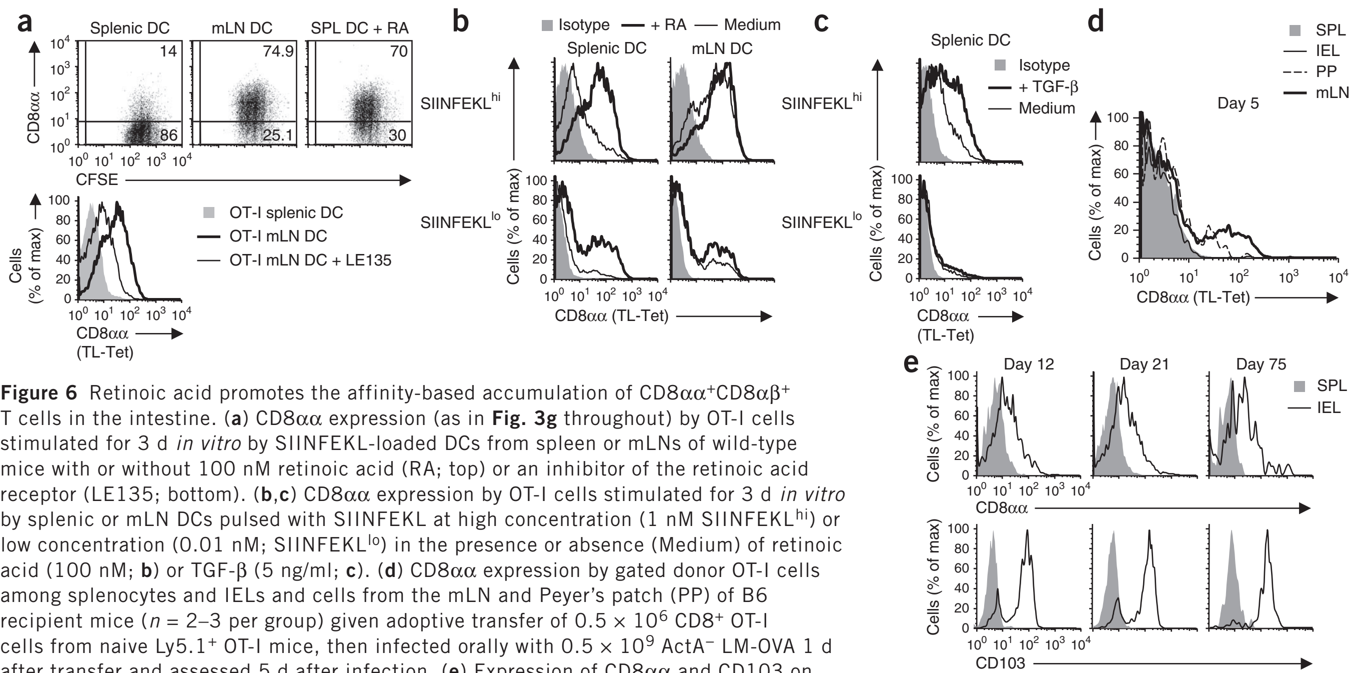

Figure 6 Retinoic acid promotes the affinity-based accumulation of CD8αα$^+$CD8αβ$^+$ T cells in the intestine. (**a**) CD8αα expression (as in **Fig. 3g** throughout) by OT-I cells stimulated for 3 d *in vitro* by SIINFEKL-loaded DCs from spleen or mLNs of wild-type mice with or without 100 nM retinoic acid (RA; top) or an inhibitor of the retinoic acid receptor (LE135; bottom). (**b**,**c**) CD8αα expression by OT-I cells stimulated for 3 d *in vitro* by splenic or mLN DCs pulsed with SIINFEKL at high concentration (1 nM SIINFEKL^{hi}) or low concentration (0.01 nM; SIINFEKL^{lo}) in the presence or absence (Medium) of retinoic acid (100 nM; **b**) or TGF-β (5 ng/ml; **c**). (**d**) CD8αα expression by gated donor OT-I cells among splenocytes and IELs and cells from the mLN and Peyer's patch (PP) of B6 recipient mice (n = 2–3 per group) given adoptive transfer of 0.5 × 10^6 CD8$^+$ OT-I cells from naive Ly5.1$^+$ OT-I mice, then infected orally with 0.5 × 10^9 ActA$^-$ LM-OVA 1 d after transfer and assessed 5 d after infection. (**e**) Expression of CD8αα and CD103 on gated donor OT-I cells among splenocytes and IELs on days 12, 21 and 75 after infection as in **d**. Data are representative of five independent experiments (**a**), more than five independent experiments (**b**,**c**) or at least three independent experiments (**d**,**e**).

