Mucosal CD8 Memory T Cells are selected in the periphery by an MHC Class I Molecule

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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 non-classical MHC class I molecule, the thymus leukemia antigen (TL), induced on dendritic cells together with CD8αα on activated CD8αβ$^+$T cells, mediates affinity-based selection of memory precursor cells. Furthermore, constitutive expression of TL on epithelial cells continues the selection of mature CD8αβ memory T cells. The TL-CD8αα-driven memory process is essential for the generation of memory CD8αβ T cells in the intestine and leads to the accumulation of highly antigen sensitive CD8αβ 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 naïve T lymphocytes, or central memory T cells (T_{CM}), that reside in lymphoid tissues, some antigen-experienced T cells gain the capacity to persist long-term as effector memory T cells (T_{EM}) in non-lymphoid tissues, such as the intestine[2-5]. T_{CM} cells, which respond with a robust clonal expansion, are effective at protecting against infections by pathogens that replicate systemically[6], but they likely are inadequate to prevent transmission of viruses, including the human immunodeficiency virus (HIV), or intracellular bacteria, which penetrate across mucosal epithelia[3, 7]. Effective resistance against transmission of such pathogens requires the presence of local antigen-specific T_{EM} prior to re-challenge[7]. Therefore, strategies aimed at inducing a powerful protective immune response that also warrants the formation of pre-existing mucosal antigen-specific T_{EM} are considered an essential goal of successful vaccinations.

Listeria monocytogenes (Lm), a Gram-positive intracellular pathogen of human and other mammals including mice, is a food borne pathogen, which upon ingestion and uptake by phagocytic cells, such as monocytes and dendritic cells (DC) disseminate from the intestine into the bloodstream and spread to various systemic tissues such as the liver[8]. In humans, ingested Lm may cause listeriosis because of its ability to also infect non-phagocytic cells such as the intestinal epithelial cells (IEC) through interaction of the molecule internalin expressed by the Lm and human E-cadherin expressed on the baso-latheral pole of the enterocytes[9]. Mice, by contrast, when infected orally do not develop listeriosis due to the inability of Lm internalin to interact with mouse E-cadherin. Instead, mice clear the infection of ingested bacteria with an effective CD8-dependent protective immune response, although bacteria that crossed the mucosal barrier can spread to the liver and other organs via the blood, as can occur in humans[8]. These observations have important implications for immunization strategies and indicate that the presence of local pre-existing mucosal immunity might be key for the induction of effective protective immunity against food borne pathogens. Despite this however, most of the current knowledge of immune memory has been gained from model systems that use systemic immunization routes and memory generation in lymphoid tissues. Although such pre-existing immunity might be highly effective to combat pathogens that enter the body systemically, it is most likely inadequate to prevent entry and systemic spreading of pathogens, which invade via the mucosal route of the intestine. Because mice can be infected by the oral route similarly to humans and because of the protective endogenous mucosal CD8-mediated immune response that is generated in mice in response to ingested bacteria, together with the emergence of genetically manipulated avirulent attenuated wild type- and recombinant ($ActA^-$) Lm strains as important vectors for vaccinations[8, 10, 11], immunizations of mice with Lm introduced via the oral route represent an optimal approach to

examine mechanisms and conditions that lead to the generation of effective pre-existing mucosal immune memory.

We showed previously that CD8 homodimers (CD8αα), induced on activated CD8αβ T cells, which maintain expression of CD8αβ, mark those primary effector cells that preferentially differentiate to memory cells[12]. In mice, memory CD8αβ T cells that co-express CD8αα are greatly enriched in the epithelium of the intestine[13]. In that same location, the high affinity ligand for CD8αα[14], a non-classical, nonpolymorphic MHC class I molecule, known as the thymus leukemia antigen but hereafter called TL, is constitutively expressed on the adjacent epithelial cells[15, 16]. This suggests a role for epithelial TL in the accumulation of mucosal CD8αα$^+$CD8αβ memory T cells, however the mechanisms that drive the CD8αα-dependent generation of mucosal immune memory remained unknown.

Using the oral Lm infection model to elicit a CD8-driven protective immune response initiated at the mucosal entry site, we define here a novel, affinity-based selection mechanism controlled by TL expression, induced on antigen presenting cells (APC), that leads to the survival and differentiation of high affinity, CD8αα$^+$CD8αβ memory precursor cells. Furthermore, the data also indicate that the constitutive expression of TL on the epithelium of the intestine continues to impose selection pressure, which contributes to the affinity maturation of the resident mucosal CD8αβ T_{EM}.

RESULTS

TL is not required for the formation of memory CD8αβ$^+$T cells.

Considering the class I-like antigen presenting molecules in the mouse genome, TL is distinguished because it has a particularly high affinity for CD8αα, due to unique amino acids

substitutions at three positions in the membrane proximal α3 domain[17]. To assess if TL, the most likely physiologic ligand for CD8αα *in vivo*[12], also plays a role in the generation of CD8αβ effector memory cells, we analyzed CD8αβ T cell memory differentiation in TL gene (*T3*^{b}) knock out (TL$^{-/-}$) mice[18]. The absence of TL did not impair, but rather enhanced, the generation of OVA-specific CD8αβ memory T cells in the spleen of TL$^{-/-}$ mice infected orally with Lm bacteria expressing OVA antigen (Lm-OVA) (**Fig. 1a**). A similar effect was observed in the epithelium of the intestine when intraepithelial lymphocytes (IEL) were analyzed (**Fig. 1a**). Likewise, naïve OVA peptide (OVAp) and H-2K^{b} specific monoclonal TCR transgenic OT-I T cells transferred to WT or TL$^{-/-}$ recipient mice that were subsequently orally infected with Lm-OVA also generated more OT-I TCR transgenic memory T cells in the absence of TL expression (**Fig. 1b**). These observations are consistent with previous published data using single chain MHC class I transgenic mice on a β2m deficient background, which also indicated that in the absence of TL, normal or slightly enhanced memory formed in response to a viral infection[19]. All together, the data show that, whereas CD8αα promotes memory differentiation of CD8αβ^{+} effector T cells[12], its high-affinity ligand TL, appears to inhibit this process.

TL negatively affects memory generation of CD8αβ effector cells.

TL has a restricted pattern of expression[20] that includes induction on antigen presenting cells (APC), such as DC, in addition to expression on epithelial cells[12]. The increase in memory CD8αβ^{+} T cells seen in the absence of TL in TL$^{-/-}$ mice suggests that, under normal conditions, TL expression on subsets of priming DC might negatively influence survival or differentiation of CD8αβ memory precursor cells. To test this possibility, we analyzed the effect of constitutive TL expression during priming using TL transgenic (TL-Tg) mice, which express an allelic form of TL

(*T18*^{d}) under the control of the promoter from the MHC class I molecule, *H-2D*^{d}. In contrast to the outcome in TL$^{-/-}$ hosts, transferred OT-I T cells which were primed *in vivo* in TL-Tg recipient mice that were orally infected with Lm-OVA failed to generate or sustain immune memory, either locally in the intestine or systemically, including in the spleen and liver (**Fig. 1c** and **Supplementary Fig. 1**). Moreover, OT-I T cells primed systemically *in vivo* using TL-Tg OVAp-loaded bone marrow (BM) DC that were adoptively transferred failed to generate memory cells in the spleen of WT hosts (**Fig. 1d**). Similarly, OT-I cells initially primed *in vitro* by TL-Tg OVAp-expressing APC, did not generate memory cells following adoptive transfer (**Fig. 1d**). These data indicate that TL expression on APC interferes with the survival and memory programming of primary CD8$\alpha\beta^{+}$ effector cells.

Under steady state conditions resting spleen DC normally do not express detectable levels of TL surface protein, although some induce it upon activation[12]. However, an analysis of different DC subsets indicated that in contrast to spleen DC, a subset of mesenteric lymph node (mLN) DC constitutively express a low level of TL. This TL^{+} subset has the phenotype of mature migratory DC (MHC class II ^{high}/CD11c^{+} and CD103^{+}CCR7^{+}) (**Supplementary Fig. 2a**), which is also typical of those DC that drive retinoic acid (RA)-based induction of gut homing receptors on the T cells they prime[21], (**Fig. 1e** and **Supplementary Fig. 2b**). The expression of TL on these mucosal DC was further upregulated during priming, and greatly enhanced in response to innate immune stimuli such as CpG oligodeoxynucleotides (**Fig. 1f**). These observations indicate that naïve T cells responding *in vivo* to gut-derived antigens are primed in the context of TL, expressed by the migratory DC and greatly upregulated under inflammatory conditions.

TL induces Fas-mediated death of activated CD8$\alpha\beta^{+}$T cells.

Although TL displays structural characteristics 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 will not permit TL peptide binding and presentation[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 CD8αβ co-receptor, despite the exclusion of TL from the TCR activation complex[14]. A similar interaction of soluble HLA class I molecules with CD8αβ TCR co-receptor, separately from TCR ligation, was previously shown to lead to Fas-FasL-induced cell death[23-26]. To investigate if the TL interaction with CD8αβ on activated T cells might also lead to cell death, we measured the survival of naïve and antigen stimulated CD8αβ$^+$ cells in the presence of constitutive TL expression in TL-Tg hosts. Whereas naïve donor cells survived similarly in WT or TL-Tg mice, activated CD8αβ$^+$ OT-I cells survived only in WT but not in TL-Tg hosts (**Fig. 2a**), supporting the notion that TL-induced cell death (TICD) targets activated CD8αβ$^+$ T cells. Activated Fas-deficient$^{lpr/lpr}$ CD8αβ$^+$ donor T cells, however, were not deleted in TL-Tg recipient mice, providing evidence that, similar to the reported death by soluble HLA-G[23, 24], TICD also involves the Fas/FasL-mediated death pathway (**Fig. 2b**).