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expanded in the spleens and intestines of wild-type recipient mice, whereas memory cells from the CD8αα^{lo-neg} effector pool were detectable only in the host spleen but not in the intestine (**Fig. 7a**). These data indicated that only CD8αα^{hi} primary memory cells persisted long term as mucosal T_{EM} cells in the proximity of TL constitutively expressed on the epithelial cells. After rechallenge, activated memory cell populations in lymphoid tissues expand and migrate as secondary effector cells to nonlymphoid tissues, such as the epithelium of the gut[35]. In agreement with that, similar numbers of effector cells derived from either CD8αα^{hi} or CD8αα^{lo-neg} primary effector OT-I cells were present in the intestine 5 d after recall (**Fig. 7b**). Nevertheless, when analyzed 45 d later, the progeny of CD8αα^{hi} effector cells were present as secondary memory cells in both the spleen and intestine, whereas the CD8αα^{lo-neg} secondary effector cells did not remain as secondary T_{EM} cells in the intestine (**Fig. 7c**). In contrast, *in vitro*–primed CD8αα^{lo-neg}CD8αβ$^+$ OT-I effector cells accumulated efficiently as T_{EM} cells did in the gut epithelium of TL$^-$ recipient mice (**Fig. 7d**), which indicated that the constitutive expression of TL continued to mediate selective pressure that prevented the accumulation of CD8αα^{lo-neg} primary and secondary effector cells as mucosal T_{EM} cells. These results also indicated that CD8αα^{lo-neg} effector cells were not intrinsically unable to convert into mucosal T_{EM} cells, but that under normal physiological conditions, only CD8αα^{hi} primary effector cells formed long-lived mucosal T_{EM} cells in proximity to TL constitutively expressed on the epithelial cells.

TL mediates the affinity maturation of memory CD8αβ$^+$ T cells

A published study using low-affinity altered peptide ligands for *in vivo* priming has suggested that the affinity maturation of memory T cells is the result of enhanced expansion and delayed contraction of the high-affinity responding effector cell populations[31]. Our results here suggested that in addition, the TL-mediated selective survival of CD8αα-expressing high-affinity memory cells might also contribute to ensuring the affinity maturation of memory populations at the mucosal border. To obtain direct evidence of this hypothesis, we used the altered-peptide-ligand approach of the published study noted above[31] to examine memory generation *in vivo* in the presence or absence of TL. We adoptively transferred naive OT-I cells into wild-type or TL$^-$ recipient mice that we subsequently orally infected with LM-Q4, which did not effectively induce CD8αα on primed OT-I effector cells (**Fig. 4d**). Fewer OT-I effector cells were detectable in the blood of wild-type mice than in the blood of TL$^-$ mice at 7 d after oral immunization with LM-Q4 (**Fig. 7e**), which suggested that TL induced on the priming mucosal APCs controlled in part the expansion of the primary effector pool. In the intestine, where TL is constitutively expressed by intestinal epithelial cells, the selective effect of TL was even greater. OT-I T_{EM} cells generated in response to the low-affinity peptide Q4 were barely detectable in the intestinal epithelium of wild-type mice, whereas they were easily detectable in TL$^-$ mice (**Fig. 7f**). These results indicated that continuous expression of TL on the epithelium of the intestine selectively eliminated low-affinity cells from the pool of mature mucosal T_{EM} cells. Along with the greater mucosal memory in TL$^-$ mice, the systemic memory pool was also larger (**Fig. 7f**), which suggested that in addition to the TL-mediated affinity selection of mature memory cells in the intestine, TL induced on mucosal APCs during priming might provide a first affinity-selection step by eliminating low-affinity or low-avidity primary effector cells from the memory precursor pool. To further test this idea, we immunized wild-type or TL$^-$ recipient mice with LM-Q4 via the intravenous route. Under these conditions, TL expression during priming had only a minimal effect on the effector or memory phase of peripheral T cells, which was similar in TL$^-$ and wild-type recipient mice (**Fig. 7g**,**h**). In contrast, however, the accumulation of T_{EM} cells at the mucosal site, where TL is constitutively expressed, remained under the TL selective pressure (**Fig. 7h**). These results indicated that T cells primed at the mucosal priming site underwent an initial selection step mediated by TL induced on the migratory DCs, whereas, regardless of where the priming occurred, the constitutive expression of TL on the mucosal