Activation-induced CD8αα rescues CD8αβ effector T cells.

In contrast to CD8αβ, CD8αα does not function as a TCR co-receptor and similar to TL, CD8αα also does not engage in the TCR activation complex[27, 28]. However, whereas TL induces death of activated CD8αβ$^+$ T cells, CD8αα, which exhibits a stronger affinity for TL compared to CD8αβ[14], promotes the survival of CD8αβ effector cells[12], suggesting that activation-induced CD8αα might interfere with TICD. To test this, we compared the survival and memory differentiation of CD8αβ effector T cells in the absence or presence of CD8αα. Due to a deletion

of CD8α enhancer region I ($E8_I^{-/-}$)[29], OT-I CD8αβ donor cells on the $E8_I^{-/-}$ background ($E8_I^{-/-}$OT-I CD8αβ^{+}) do not induce detectable levels of activation-dependent CD8αα[12]. When analyzed *in vitro*, $E8_I^{-/-}$OT-I CD8αβ T cells primed by OVA-loaded mLN DC, which constitutively express TL (**Fig. 1e**), showed increased activation induced death compared to their counterparts primed by spleen DC (**Fig. 3a**). However, the increase in cell death induced by these mucosal APC was not observed when mLN and spleen DC isolated from $TL^{-/-}$ mice were compared (**Fig. 3a**).

Furthermore, similar to our previous published results showing impaired spleen memory generation by $E8_I^{-/-}$ CD8αβ T cells in response to a systemic immunization with *Lymphocytic Choriomeningitis* virus (LCMV)[12], $E8_I^{-/-}$OT-I CD8αβ donor cells primed *in vivo* with Lm-OVA via the oral route, also failed to generate detectable memory cells in the spleen or the intestine of WT recipient mice (**Fig. 3b**). In contrast, in another study by Chandele et al., using the same systemic LCMV immunization approach, $E8_I^{-/-}$ CD8αβ T cells did generate systemic memory[30]. However in that case, a drastic downregulation of CD8αβ was noticed during the initial priming phase, which was not seen on either WT CD8αβ T cells or the LCMV primed $E8_I^{-/-}$ CD8 T cells in our published study[12]. It is possible that stronger activation conditions in the Chandele study might have caused the downregulation of CD8αβ, which likely resulted in similar effects on survival as mediated by activation-induced CD8αα on normal WT CD8αβ T cells. To test this, we analyzed the memory response of $E8_I^{-/-}$OT-I CD8αβ^{+} T cells primed systemically with Lm-OVA introduced intravenously (i.v.), which induces a much more potent immune response as compared to the oral route[8]. In contrast to the oral immunization, systemic priming of the $E8_I^{-/-}$OT-I CD8αβ^{+} T cells with Lm-OVA resulted in a clear memory response in the spleen and in the intestine comparable to that of WT OT-I cells (**Fig. 3c**). Furthermore, similar to the Chandele study[30], a drastic downregulation of CD8αβ was noticed during the potent priming of $E8_I^{-/-}$OT-I CD8αβ^{+} T cells

activated via the i.v. route, whereas, such downregulation was not observed when $E8I^{-/-}$OT-I CD8αβ^{+} T cells were primed via the oral route (**Fig. 3d**). Although the activation-induced downregulation of CD8αβ could explain the survival of the $E8I^{-/-}$OT-I CD8αβ^{+} T cells during the systemic priming, it does not explain the longterm survival of these cells in the intestine where TL is constitutively expressed on the IEC. To further investigate this, we analyzed the CD8αβ expression of the $E8I^{-/-}$OT-I CD8αβ^{+} memory T cell subsets in the spleen and the intestine. Interestingly, whereas $E8I^{-/-}$OT-I CD8αβ^{+} memory cells in the periphery expressed normal levels of CD8αβ, memory $E8I^{-/-}$OT-I CD8αβ^{+} IEL that persist in the presence of TL expressed much reduced levels of CD8αβ (**Fig. 3e**). These results indicate that the mechanism of TICD continuously and selectively shapes the repertoire of the memory cells that accumulate at the mucosal site of the intestine. In support of this, in the absence of TL expression in the intestine of $TL^{-/-}$ hosts, similar to the systemic priming, there was no difference in the efficiency of memory formation in the spleen or the intestine when WT OT-I or $E8I^{-/-}$OT-I CD8αβ^{+} T cells were primed via the oral route (**Fig. 3f**). Furthermore, there was also no selective accumulation of CD8αα expressing WT OT-I CD8αβ IEL in the $TL^{-/-}$ hosts, indicating that TL on the epithelial cells in the intestine imposes a selective pressure to promote the local accumulation of CD8αα-expressing effector memory T cells (**Fig. 3g**). These results indicate that the requirement for activation-induced CD8αα on primary effector cells is in part determined by the presence of TL. The data are consistent with a role for activation-induced CD8αα in sequestering TL ligand away from the CD8αβ co-receptor, thereby avoiding TICD of the CD8αβ^{+} primary effector T cells.

CD8αα expression is linked to the intensity of TCR activation.

Due to the relatively high affinity interaction of CD8αα with TL, activation-induced expression of CD8αα can be distinguished from CD8αβ co-receptor expression using TL tetramers[12]. Activated CD8αβ T cells do not all induce CD8αα to the same extent, and using TL tetramers a variegated expression pattern of high (CD8αα^{hi}) and low (CD8αα$^{lo/-}$) expression is typically observed. 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 this, TL tetramer staining of polyclonal CD8αβ^{+} WT T lymphocytes activated *in vitro* with variable concentrations of soluble α-CD3 and α-CD28 antibodies showed graded increase in CD8αα expression with higher concentrations of α-CD3/CD28 mAbs (**Fig. 4a**). Furthermore, OT-I T cells stimulated with different altered peptide ligands (APL) that bind equally well to the K^{b} class I molecule as the original OT-I ligand, SIINFEKL (N4), but which have different antigenic potencies[31], also showed a tight association between the level of CD8αα induction and the degree of TCR activation. Therefore the high affinity N4 ligand induced the highest amounts of CD8αα expression compared to the lower-affinity altered peptide ligands (**Fig. 4b**). Similar results of affinity-based induction of CD8αα were obtained *in vivo* when mice were analyzed that were infected orally with Lm-OVA after they received either a high- or a low-precursor frequency of naïve donor OT-I cells, which because of antigenic competition will model a low or high antigen dose, respectively (**Fig. 4c**). Additionally, we analyzed recipient mice adoptively transferred with equal amounts of OT-I precursor cells, but orally infected with bacteria expressing either the low-affinity Q4 (Lm-Q4)- or the high-affinity N4 (Lm-N4)-antigen. Mice receiving higher affinity N4 as compared to Q4 antigen generated more CD8αα-expressing CD8αβ T cells (**Fig. 4d**). In each of these approaches, the results

consistently indicated that the level of CD8αα induction represents a sensitive measurement for the intensity of the signal strength received through the activated TCR.

Similar to activation-induced CD8αα; IL-7 receptor (IL-7Rα) expression also has been proposed as a marker for memory precursor cells[32]. However, in contrast to CD8αα, IL-7R expression did not correlate with TCR activation, and instead, IL-7R was constitutively expressed on naïve cells and initially down regulated during activation at the time when CD8αα is first up regulated (**Supplementary Fig. 3a**). The reciprocal expression of CD8αα and IL-7R suggests different roles for these molecules in memory programming and/or survival. Consistent with this, a mutation in the cytoplasmic IL-7Rα Y449XXM motif (IL-7Rα^{449F}), 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/ IL-7Rα^{449F} memory T cells in response to an oral immunization with Lm-OVA (**Supplementary Fig. 3b**). These data indicate 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/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 avoid TICD therefore represents a mechanism to selectively preserve the most avid CD8αβ primary effector cells as part of the memory precursor pool.

CD8αα expression marks high affinity CD8αβ T cells in humans.

Previous evidence indicated that the mouse TL tetramers also detect expression of human CD8αα homodimers[12, 17]. Consistent with activation-induced expression of CD8αα, human CD8αβ effector cells also stained with TL tetramers, whereas naïve T cells did not (**Fig. 5a**). TL

tetramer staining could be blocked with an anti-human CD8α antibody, but not with an anti-CD8β antibody, confirming the specificity of the TL tetramer for human CD8αα (**Fig. 5b** and **Supplementary Fig. 4**). Furthermore, the subset of immuno-dominant, high affinity, CMV-pp65-specific CD8αβ T cells[34], isolated from *Cytomegalovirus* (CMV)-sero-positive individuals, stained almost exclusively with TL tetramer, which could be blocked with anti-CD8α (**Fig. 5c**), indicating that CD8αα expression on human CD8αβ effector T cells also is associated with high affinity effector cells.

Retinoic acid and TGF-β promote affinity-based accumulation of CD8αα$^+$CD8αβ T cells.