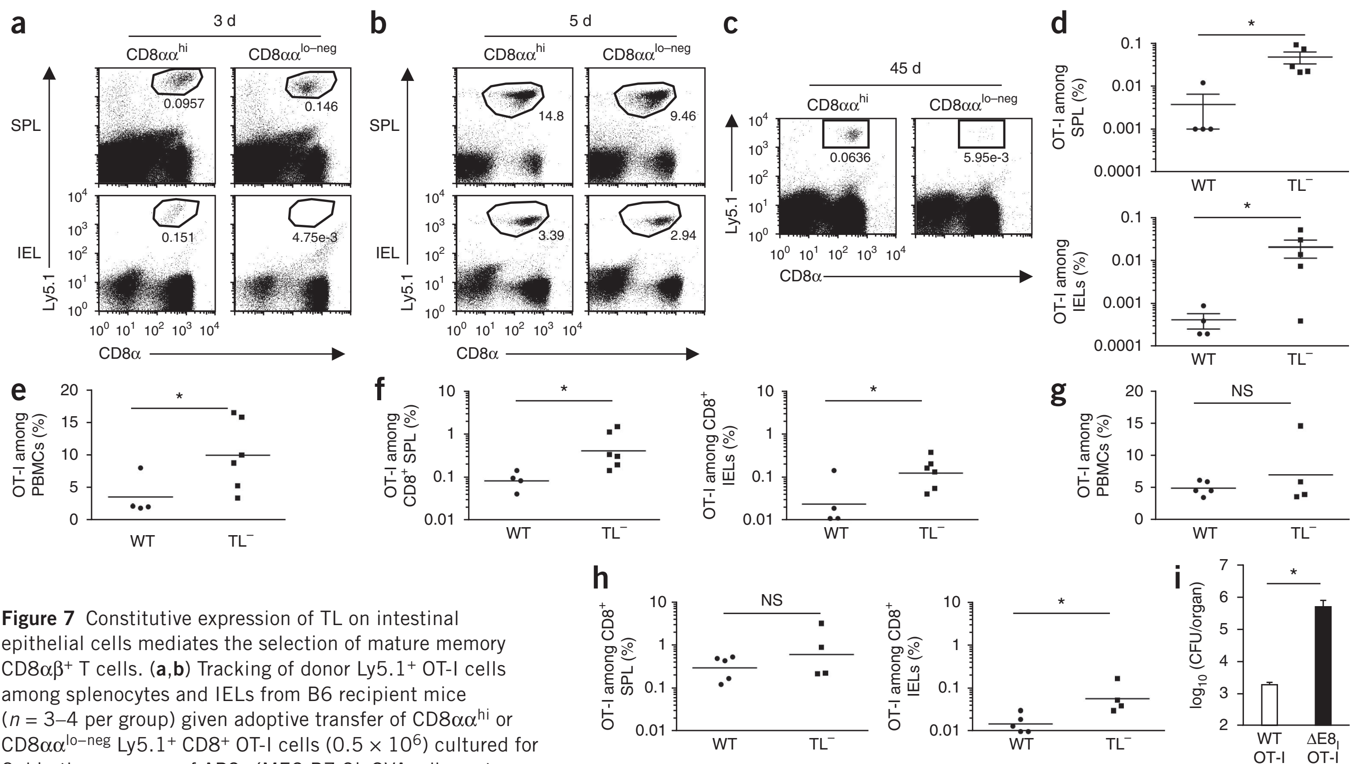

Figure 7 Constitutive expression of TL on intestinal epithelial cells mediates the selection of mature memory CD8αβ⁺ T cells. (**a**,**b**) Tracking of donor Ly5.1⁺ OT-I cells among splenocytes and IELs from B6 recipient mice (n = 3–4 per group) given adoptive transfer of CD8αα^{hi} or CD8αα$^{lo–neg}$ Ly5.1⁺ CD8⁺ OT-I cells (0.5 × 10^6) cultured for 2 d in the presence of APCs (MEC.B7.SigOVA adherent fibroblasts, which express the H-2K^b-restricted SIINFEKL epitope and B7.1), then sorted as CD8αα^{hi} and CD8αα$^{lo–neg}$ cells and cultured for 3 d *in vitro*; recipient mice were infected orally with 5 × 10^8 ActA⁻ LM-OVA1 month after transfer and assessed 3 d (**a**) or 5 d (**b**) after infection. (**c**) Flow cytometry of secondary OT-I memory cells among IELs 45 d after infection as in **a**. (**d**) Tracking of memory OT-I cells among splenocytes and IELs from wild-type or TL⁻ recipients of sorted, *in vitro*–activated Ly5.1⁺ CD8αα$^{lo–neg}$ OT-I primary effector cells (0.5 × 10^6) cultured for 3 d; recipient mice were infected orally with 5 × 10^8 ActA⁻ LM-OVA 1 month after transfer and assessed 4 months after infection (presented as in **Fig. 1a–d**). (**e**–**h**) Analysis of effector OT-I cells in the peripheral blood (PBMCs; **e**,**g**) and memory OT-I cells among splenocytes and IELs (**f**,**h**) of Ly5.1⁺ wild-type or Ly5.1⁺ TL⁻ recipient mice given 5 × 10^4 naive CD8⁺ OT-I cells and infected orally (**e**,**f**) or intravenously (**g**,**h**) with LM-Q4 1 d after transfer, assessed 7 d after infection (effector) or 2 months after infection (memory) and presented as in **d**. (**i**) Bacterial load in the liver of Ly5.1⁺ mice (n = 6) given adoptive transfer of 5 × 10^4 naive wild-type or ΔE8$_I$ OT-I cells, then immunized orally with 1 × 10^9 ActA⁻ LM-OVA and rechallenged orally with 1 × 10^{10} wild-type LM-OVA 2 months after the initial immunization and assessed 3 d later. CFU, colony-forming units. NS, not significant; *P < 0.05 (unpaired t-test). Data are representative of at least five (**a**,**b**), three (**c**) or two (**d**) independent experiments or three independent experiments (**e**–**i**; mean and s.e.m. in **i**).

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epithelium provided additional and constant selection pressure that drove continuous affinity maturation of the mature memory pool that accumulated long term at the mucosal interface of the intestine. (**Supplementary Fig. 6**)

To evaluate the importance of preexisting TL-CD8αα–selected intestinal T_{EM} cells, we assessed the resistance to oral infection with *L. monocytogenes* in mice with or without affinity-selected memory cells. For this, we transferred wild-type or ΔE8$_I$ OT-I cells into mice that we then orally immunized with the attenuated ActA⁻ LM-OVA strain (1 × 10^9 colony-forming units). We subsequently rechallenged the mice orally with a higher dose (1 × 10^{10} colony-forming units) of wild-type LM-OVA and analyzed systemic spreading of the pathogen. In contrast to the efficient resistance observed in mice that received wild-type OT-I precursor cells, which generated memory cells in the intestine and elsewhere, preimmunized mice that initially received ΔE8$_I$ OT-I precursor cells failed to generate affinity-selected memory and had significantly less resistance to systemic spreading of the orally introduced pathogen (**Fig. 7i**). These results emphasize the importance of the selective immune memory differentiation process as a critical mechanism for the efficient generation of preexisting immunity at critical mucosal interfaces that form the main entry sites for invading pathogens.