CD8αα-expressing CD8αβ T cells are highly enriched in the epithelium of the intestine, suggesting that a selective process based on CD8αα expression might drive this localized accumulation. Consistent with this, when primed *in vitro* CD8αα induction was stronger using mLN DC compared to spleen DC (**Fig. 6a,b**). In the presence of exogenous RA, normally released by mLN DC during priming[21], spleen DC also mediated strong induction of CD8αα on the OT-I T cells they primed (**Fig. 6a,b**). Whereas a retinoic acid receptor (RAR) inhibitor reduced CD8αα expression on T cells primed by mLN DC (**Fig. 6a**). Likewise, TGF-β known to be an important modulator of mucosal T cell differentiation also increased CD8αα induction on activated CD8αβ primary effector cells (**Fig. 6c**). Both, RA- and TGF-β-mediated enhanced induction of CD8αα were observed, however, only under strong antigen activation conditions, suggesting that they influence CD8 effector differentiation by further promoting the selective marking of high affinity effector cells by CD8αα (**Fig. 6b, c**).

The constitutive and enhanced expression of TL on the mucosal migratory DC known to release RA and TGF-β (**Fig. 1e, f**), together with the increased CD8αα induction on high affinity effectors in the presence of RA as well as TGF-β, indicate that the selective rescue of high affinity memory precursor cells is geared toward effector cells that home to the gut. In agreement with this, CD8αα-expressing OT-I cells were first detectable at mucosal induction sites, such as the mLN and Peyer's patches, early after oral antigen exposure (**Fig. 6d**) and gradually accumulated during the contraction and memory phase within the pool of $\alpha_E\beta_7$ integrin ($CD103^+$) T_{EM} in the gut epithelium, but not in the $CD103^-$ memory cells that persist systemically (**Fig. 6e**).

Constitutive TL on epithelial cells continues to select mature CD8αβ T_{EM}.

The constitutive TL expression on intestinal epithelial cells[15, 16], suggests that TL might continuously shape the resident mucosal memory $CD8\alpha\beta^+$ T cell population even after rechallenge. To investigate this, we examined the fate of primary and secondary $CD8\alpha\alpha^{hi}$- or $CD8\alpha\alpha^{lo/-}$-$CD8\alpha\beta^+$ effector T cells *in vivo*. OT-I cells initially primed *in vitro* in the absence of TL, using TL negative APC, were sorted into $CD8\alpha\alpha^{hi}$- and $CD8\alpha\alpha^{lo/-}$-primary effector T cells and adoptively transferred to WT recipient mice. Both subsets of primary effector cells displayed a similar short term homing capacity (**Supplementary Fig. 5a**), and both effector T cell types responded alike when tested *in vitro* for IFNγ production (**Supplementary Fig. 5b**) or *in vivo* for cytotoxicity (**Supplementary Fig. 5c**). However, in response to an oral re-challenge with Lm-OVA, primary memory OT-I T cells derived from the $CD8\alpha\alpha^{hi}$ precursors expanded in the spleen and in the intestine of the WT recipient mice, whereas memory cells from the $CD8\alpha\alpha^{lo/-}$ effector pool were only detectable in the host spleen, but not in the intestine (**Fig. 7a**). These data indicate that only $CD8\alpha\alpha^{hi}$ primary memory cells can persist long-term as mucosal T_{EM} in proximity of TL

constitutively expressed on the epithelial cells. Upon re-challenge, activated memory cells in lymphoid tissues expand and migrate as secondary effector cells to non-lymphoid tissues, such as the epithelium of the gut[35]. In agreement with this, comparable numbers of effector cells derived from either CD8αα^{hi} or CD8αα$^{lo/-}$primary effector OT-I cells were present in the intestine 5 days after recall (**Fig. 7b**). Nevertheless, when analyzed 45 days later, progeny of CD8αα^{hi} effector cells were present as secondary memory cells in both the spleen and the intestine, whereas the CD8αα$^{lo/-}$ secondary effector cells did not remain as secondary T_{EM} in the intestine (**Fig. 7c**). In contrast, *in vitro* primed CD8αα$^{lo/-}$CD8αβ^{+} OT-I effector cells accumulated efficiently as T_{EM} within the gut epithelium of TL$^{-/-}$ recipient mice (**Fig. 7d**), indicating that the constitutive expression of TL continues to mediate selective pressure that prevents the accumulation of CD8αα$^{lo/-}$ primary and secondary effector cells as mucosal T_{EM}. These results also specify that CD8αα$^{lo/-}$effector cells are not intrinsically incapable of converting to mucosal T_{EM}, but that under normal physiological conditions, only CD8αα^{hi} primary effector cells form long-lived mucosal T_{EM} in proximity to TL constitutively expressed on the epithelial cells.

Constitutive TL mediates affinity maturation of mucosal memory CD8αβ T cells.

A recent study, using low-affinity APL for *in vivo* priming, suggested that affinity maturation of memory T cells was the result of enhanced expansion and delayed contraction of the high affinity responding effector cells[31]. Our results here suggest that, in addition, the TL-mediated selective survival of CD8αα-expressing high affinity memory cells might also contribute to ensure affinity maturation of memory populations at the mucosal border. To provide direct evidence for this, we used the same APL approach[31] to examine memory generation *in vivo* in the presence or absence of TL. Naïve OT-I cells were adoptively transferred to either WT or TL$^{-/-}$ recipient mice

that were subsequently orally infected with bacteria expressing the low affinity APL, Q4 (Lm-Q4), which does not effectively induce CD8αα on primed OT-I effector cells (**Fig. 4d**). Fewer OT-I effector cells were detectable in the blood of WT mice compared to $TL^{-/-}$ animals when analyzed 7 days after oral immunization with Lm-Q4 (**Fig. 7e**), suggesting that TL induced on the priming mucosal APC controls in part the expansion of the primary effector pool. In the intestine, where TL is constitutively expressed by the IEC, the selective impact of TL was even more pronounced and OT-I memory IEL generated in response to the low affinity peptide Q4 are barely detectable in WT animals whereas they were readily present in the $TL^{-/-}$ mice (**Fig. 7f**). These results indicate that the continuous expression of TL on the epithelium of the intestine selectively eliminates low affinity cells from the pool of mature mucosal T_{EM}. Together with the increase in mucosal memory in the $TL^{-/-}$ mice, also the systemic memory pool was increased (**Fig. 7f**), suggesting that in addition to the TL-mediated affinity selection of mature memory cells in the intestine, TL induced on the mucosal APC during priming might already provide a first affinity- selection step by eliminating low affinity/avidity primary effector cells form the memory precursor pool. To further test this, we immunized WT or $TL^{-/-}$ recipient mice with Lm-Q4 via the i.v. 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 comparable between the $TL^{-/-}$ and WT recipient mice (**Figs. 7g and 7h**). In sharp contrast however, the accumulation of T_{EM} 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 undergo an initial selection step mediated by TL induced on the migratory DC, whereas the constitutive expression of TL on the mucosal epithelium provides for additional and constant selection pressure that drives continuous affinity

maturation of the mature memory pool that accumulates longterm at the mucosal interface of the intestine.

To evaluate the importance of pre-existing TL-CD8αα-selected mucosal memory T cells residing at this entry port, we examined the resistance against an oral infection with Lm in mice with or without affinity-selected memory cells. To this end, mice transferred with either WT or $E8_I^{-/-}$ OT-I cells were orally immunized with an attenuated strain of Lm-OVA ($ActA^-$ Lm-OVA) (10^9 cfu). The mice were subsequently re-challenged orally with a higher dose (10^{10} cfu) of WT Lm-OVA and analyzed for systemic spreading of the pathogen. In contrast to the efficient resistance observed in mice which received the WT OT-I precursor cells, which generated memory cells in the intestine and elsewhere, pre-immunized mice that initially received the $E8_I^{-/-}$OT-I precursor cells failed to generate affinity selected memory and they showed significantly less resistance to prevent systemic spreading of the orally introduced pathogen (**Fig. 7i**). These results underscore the importance of the selective immune memory differentiation process as a critical mechanism for the efficient generation of pre-existing immunity at critical mucosal interfaces that form the main entry sites for invading pathogens.

DISCUSSION

The data presented here define a fundamentally new concept for our understanding of immune memory differentiation and in particular mucosal immunity. The data demonstrate that an affinity-based selective process operates *in vivo* that preserves the optimal effector cells to become long-lived memory T cells that form pre-existing and heightened protective immunity. This mechanism is especially geared to generate high affinity T_{EM} that line the mucosal barrier of the intestine, where most pathogens enter the body.

The results also demonstrate a functional role TL, a non-classical MHC class I-like molecule, in mediating TICD of CD8αβ effector T cells that fail to induce CD8αα. Because TL is a much stronger ligand for CD8αα as compared to CD8αβ, CD8αα likely can sequester TL away from CD8αβ and prevent TICD (**Supplementary Fig. 6**). The induction of CD8αα is directly linked to the degree of TCR signal strength, which leads to the selective survival of the most avid effector cells to become memory T cells. The constitutive expression of TL on the epithelial cells in the intestine continues selective pressure which mediates affinity maturation of the mucosal T_{EM} population in response to repeated re-challenges (**Supplementary Fig. 6**).

The data here, using the Lm model are consistent with our previously published study using the LCMV model system, which also indicated a critical role for activation-induced CD8αα in the generation of CD8αβ memory T cells, Nevertheless, two subsequent studies challenged this conclusion and provided evidence that under certain conditions CD8αβ^{+} E8$_{I}^{-/-}$ memory T cells could also be generated in a CD8αα-independent fashion[30, 36]. In those two studies however and similar to what we observed here using Lm immunizations via the iv route, there was a pronounced down regulation of CD8αβ expression during the priming of the E8$_{I}^{-/-}$ CD8αβ T cells, which was not observed in our previous study using the LCMV system or during oral immunizations using the Lm system as shown here. The fact that in the absence of CD8αβ downregulation in both of those cases, E8$_{I}^{-/-}$ CD8αβ T cells failed to accumulate as memory cells, indicates that strong activation signals which result in down regulation of CD8αβ co-receptor on the E8$_{I}^{-/-}$mutant cells may rescue CD8αβ memory precursor cells similarly to the biological activity of activation-induced CD8αα on normal WT CD8αβ effector cells.