DISCUSSION

The data presented here have defined a fundamentally new idea for the understanding of the mechanism of immune memory and in particular of the generation of mucosal immunity. Our data have demonstrated that an affinity-based selective process operates *in vivo* that preserves the optimal effector cells so they can become long-lived memory T cells that form preexisting and heightened protective immunity. This mechanism is especially geared to generate the high-affinity T_{EM} cells that line the mucosal barrier of the intestine, where most pathogens enter the body.

Our results have also demonstrated a function for TL, a nonclassical MHC class I–like molecule, in mediating TL-induced cell death of CD8αβ⁺ effector T cells that failed to induce affinity-based CD8αα expression. Because TL is a much stronger ligand for CD8αα than for CD8αβ, CD8αα can probably sequester TL away from CD8αβ and prevent TL-induced cell death. As the induction of CD8αα was directly linked to the degree of TCR signal strength, this led to the selective survival of the most avid effector cells that then further differentiated into memory T cells. The constitutive expression of TL on the epithelial cells in the intestine continued selective pressure, which mediated affinity maturation of the mucosal T_{EM} population in response to repeated rechallenge.

The data presented here obtained with the *L. monocytogenes* model are consistent with a published study using the LCMV model system, which has also indicated a critical role for activation-induced CD8αα in the generation of CD8αβ$^+$ memory T cells[12]. Nevertheless, two subsequent studies have challenged that conclusion and have provided evidence that under certain conditions, CD8αβ$^+$ ΔE8$_I$ memory T cells can also be generated in a CD8αα-independent way[30,36]. In those two studies[30,36], however, and similar to what we observed here with immunization with *L. monocytogenes* via the intravenous route, there was substantial downregulation of CD8αβ expression during the priming of the ΔE8$_I$ CD8αβ$^+$ T cells, which was not observed in the earlier study with the LCMV system[12] or during oral immunization with the *L. monocytogenes* system, as shown here. The fact that in the absence of CD8αβ downregulation in both of those cases, ΔE8$_I$ CD8αβ$^+$ T cells failed to accumulate as memory cells indicates that strong activation signals that result in downregulation of CD8αβ on ΔE8$_I$ cells may 'rescue' CD8αβ$^+$ memory precursor cells, as does the biological activity of activation-induced CD8αα on normal wild-type CD8αβ$^+$ effector cells.

Although a sequence-based homolog of TL has not been identified in humans, TL and the nonclassical MHC class I molecule HLA-G have notable similarities, including their restricted pattern of expression, limited antigen-presentation ability, greater affinity for CD8αα than for CD8αβ and ability to induce the death of activated CD8αβ$^+$ T cells mediated by Fas and its ligand[25,37,38]. Such parallels suggest that like other nonclassical MHC or HLA pairs, TL and HLA-G might represent functional homologs of each other, and they further suggest that an affinity-based selective memory differentiation program probably operates in humans as well. Consistent with that, we found that activated human CD8αβ$^+$ T cells coexpressed CD8αα but naive cells did not, and that CD8αα expression coincided with the high-affinity CD8αβ$^+$ effector T cells specific for the dominant epitope pp65 of cytomegalovirus. Another study has indicated that the endogenous protective immune response to HIV is characterized by a CD8αα-expressing CD8αβ subpopulation with strong antiviral activity, and high-avidity HIV-specific CD8$^+$ T cell clonotypes are largely preserved in patients who control HIV viremia but not in progressive chronic HIV infection[39]. Together these observations suggest that the induction of CD8αα on human CD8αβ$^+$ T cells is also an activation-induced and affinity-based process that marks the avid effector cells.

Most infections, not only infection with *L. monocytogenes* but also many viral infections such as infection with HIV or simian immunodeficiency virus, are acquired across mucosal barriers, and several studies have demonstrated that CD8$^+$ cytotoxic T lymphocyte responses have a crucial role in the initial containment and early control of pathogen replication[40–44]. Although such responses may be unable to provide sterilizing protection, they can control the pathogen load and delay or even prevent spreading and the onset of disease, as well as diminish the potential for secondary transmission. Therefore, the finding that an endogenous TCR quality–based mechanism may select for the most avid effector cells so they form immune memory cells able to reside long term at mucosal interfaces has important implications for the design of new and improved strategies to induce effective preexisting protective immunity, not only systemically but also locally at the main and most vulnerable entry sites for pathogens.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS
Y.H. and Y.P., conceptual development and execution of the studies and preparation of the manuscript; Y.W.-Z., A.L., R.A. and I.B., technical assistance and input into data analyses; D.O.-V. and L.V.K., generation of TL-deficient mice; M.A.T., generation and backcrossing of TL-transgenic mice; D.H.-B. and B.G.-L., experiments with human samples; N.A., mice with mutation in the sequence encoding IL-7Rα Y449XXM; S.P.S., help with *in vitro* culture experiments; M.K., participation in discussions of the data and preparation of the manuscript; H.C., conception of ideas, generation of TL transgenic mice with the assistance of M.A.T., manuscript authorship and experiment supervision.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Mice. C57BL/6 CD45.2$^+$ (Ly5.2$^+$) mice (wild-type) and CD45.1$^+$ (B6.SJL, Ly5.1) congenic mice, B6.MRL-*Fas*^{lpr}/J mice and B6.Smn.C3-*Fasl*^{gld}/J mice were from The Jackson Laboratory. OT-I TCR–transgenic mice were crossed onto the Ly5.1$^+$ congenic background. Additionally, $\Delta E8_I$ mice with mutation in sequence encoding the IL-7Rα Y449XXM motif and mice were crossed to OT-I mice. TL-transgenic mice were generated by forced expression of the gene encoding TL under control of the *H2-D* promoter and were backcrossed to the B6 background for more than 12 generations. TL$^-$ mice were generated by deletion of the gene encoding TL on the B6 background. Mice were maintained by being bred under specific pathogen–free conditions in the animal facility of the La Jolla Institute for Allergy & Immunology. Unless noted otherwise, mice were maintained under specific pathogen–free conditions. Sentinel mice from the colony of mice deficient in recombination-activating gene 1 were determined to be negative for *Helicobacter* species and *Citrobacter rodentium*. Animal care and experimentation were consistent with the guidelines of the US National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the La Jolla Institute for Allergy & Immunology.

T cell isolation, cell sorting, CFSE labeling and adoptive transfer. CD8$^+$ OT-I cells were purified by magnetic negative selection with the MACS CD8α$^+$ T cell Isolation kit according to the manufacturer's protocol (Miltenyi Biotec). CD44^{lo} cells were sorted as naive CD8$^+$ OT-I cells with a FACSAria (Becton Dickinson). In some cases, sorted cells were labeled with CFSE (carboxyfluorescein diacetate succinimidyl ester). OT-I cells were resuspended at a density of 10×10^6 cells per ml in PBS and CFSE was added to a final concentration of 5 μM (Invitrogen). After 10 min of incubation at 37 °C, labeling was quenched with ice-cold DMEM medium with 10% (vol/vol) FCS. Cells were washed with PBS three times and were injected intravenously into the recipient mice.

DC isolation and activation. Pooled spleens or mLNs were cut into small pieces and then digested for 30 min at 23 °C with collagenase D (Roche); EDTA (final concentration, 5 mM) was added for the final 5 min. Digested tissues were further homogenized and then enriched for CD11c$^+$ DCs by positive selection with a MACS cell separator (Miltenyi Biotec). For DC activation *in vitro*, freshly isolated splenic DCs were cultured for 1 d with 1 mM CpG DNA (ODN 1826; Integrated DNA Technologies) and TL expression was analyzed with anti-TL (HD168; a gift from E. Stockert and L. Old).