Although, a sequence-based homologue for TL in humans has not been identified, TL and the non-classical MHC class I molecule, HLA-G, display striking similarities, including their

restricted pattern of expression, the limited antigen presentation capacity, the relative strong affinity for CD8αα compared to CD8αβ and the ability to induce Fas-FasL cell death of activated CD8αβ T cells[25, 37, 38]. These parallels suggest that, similar to other non-classical MHC/HLA pairs, TL and HLA-G might represent functional homologues of each other, and they further suggest that an affinity-based selective memory differentiation program likely operates in humans as well. Consistent with this, we showed that activated but not naïve human CD8αβ T cells co-express CD8αα, and CD8αα expression also coincided with the high affinity CMVpp65-specific CD8αβ effector T cells. In addition, a recent study indicated that the endogenous protective immune response against HIV was characterized by a CD8αα-expressing CD8αβ subpopulation that exhibited strong antiviral activity[39] and high-avidity HIV-specific $CD8^+$ T cell clonotypes were largely preserved in patients who control viremia, but not in progressive chronic HIV infections. All together, these observations suggest that CD8αα induction on human CD8αβ T cells is also an activation-induced and affinity-based process that marks the avid effector cells.

Most infections, including Lm but also many viral infections such as HIV and SIV infections, are acquired across mucosal barriers, and several studies have demonstrated that $CD8^+$ CTL responses play 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 onset of disease, as well as reduce the potential for secondary transmission. Therefore, the finding that an endogenous TCR quality-based mechanism may select for the most avid effector cells to form immune memory cells that have the capacity to reside longterm at mucosal interfaces, has significant implications for the design of new and improved strategies to induce effective pre-existing protective immunity, not only systemically but also locally at the major and most vulnerable entry sites for pathogens.

METHODS

Mice. C57BL/6 CD45.2 (Ly5.2), referred to as WT, and CD45.1 (B6.SJL, Ly5.1) congenic, B6.MRL-*Fas*^{lpr}/J and B6.Smn.C3-*FasL*^{gld}/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). OT-I TCR-transgenic mice were crossed onto the Ly5.1 congenic background. Additionally, E8$_I^{-/-}$, IL-7Rα^{449F}, and TL-Tg mice were crossed to OT-I mice. TL-Tg mice were generated by forced expression of the *T18*^{d} gene under the *D*^{d} promoter and backcrossed to the C57BL/6 background for more than 12 generations. TL$^{-/-}$ mice were generated by deletion of the *T3*^{b} gene in the C57BL/6 background. Mice were maintained by breeding under specific pathogen-free conditions in the animal facility of the La Jolla Institute for Allergy & Immunology. Unless otherwise noted, mice were maintained under specific pathogen-free conditions. Sentinel mice from the *RAG1*$^{-/-}$ colony were tested to be negative for *Helicobacter spp*. and *Citrobacter rodentium*. Animal care and experimentation were consistent with the NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the La Jolla Institute for Allergy and Immunology.

T cell isolation, cell sorting, CFSE labeling and adoptive transfer. CD8$^+$ OT-I cells were purified by magnetic negative selection using the MACS CD8α^+ T cell isolation kit according to the manufacturer's protocol (Miltenyi Biotec). CD44^{low} cells were sorted as naïve CD8 OT-I cells by FACSAria (Becton Dickinson). In some cases, sorted cells were CFSE-labeled. OT-I cells were resuspended at a concentration of 10×10^6 cells/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% FCS. Cells were washed with PBS three times and intravenously injected into the recipient mice.

DC isolation and activation. Pooled spleens or MLNs were cut into small pieces and digested with collagenase D (Roche) at room temperature for 30 min and EDTA (5 mM in final concentration) was added for the last 5 min. Digested tissues were further homogenized and then enriched for $CD11c^+$ DC by positive selection using a MACS cell separation (Miltenyi Biotec). For DC activation *in vitro*, freshly isolated spleen DC were cultured with 1 mM CpG DNA (1826, IDT) for 1 day and analyzed for TL expression by using anti-TL antibody (HD168).

In vitro **T cell stimulation culture.** For total splenocyte culture, 5×10^5 total splenocytes were cultured in 96-well plates in the presence of anti-CD3 and anti-CD28 antibodies. For artificial APC/OT-I cultures, either the transfected adherent fibroblast cell line (MEC.B7) expressing the costimulatory molecule B7.1, or the OVA-expressing cell line (APC, MEC.B7.SigOVA) having the OVA-derived, H-$2K^b$-restricted peptide epitope $OVA_{257\text{-}264}$ (SIINFEKL), along with B7.1, were used. Irradiated APCs were cultured at 150,000 cells per well in 24-well plates overnight to establish a single layer. Naïve OT-I cells (5×10^5) were added to the monolayer of APCs in 2 ml of medium. The TL expressing APC (APC-TL) were generated by transfection of MEC.B7.SigOVA cells with a plasmid expressing $T18^d$ gene. TL expression was confirmed by staining with a TL-specific antibody (HD168). For DC/OT-I culture, purified DC were incubated with 1 nM SIINFEKL for 2 h and then washed. 2×10^5 CFSE-labeled naïve OT-I cells were cultured with 4×10^4 peptide-pulsed DC, with or without 100 nM all-trans RA (Sigma), in 96-well plates for 3-4 d. RA receptor antagonist LE135 (TOCRIS bioscience) was used for some cultures.

Bone marrow-derived DC immunization. Bone marrow was flushed from the tibia and femurs of 8-10-week-old mice and red blood cells were lysed. Bone marrow cells were plated at 5×10^5 cells/ml in complete IMDM with 20 ng/ml rmGM-CSF (Kyowa-Hakko Kirin California). Fresh media containing rmGM-CSF were added on day 3 and half of the media was gently replaced on day 6. 100 ng/ml LPS (Sigma) was added on day 6 for 1 d to induce DC maturation and then 1 mM SIINFEKL was added to the culture 2 h before collecting cells. After extensive washing, 5×10^5 DC were injected *i.v.* in mice, which had previously received naïve OT-I cells 1 d earlier.

qRT-PCR. RNA from the FACS-sorted DC were extracted with TRIZol (Invitrogen) and complementary DNA (cDNA) were synthesized using the iScript cDNA Synthesis kit (Bio-Rad). Real time RT-PCR was performed with the Roche 480 real-time PCR System. Values were normalized by the amount of L32 in each sample. The primers for qPCR are as following: TL-forward 5'-TGT ATG GCT GTG AGG TGG AG-3', TL-reverse 5'-GCT CCC ACT TGC TTC TGG T-3'; L32-forward 5'-GAA ACT GGC GGA AAC CCA-3', L32-reverse 5'-GGA TCT GGC CCT TGA ACC TT-3'.

Bacterial infection and bacterial counts in organs. ActA$^-$ Lm-OVA for immunization were prepared from cultures in brain heart infusion broth. Lm-N4OVA and Lm-Q4OVA strains stably express chicken ovalbumin ($AA_{134–387}$) containing either the native ligand $SIINFEKL_{257–264}$ (N4) or the altered peptide ligand SIIQFEKL (Q4). Bacteria were washed and resuspended in Hanks' balanced salt solution (HBSS) prior to oral infection by gavage. Bacterial injection stocks were plated to confirm the CFU. To count the bacteria in organs, livers were sterilely dissected,

homogenized and lysed with 0.1% Triton X-100. Serial dilutions were plated onto BHI plates and bacterial colonies were counted 24 h after incubation at 37 °C.

IEL preparation. In brief, small intestines were removed and separated from Peyer's patches. They were cut longitudinally and then into 0.5 cm pieces. The pieces were shaken for 40 min in Mg^{2+}-free, Ca^{2+}-free HBSS supplemented with 1 mM dithiothreitol and 5% FCS. Cells were collected from the washes and passed over a discontinuous 40/70% Percoll (Pharmacia Biotech) gradient at 900 *g* for 20 min. IEL 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 pre-incubated with anti-CD16/CD32 Fc-receptor antibody (2.4G2) to block Fc-antibody binding. Then, cells were stained in cold PBS containing 0.5% FBS and 0.05% sodium azide with the relevant labeled antibodies and tetramers. The following antibodies were used: CD8α (clone 53.6.7), CD8β (clone 53-5.8), CD44 (clone IM7), CD45.1 (clone A20), CD45.2 (clone 104), CD103 (clone M290), CD127 (IL-7Rα , clone A7R34), and IFN-γ (clone XMG1. 2). All primary antibodies were directly conjugated to fluorophores (BD Biosciences or eBioscience). CD8αα was detected with PE-labeled TL-tetramers. OVA-specific CD8 T cells were detected with PE-labeled K^b/ $OVA_{257-264}$ tetramers. For intracellular staining of IFNγ, splenocytes or IEL were stimulated with $OVA_{257-264}$ (5 μg/ml) for 5 h in the presence of brefeldin A at 37°C. After surface staining, intracellular cytokine staining for IFNγ was performed using a Cytofix/Cytoperm Kit (BD Biosciences), according to the manufacturer's directions. For detection of cell apoptosis and death, cells were stained with Annexin V-APC (BD Biosciences) using the