***In vitro* T cell–stimulation culture.** For culture of total splenocytes, 5×10^5 total splenocytes were cultured in 96-well plates in the presence of anti-CD3 (145-2C11; eBioscience) and anti-CD28 (37.51; eBioscience). For artificial APC–OT-I cell cocultures, either the transfected adherent fibroblast cell line MEC.B7 (which expresses the costimulatory molecule B7.1) or MEC.B7.SigOVA (which expresses the H-2K^b-restricted SIINFEKL epitope and B7.1) was used as APCs. Irradiated APCs were cultured at a density of 1.5×10^5 cells per well in 24-well plates to establish a single layer. Naive OT-I cells (5×10^5) were added to the monolayer of APCs in 2 ml medium. TL-expressing APCs were generated by transfection of MEC.B7.SigOVA cells with a plasmid expressing TL. TL expression was confirmed by staining with a TL-specific antibody (HD168). For the culture of DCs with OT-I cells, purified DCs were incubated for 2 h with 1 nM SIINFEKL and then washed. CFSE-labeled naive OT-I cells (2×10^5) were cultured for 3–4 d in 96-well plates with peptide-pulsed DCs (4×10^4) with or without 100 nM all-*trans* retinoic acid (Sigma). The retinoic acid receptor antagonist LE135 (Tocris Bioscience) was used in some cultures.

Immunization with bone marrow–derived DCs. Bone marrow was flushed from the tibia and femurs of 8- to 10-week-old mice and red blood cells were lysed. Bone marrow cells were plated at a density of 5×10^5 cells per ml in Iscove's modified Dulbecco's medium with recombinant mouse granulocyte-macrophage colony-stimulating factor (20 ng/ml; Kyowa-Hakko Kirin). Fresh medium containing recombinant mouse granulocyte-macrophage colony-stimulating factor was added on day 3 and half of the medium was gently replaced on day 6. LPS (100 ng/ml; Sigma) was added for 1 d on day 6 to induce DC maturation and then 1 mM SIINFEKL was added to the culture 2 h before collection of cells. After extensive washing, 5×10^5 DCs were injected intravenously into mice that had received naive OT-I cells 1 d earlier.

Quantitative RT-PCR. DCs were sorted by flow cytometry, then RNA was extracted with TRIzol (Invitrogen) and cDNA was synthesized with the iScript cDNA Synthesis kit (Bio-Rad). A 480 Real-Time PCR System (Roche) was used for real-time RT-PCR. Values were normalized to the amount of *Rpl32* (which encodes the ribosomal protein L32) in each sample. The primers for quantitative PCR were as follows: TL forward, 5′-TGTATG GCTGTGAGGTGGAG-3′, and reverse, 5′-GCTCCCACTTGCTTCTGGT-3′; and L32 forward, 5′-GAAACTGGCGGAAACCCA-3′, and reverse, 5′-GGA TCTGGCCCTTGAACCTT-3′.

Bacterial infection and quantification of bacteria in organs. ActA$^-$ LM-OVA for immunization was prepared from cultures in brain-heart–infusion broth. LM-N4 and LM-Q4 stably express chicken ovalbumin (amino acids 134–387) containing either the native ligand SIINFEKL (N4) or the altered peptide ligand SIIQFEKL (Q4). Bacteria were washed and resuspended in Hanks' balanced-salt solution before oral infection by gavage. Bacterial stocks for injection were plated to confirm the colony-forming units. For quantification of bacteria in organs, livers were dissected by sterile methods, then were homogenized and lysed with 0.1% (vol/vol) Triton X-100. Serial dilutions were plated onto brain-heart–infusion plates and bacterial colonies were counted after 24 h of incubation at 37 °C.

IEL preparation. Small intestines were removed and separated from Peyer's patches, then were cut longitudinally and then into pieces 0.5 cm in length. Pieces were shaken for 40 min in magnesium-free, calcium-free Hank's balanced-salt solution supplemented with 1 mM dithiothreitol and 5% (vol/vol) FCS. Cells were collected from the washes and were passed over a discontinuous 40–70% (vol/vol) gradient of Percoll (Pharmacia Biotech) for 20 min at 900*g*. IELs were then isolated from the Percoll gradient interface and washed free of Percoll.