manufacturer's protocol and cells were analyzed immediately after staining. For human samples, peripheral blood samples were obtained from healthy volunteers (26-78 years old). Informed written consent was obtained from all participants and the blood collection was approved by the ethics committee of the Innsbruck Medical University. Isolation of peripheral blood mononuclear cells (PBMC) was performed by density gradient centrifugation using Ficoll-Hypaque (Amersham Biosciences). Immunofluorescence surface staining of PBMC was performed using the following conjugated antibodies: TCRαβ (FITC), CD3 (PerCP or APC-Cy7), CD4 (PerCP), CD8α (PerCP), CD8β (PE or APC), CD16 (FITC), CD28 (APC or PE-Cy7), CD45RA (FITC) and CD45RO (FITC) (BD Biosciences), CMVpp65$_{495-503}$ pentamer (PE or APC; ProImmune) and TL tetramer (PE). PBMC were pre-incubated with unlabeled anti-CD8α (clone SK1; 2.5 μg/mL; BD Biosciences) or anti-CD8β (polyclonal; 100 μg/mL; Abcam or clone 2ST8.5H7; 25 μg/mL; BD Biosciences) for 15 min at room temperature. After a washing step with PBS, TL tetramer (1:100) was incubated for 10 min at room temperature. Thereafter, PBMC were co-stained with the relevant antibodies for 30 min at 4-8°C. Plasma CMV IgG titers were analyzed by ELISA using Enzygnost® (Dade Behring). All the stained cells were analyzed on a FACSCalibur or FACSCanto II® flow cytometer (Becton Dickinson) and FlowJo software (Three Star) or FACSDiva® Software (BD Biosciences).

***In vivo* cytotoxicity assays.** *In vivo* cytolytic activity was determined using B6 splenocytes differentially labeled with CFSE. The cells highly labeled (CFSE^{high}) were used as target cells and pulsed with OVA$_{257-264}$ (0.5 μg/ml; 90 min at 37°C, 5% CO_2), whereas CFSE^{low} labeled cells were pulsed with a negative control peptide, TRP-2$_{180-188}$ (0.5 μg/ml). Peptide-pulsed target cells were extensively washed to remove free peptide and then co-injected intravenously in a 1:1 ratio to

recipient mice. Sixteen hours later, spleens were removed and the ratio of $CFSE^{low}/CFSE^{high}$ cells was determined by flow cytometry.

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FIGURE LEGENDS

Figure 1: TL negatively affects memory generation of CD8αβ$^+$ T cells. (**a**) WT or TL$^{-/-}$ mice were orally infected with 1×10^9 ActA$^-$ Lm-OVA. 30 days p.i., splenocytes (SPL) and IEL were isolated for IFN-γ intracellular staining after *ex vivo* re-stimulation with $OVA_{257-264}$ peptide. Graph depicts pooled data ± s.e.m.. (**b**) 5×10^4 naïve Ly5.1$^+$ CD8$^+$ OT-I cells were adoptively transferred into WT or TL$^{-/-}$ recipients. One day after transfer, mice were orally infected with 1×10^9 ActA$^-$ Lm-OVA. Donor OT-I cells were tracked in the spleen and IEL 2 months p.i. Graph depicts pooled data ± s.e.m.. (**c**) 1×10^6 naïve Ly5.1$^+$ OT-I cells were transferred into WT or TL-Tg recipients. One day after transfer, mice were orally infected with 1×10^9 ActA$^-$ Lm-OVA. The donor OT-I cells in the spleens and IEL were tracked two months p.i. Graph depicts pooled data ± s.e.m.. (**d**) 5×10^4 Ly5.1$^+$ naïve OT-I cells were transferred into C57BL/6 mice which were subsequently immunized i.v. with 5×10^5 OVAp-loaded DCs generated from bone marrow cells (BMDC) of WT or TL-Tg mice (top). Alternatively, OT-I cells were primed *in vitro* with TL negative APC or APC transfected with TL and then transferred to B6 recipients (bottom). Memory OT-I cells in the spleen were analyzed 2 months after DC immunization or after transfer of *in vitro* activated OT-I cells. Graph depicts pooled data ± s.e.m.. (**e**) Migratory mLN DCs express a high level of TL. Spleen or mLN DC were sorted based on the CD11c and MHC II expression and assessed for TL expression by flow cytometry. Data are representative from three independent experiments. (**f**) Freshly isolated SPL or mLN DC were activated with CpG for 1 d and then analyzed for TL expression. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (unpaired *t*-test).

Figure 2: TL mediates death of activated CD8αβ$^+$ T cells. (**a**) 1×10^6 naïve Ly5.1$^+$ OT-I cells were transferred into WT or TL-Tg recipients and the donor cells in the spleens were tracked one

month after transfer (top, n = 5 per group). 1 × 10^6 *in vitro* activated Ly5.1$^+$ OT-I cells were transferred into WT or TL-Tg recipients and the donor cells in the spleen were tracked one month after transfer (bottom, n = 8 per group). (**b**) Naïve CD8 T cells sorted from WT, FasL^{gld} or Fas^{lpr} mice were stimulated *in vitro* by anti-CD3/-CD28 beads for 3 d. 0.5 × 10^6 activated CD8 T cells were transferred into Ly5.1$^+$ WT or TL-Tg recipients and the donor cells in the spleens were tracked 1 month after transfer. Representative data are shown of four mice analyzed in each group. Two independent experiments were performed in each case.

Figure 3: Activation-induced CD8αα rescues CD8αβ primary effector T cells from TICD. (**a**) 2 × 10^5 CFSE-labeled naïve E8$_I^{-/-}$ OT-I cells were cultured with 4 × 10^4 OVAp-pulsed DCs from SPL or mLN of WT or TL$^{-/-}$ mice that were previously orally infected with ActA$^-$ Lm-OVA. After 2 d culture, OT-I cells were harvested and analyzed for cell death by Annexin V staining. Data are representative of three independent experiments. (**b**) 5 × 10^4 naïve Ly5.2$^+$ WT or Ly5.2$^+$ E8$_I^{-/-}$ OT-I cells were adoptively transferred into Ly5.1 recipient mice. 1 d after transfer, mice were orally infected with 1 × 10^9 ActA$^-$ Lm-OVA. Donor OT-I cells were tracked in the spleen and IEL, 2 months p.i.. Graph depicts pooled data ± s.e.m.. (**c**) 5 × 10^4 naïve Ly5.2$^+$ WT or Ly5.2$^+$ E8$_I^{-/-}$ OT-I cells were adoptively transferred into Ly5.1 recipient mice. 1 d after transfer, mice were intravenously infected with 2.5 × 10^5 ActA$^-$ Lm-OVA. Donor OT-I cells were tracked in the spleen and IEL, 2 months p.i.. Graph depicts pooled data ± s.e.m.. (**d**) As described in (b) and (c), CD8β expression was measured on effector WT OT-I or E8$_I^{-/-}$ OT-I cells 7 days post oral infection (left panel) or i.v. infection (right panel). (**e**) As described in (c), CD8β expression was measured on memory WT OT-I or E8$_I^{-/-}$ OT-I cells 2 m post i.v. infection in the spleen and IEL (**f, g**) 5 × 10^4 naïve Ly5.1$^+$Ly5.2$^+$ WT OT-I and 5 × 10^4 naïve Ly5.2$^+$ E8$_I^{-/-}$ OT-I cells were co-transferred into

Ly5.1$^+$ WT or Ly5.1$^+$ TL$^{-/-}$ mice. One day after transfer, the mice were orally infected with 1×10^9 ActA$^-$ Lm-OVA. Two months p.i., memory OT-I cells were tracked in the spleen and IEL. (f) Graph depicts pooled data ± s.e.m. (n = 5 per group). (g) Staining for CD8αα expression, using TL-tetramers, on gated memory WT OT-I cells in IEL of WT or TL$^{-/-}$ recipient mice 2m p.i. Representative of at least two independent experiments.

Figure 4: CD8αα expression correlates with the intensity of TCR activation. (**a**) Total splenocytes were cultured in the presence of graded concentration of soluble anti-CD3 and anti-CD28 antibodies. CD8αα expression, as measured by TL tetramer staining, was analyzed 3 d after *in vitro* culture. Representative data on gated CD8$^+$ T cells are shown. Three independent experiments were performed. (**b**) Naïve OT-I cells were cultured with artificial APC (MEC.B7) in the presence of graded concentration of $OVA_{257-264}$ SIINFEKL (N4) or altered peptide ligands (Q4R7 and Q4). CD8αα expression was detected 2 d after *in vitro* culture. Three independent experiments were performed. (**c**) 100,000 or 1,000 sorted naïve Ly5.1$^+$ CD8$^+$ OT-I cells were transferred into B6 recipient mice. 1 d after transfer, mice were orally infected with 1×10^9 ActA$^-$ Lm-OVA. 7 d p.i., CD8αα expression was analyzed on Ly5.1$^+$ CD8$^+$ OT-I cells from the spleen and IEL (n = 4 mice per group). (**d**) 5×10^4 naïve Ly5.1$^+$ CD8$^+$ OT-I cells were transferred into WT recipient mice. 1 d after transfer, mice were orally infected with 2×10^8 WT Lm-Q4OVA or Lm-N4OVA. 7 d p.i., CD8αα expression was analyzed on donor OT-I cells from the spleen and IEL (n = 5 mice per group).

Figure 5: CD8αα expression marks effector memory CD8αβ T cells in humans. (**a**) Expression of CD8αα on polyclonal human naive (T_N; CCR7$^+$CD45RA$^+$), recently activated

effector-memory (T_{EMRA}; $CCR7^{-}CD45RA^{+}$), effector-memory (T_{EM}; $CCR7^{-}CD45RA^{-}$) and central-memory (T_{CM}; $CCR7^{+}CD45RA^{-}$) $CD8^{+}$ T cells was measured by TL-tetramer staining. The numbers indicate the percentage of TL-tetramer^{hi} cells. Graph depicts pooled data ± s.e.m. on percentage of CD8αα expression on human $CD8^{+}$ T cells ($n = 9$). *** $P < 0.001$ (unpaired *t*-test). (**b**) TL-tetramer staining of human T_{EMRA} $CD8^{+}$ T cells is blocked by anti-CD8α but not anti-CD8β antibody. Data are representative of two independent experiments. (**c**) CMV_{pp65}-specific $CD8^{+}$ T cells display a T_{EM}/T_{EMRA} phenotype and persist at high frequency in humans. Data from two representative donors from a total of six persons are shown. The TL-tetramer staining was absent on naive $CD8^{+}$ T cells and was blocked by an anti-CD8α antibody.

Figure 6: Retinoic acid promotes the affinity-based accumulation of CD8αα^{+}CD8αβ T cells in the intestine. (**a**) OT-I cells were stimulated by OVAp-loaded DCs from SPL or mLN of WT mice with or without 100 nM RA (dot plots) or with LE135 (histogram) *in vitro* for 3 d and CD8αα expression was analyzed. Data are representative of five independent experiments. (**b,c**) OT-I cells were stimulated by SPL or mLN DCs pulsed with OVAp (high, 1 nM; low, 0.01 nM) in the presence or absence of 100 nM RA (**b**) or 5 ng/ml TGF-β (**c**) *in vitro* for 3 d and CD8αα expression was analyzed. Data are representative of more than five independent experiments. (**d,e**) 0.5×10^{6} $CD8^{+}$ OT-I cells isolated from naïve $Ly5.1^{+}$ OT-I^{+} $Rag^{-/-}$ mice were adoptively transferred into B6 recipient mice. 1 d after transfer, mice were orally infected by 0.5×10^{9} $ActA^{-}$ Lm-OVA. CD8αα expression was measured on gated donor OT-I cells from the SPL, mLN, PP and IEL, 5 d p.i. (**d**). CD8αα and CD103 expression is shown on gated donor OT-I cells from the spleen and IEL on days 12, 21 and 75 p.i. (**e**). Representative data from two to three mice per group are shown. At least three independent experiments were performed.

Figure 7: Constitutive expression of TL on intestinal epithelial cells mediates selection of mature memory CD8αβ T cells. (**a,b**) Naïve Ly5.1^{+} CD8^{+}OT-I cells were cultured in the presence of APC (MEC.B7.SigOVA). After 2 days' culture, CD8αα^{hi} and CD8αα$^{lo/-}$ OT-I cells were sorted and cultured for 3 more days *in vitro*. Then 0.5 × 10^{6} CD8αα^{hi} or CD8αα$^{lo/-}$ cells were adoptively transferred into B6 recipients. One month after transfer, mice were orally infected with 5 × 10^{8} ActA^{-} Lm-OVA. Donor Ly5.1^{+} OT-I cells were tracked in the spleen and IEL 3 d (**a**) and 5 d (**b**) p.i.. Representative data from 3-4 mice in each group are shown. At least five independent experiments were performed. (**c**) As shown in (**a**), secondary OT-I memory cells were assessed in the IEL 45 d p.i.. Representative data from three to four mice in each group are shown. At least three independent experiments were performed. (**d**) Sorted *in vitro* activated Ly5.1^{+} CD8αα$^{lo/-}$ OT-I cells were cultured for 3 d and 0.5 × 10^{6} primary effector cells were transferred into WT or TL$^{-/-}$ recipients. One month after transfer, mice were orally infected with 5 × 10^{8} ActA^{-} Lm-OVA. 4 months p.i., memory OT-I cells were tracked in the spleens and IEL. Pooled data ± s.e.m. are shown. At least two independent experiments were performed. (**e, f**) 5 × 10^{4} naïve CD8^{+} OT-I cells were transferred into Ly5.1^{+} WT or Ly5.1^{+} TL$^{-/-}$ recipient mice. 1 d after transfer, mice were orally infected with Lm-Q4OVA. Effector OT-I cells in the peripheral blood (7 d p.i.) and memory OT-I cells (2 m p.i.) in the spleen and IEL were analyzed. Pooled data ± s.e.m. are shown. (**g, h**) 5 × 10^{4} naïve CD8^{+} OT-I cells were transferred into Ly5.1^{+} WT or Ly5.1^{+} TL$^{-/-}$ recipient mice. 1 d after transfer, mice were intravenously infected with Lm-Q4OVA. Effector OT-I cells in the peripheral blood (7 d p.i.) and memory OT-I cells (2 m p.i.) in the spleen and IEL were analyzed. Pooled data ± s.e.m. are shown. At least three independent experiments were performed. (**i**) Ly5.1 mice adoptively transferred with 5 × 10^{4} naïve WT or E8$_{I}^{-/-}$ OT-I cells were orally immunized with 1 ×

10^9 ActA$^-$ Lm-OVA. Two months after immunization, mice were re-challenged orally with 1×10^{10} WT Lm-OVA. Bacterial loads in the livers were assessed day 3 p.i.. Pooled data ± s.e.m. are shown ($n = 6$). Representative data are shown of three independent experiments. * $P < 0.05$; ns: no significant difference (unpaired *t*-test).

Mucosal CD8 Memory T Cells are selected in the periphery by an MHC Class I Molecule

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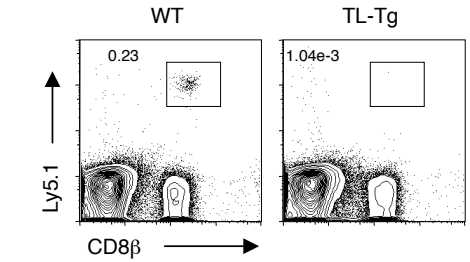
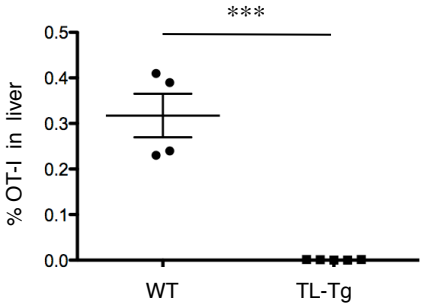
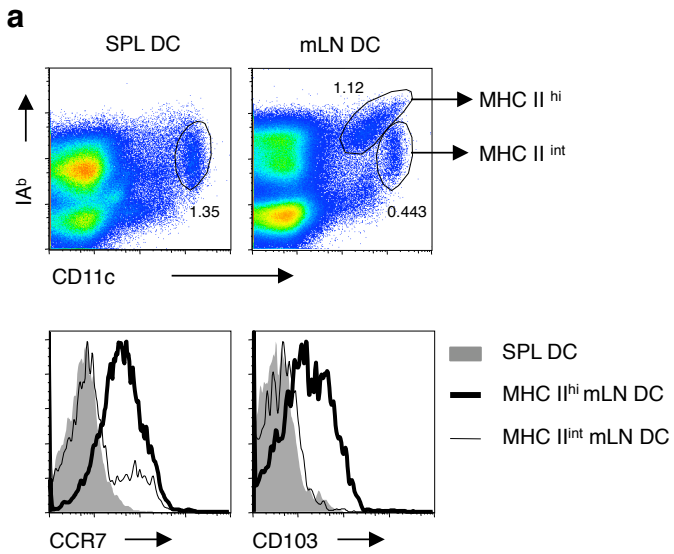
Supplementary Figures 1-6