Immunofluorescence staining and flow cytometry. For mouse samples, a standard surface-staining protocol included preincubation with anti-CD16-CD32 (2.4G2; prepared 'in-house') to block binding of antibody to the Fc receptor, then cells were stained in cold PBS containing 0.5% (vol/vol) FBS and 0.05% (wt/vol) sodium azide with the relevant labeled antibodies and tetramers. The following antibodies were used: anti-CD8α (53.6.7), anti-CD8β (53-5.8), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD103 (M290) and anti-CD127 (IL-7Rα; A7R34; all from BD Biosciences); and anti-interferon-γ (XMG1. 2; eBioscience). CD8αα was detected with phycoerythrin-labeled TL tetramers. OVA-specific CD8$^+$ T cells were detected with phycoerythrin-labeled H-2K^b–SIINFEKL tetramers. For intracellular staining of interferon-γ, splenocytes or IELs were stimulated for 5 h at 37 °C with SIINFEKL (5 μg/ml) in the presence of brefeldin A. A Cytofix/Cytoperm kit was used according to the manufacturer's directions (BD Biosciences) for intracellular staining of interferon-γ after surface staining. For detection of cell apoptosis and death, cells were stained with annexin V–allophycocyanin according to the manufacturer's protocol (BD Biosciences) and cells were analyzed immediately after staining. For human samples, peripheral blood samples were obtained from healthy volunteers (26–78 years of age). Informed written consent was provided by all participants and the blood collection was approved by the ethics committee of the Innsbruck Medical University. Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation with Ficoll-Hypaque (Amersham Biosciences). The following conjugated antibodies were used for immunofluorescence surface staining of PBMCs: fluorescein isothiocyanate (FITC)-conjugated anti-TCRαβ (T10B9.1A-31), peridinin chlorophyll protein– or allophycocyanin-indotricarbocyanine–conjugated anti-CD3 (SK7), peridinin chlorophyll protein–conjugated anti-CD4 (SK3), peridinin chlorophyll protein–conjugated anti-CD8α (SK1), phycoerythrin- or allophycocyanin-conjugated anti-CD8β (2ST8.5H7), FITC-conjugated anti-CD16 (B73.1), FITC-conjugated anti-CD45RA (HI100), FITC-conjugated anti-CD45RO (UCHL1) and allophycocyanin- or phycoerythrin-indotricarbocyanine–conjugated anti-CD28 (CD28.2; all from BD Biosciences); phycoerythrin- or allophycocyanin-conjugated CMV pp65 (amino acids 495–503) pentamer (ProImmune) and phycoerythrin-conjugated TL tetramer.

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PBMCs were preincubated for 15 min at 23 °C with unlabeled anti-CD8α (2.5 µg/ml (SK1; BD Biosciences)) or anti-CD8β (100 µg/ml (ab71530; Abcam) or 25 µg/ml (2ST8.5H7; BD Biosciences)). After a washing step with PBS, TL tetramer (dilution, 1:100) was incubated for 10 min at 23 °C. Thereafter, PBMCs were stained with the relevant antibodies for 30 min at 4–8 °C. Plasma titers of immunoglobulin G antibody to cytomegalovirus were analyzed by enzyme-linked immunosorbent assay with Enzygnost (Dade Behring). All stained cells were analyzed on a FACSCalibur or FACSCanto II (Becton Dickinson) with FlowJo (TreeStar) or FACSDiva (BD Biosciences).

***In vivo* cytotoxicity assays.** *In vivo* cytolytic activity was determined with B6 splenocytes labeled with different amounts of CFSE. Cells with more labeling ($CFSE^{hi}$) were used as target cells and were pulsed with SIINFEKL (0.5 µg/ml; 90 min at 37 °C and 5% CO_2); $CFSE^{lo}$ cells were pulsed with a negative control peptide (amino acids 180–188 of tyrosinase-related protein 2; 0.5 µg/ml). Peptide-pulsed target cells were washed extensively for the removal of free peptide and then were injected intravenously into recipient mice together at a ratio of 1:1. Then, 16 h later, spleens were removed and the ratio of $CFSE^{lo}$ cells to $CFSE^{hi}$ cells was determined by flow cytometry.

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