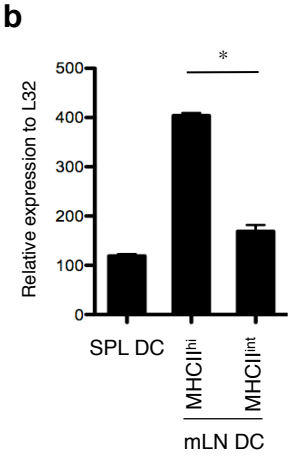
Supplementary Figure 1



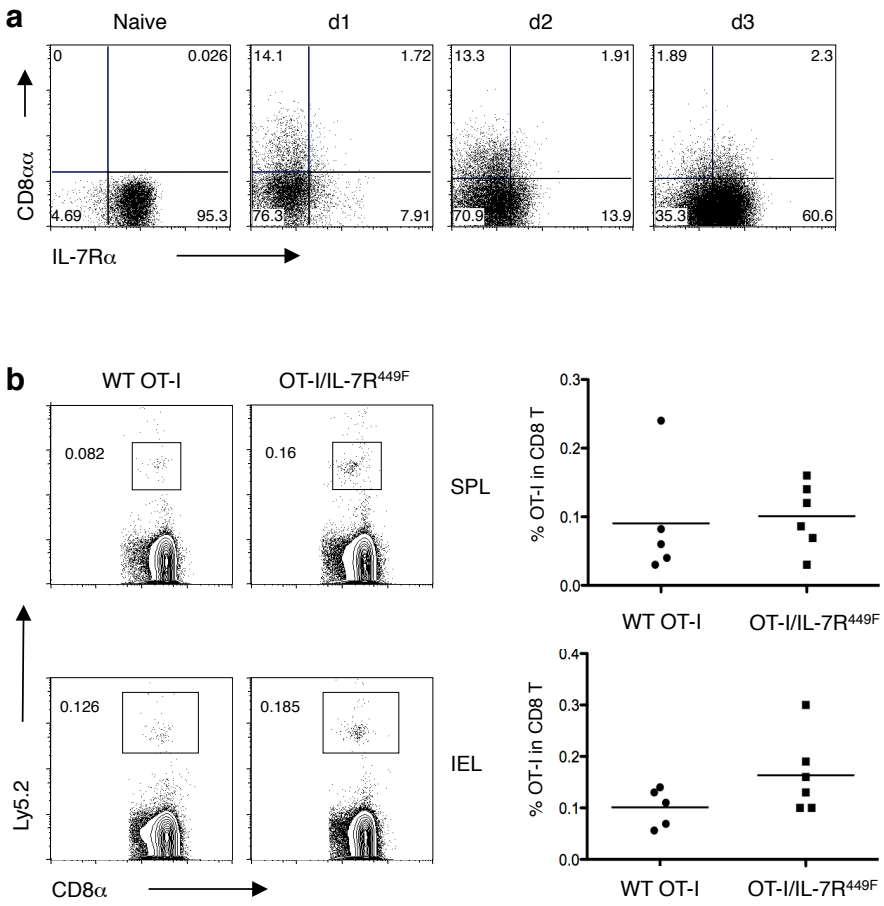


Supplementary Figure 2

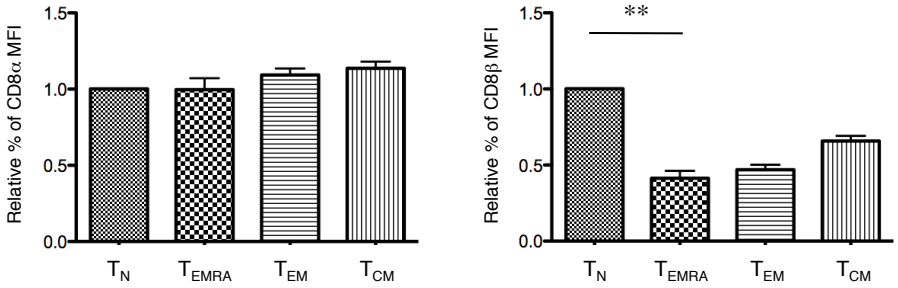




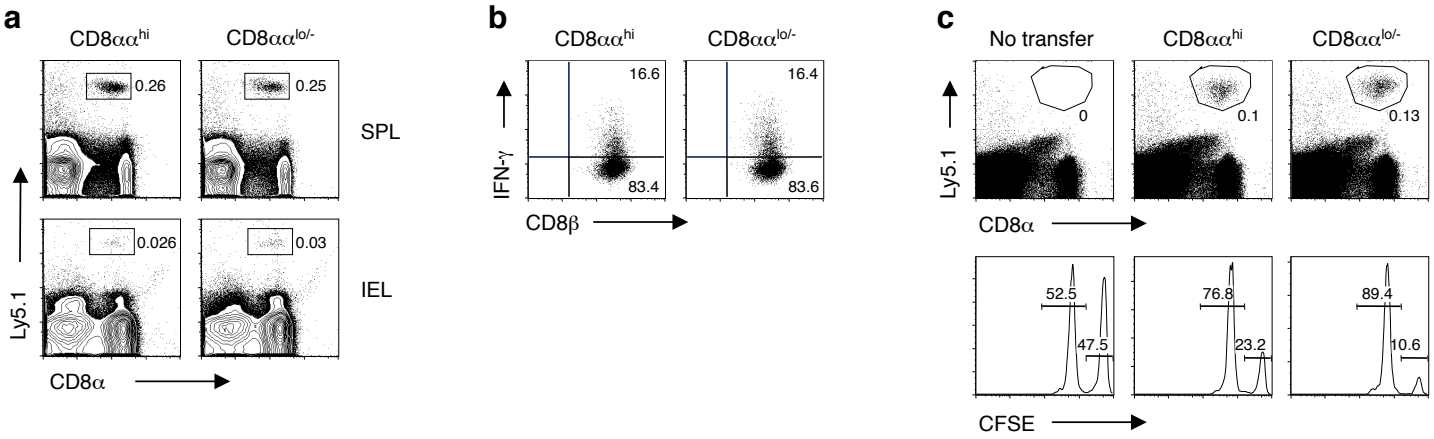
Supplementary Figure 3



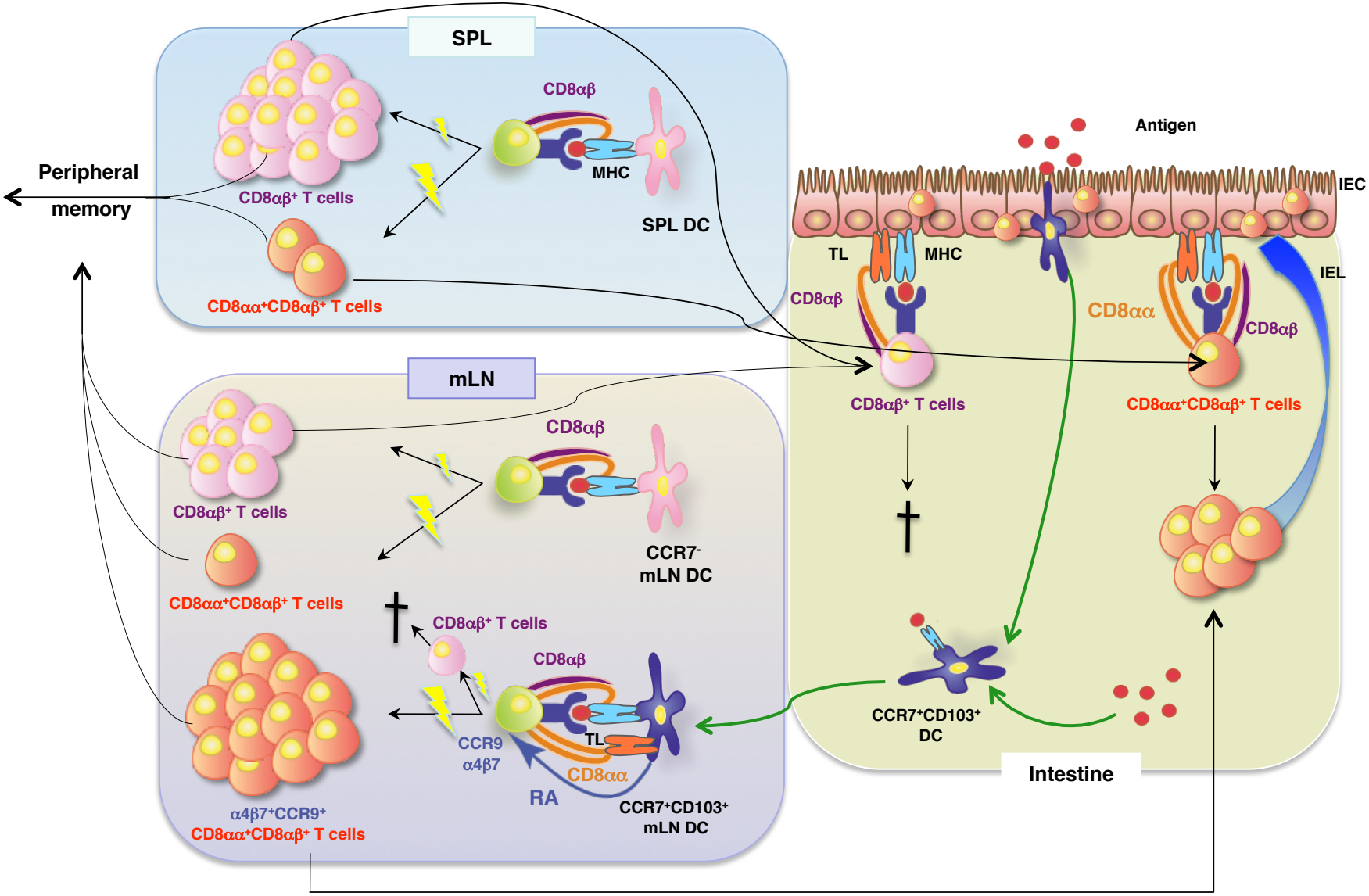
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure Legends

Supplementary Figure 1. TL deletes activated CD8αβ$^+$ T cells. 1 × 10^6 naïve Ly5.1$^+$ OT-I cells were transferred into WT or TL-Tg recipients. One day after transfer, mice were orally infected with 1 × 10^9 ActA$^-$ Lm-OVA. Memory CD8$^+$ OT-I cells in the livers were analyzed 2 month p.i.. Graph depicts pooled data ± s.e.m.. *** $P < 0.001$ (unpaired *t*-test).

Supplementary Figure 2. MHC class II^{hi}, CD11c$^+$ MLN DC have the phenotype of CCR7$^+$ CD103$^+$ migratory DC. (**a**) mLN contain a distinct MHC class II^{hi} DC subset that is absent in the spleen (dot plots). Surface expression of CCR7 and CD103 were analyzed on CD11c$^+$MHC II$^+$ DCs from SPL and CD11c$^+$MHC II^{hi} or CD11c$^+$MHC II^{int} DCs from mLN (histograms). Data are representative from at least three independent experiments. (**b**) Spleen or mLN DCs were sorted based on the CD11c and MHC II expression and used for mRNA quantification for TL expression by qRT-PCR. Data are representative from three independent experiments.

Supplementary Figure 3. Affinity-based selective programming of memory precursor cells does not require IL-7R signals. (**a**) Naïve CD8$^+$ OT-I cells were cultured in the presence of APC (MEC.B7.SigOVA). CD8αα and IL-7Rα expression on OT-I cells was determined by flow cytometry at different time points. (**b**) 1 × 10^4 naïve CD8$^+$ WT OT-I cells or IL-7Rα^{449F} OT-I cells were adoptively transferred into Ly5.1 WT recipient mice. 1 d after transfer, mice were orally infected with 1 × 10^9 ActA$^-$ Lm-OVA. Donor OT-I cells were tracked in the spleens and IEL 2 months p.i.. At least three independent experiments were performed.

Supplementary Figure 4. Expression of CD8α and CD8β on human $CD8^+$ T cell subsets. The bar graphs show the relative percentage of CD8α and CD8β mean fluorescence intensity (MFI) in human $CD8^+$T cell subsets ($n = 9$). ** $P < 0.01$ (unpaired *t*-test).

Supplementary Figure 5. Characterization of *in vitro* activated $CD8\alpha\alpha^{hi}$ and $CD8\alpha\alpha^{lo/-}$ $CD8\alpha\beta^+$ T cells. (**a**) Naïve $Ly5.1^+$ $CD8^+$ OT-I cells were cultured in the presence of APC (MEC.B7.SigOVA). After two days' culture, $CD8\alpha\alpha^{hi}$ and $CD8\alpha\alpha^{lo/-}$ OT-I cells were sorted and cultured for 3 more days *in vitro*. Then 0.5×10^6 $CD8\alpha\alpha^{hi}$ or $CD8\alpha\alpha^{lo/-}$ were adoptively transferred into B6 recipients. 3 d after transfer, effector OT-I cell were tracked in the spleen and IEL. (**b**) Sorted $CD8\alpha\alpha^{hi}$ and $CD8\alpha\alpha^{lo/-}$ OT-I cells were directly stimulated with $OVA_{257-264}$ peptide for 5 h and intracellular staining for IFN-γ was performed. (**c**) 3 d after transfer of $CD8\alpha\alpha^{hi}$ or $CD8\alpha\alpha^{lo/-}$, *in vivo* cytotoxicity assay was performed in recipient mice. B6 splenocytes labeled as $CFSE^{hi}$ and pulsed with $OVA_{257-264}$ peptide were used as target cells and splenocytes labeled as $CFSE^{low}$ and pulsed with the control peptide were used as control target cells. Data are representative from three independent experiments.

Supplementary Figure 6. Proposed model for the roles of TL and CD8αα on selection of memory CD8αβ T cells. CD8αα expression is selectively induced on high affinity/avidity primary effector $CD8\alpha\beta^+$ T cells and further enhanced by RA released by mucosal migratory ($CCR7^+$) DC, which also promote gut-tropism ($\alpha4\beta7^+CCR9^+$) of the effector T cells. Activated migratory DC express the CD8αα high affinity ligand, TL, which when interacting with CD8αβ on activated T cells leads to TICD. High affinity primary effector cells that induce CD8αα, escape TICD by sequestering TL away from CD8αβ via CD8αα leading to affinity-based selective survival. TL

constitutively expressed on the intestinal epithelial cells (IEC) mediates affinity maturation of the mucosal T_{EM} and eliminates low affinity/avidity primary and secondary effector cells that home to the gut and fail to induce CD8αα.

Figure 1

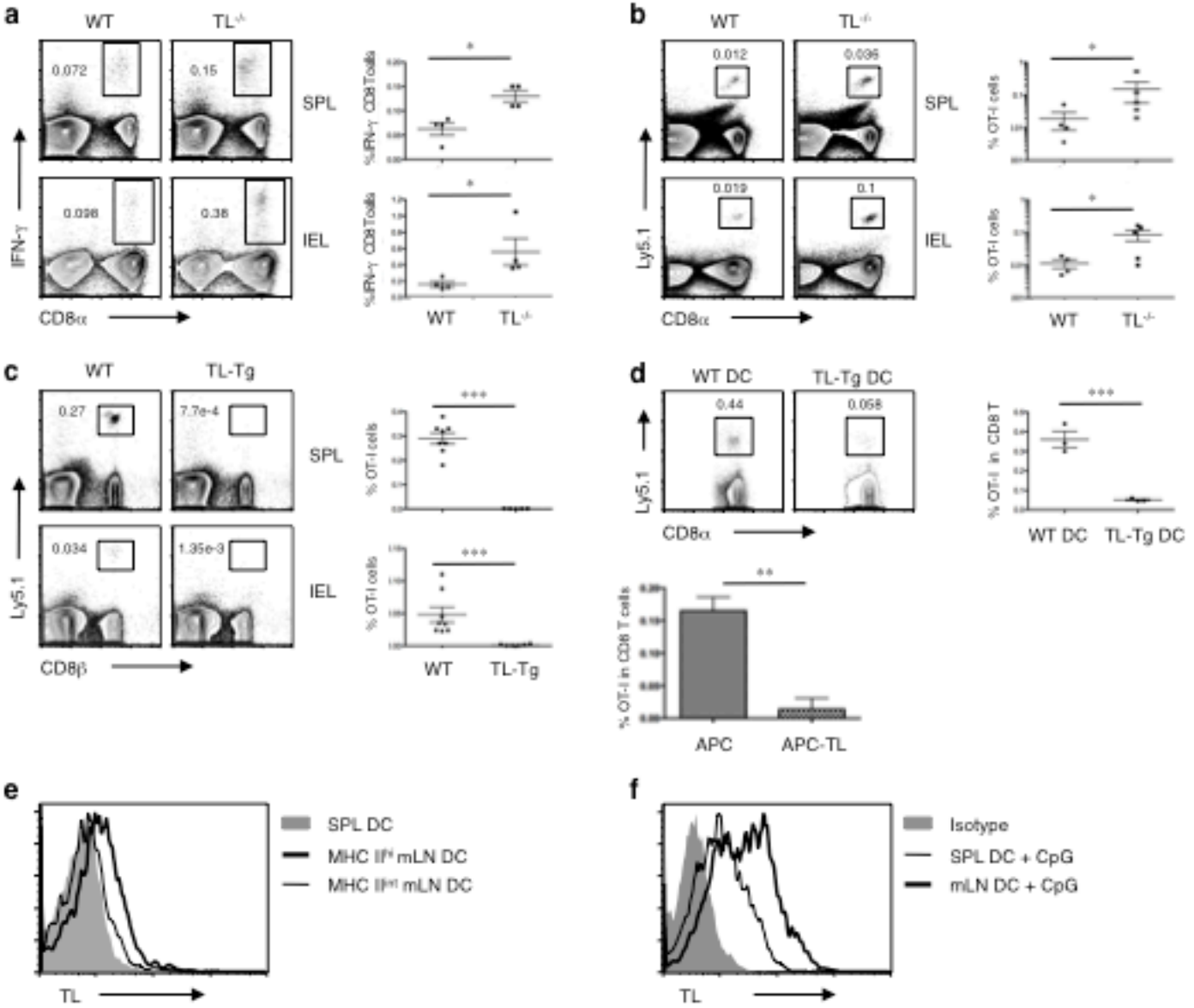

a
WT
TL-/-
0.072
0.15
SPL
0.036
0.38
IEL
IFN-γ
CD8α
%IFN-γ CD8 T cells
WT
TL-/-
b
WT
TL-/-
0.012
0.036
SPL
0.019
0.1
IEL
Ly5.1
CD8α
% OT-I cells
WT
TL-/-
c
WT
TL-Tg
0.27
7.7e-4
SPL
0.034
1.35e-3
IEL
Ly5.1
CD8β
% OT-I cells
WT
TL-Tg
d
WT DC
TL-Tg DC
0.44
0.058
Ly5.1
CD8α
% OT-I in CD8 T
WT DC
TL-Tg DC
% OT-I in CD8 T cells
APC
APC-TL
e
SPL DC
MHC IIhi mLN DC
MHC IIint mLN DC
TL
f
Isotype
SPL DC + CpG
mLN DC + CpG
TL

Figure 2

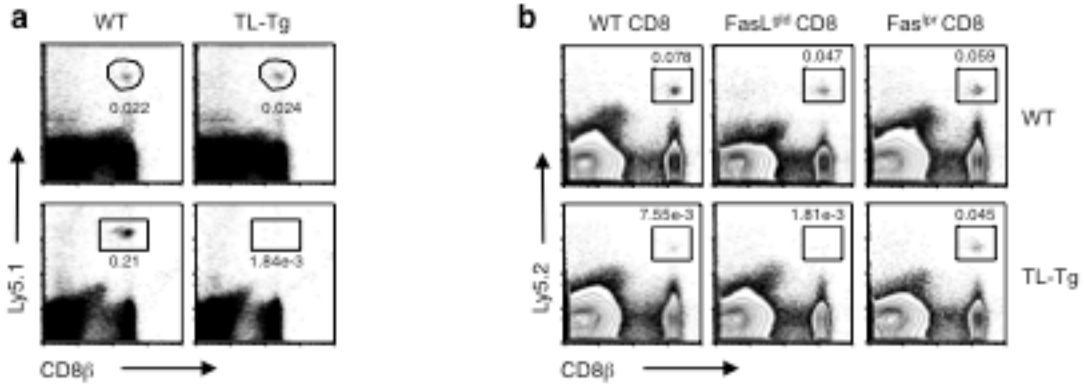

a
WT
TL-Tg
0.022
0.024
0.21
1.84e-3
Ly5.1
CD8β
b
WT CD8
FasL^{gld} CD8
Fas^{lpr} CD8
0.078
0.047
0.059
7.55e-3
1.81e-3
0.045
WT
TL-Tg
Ly5.2
CD8β

Figure 3

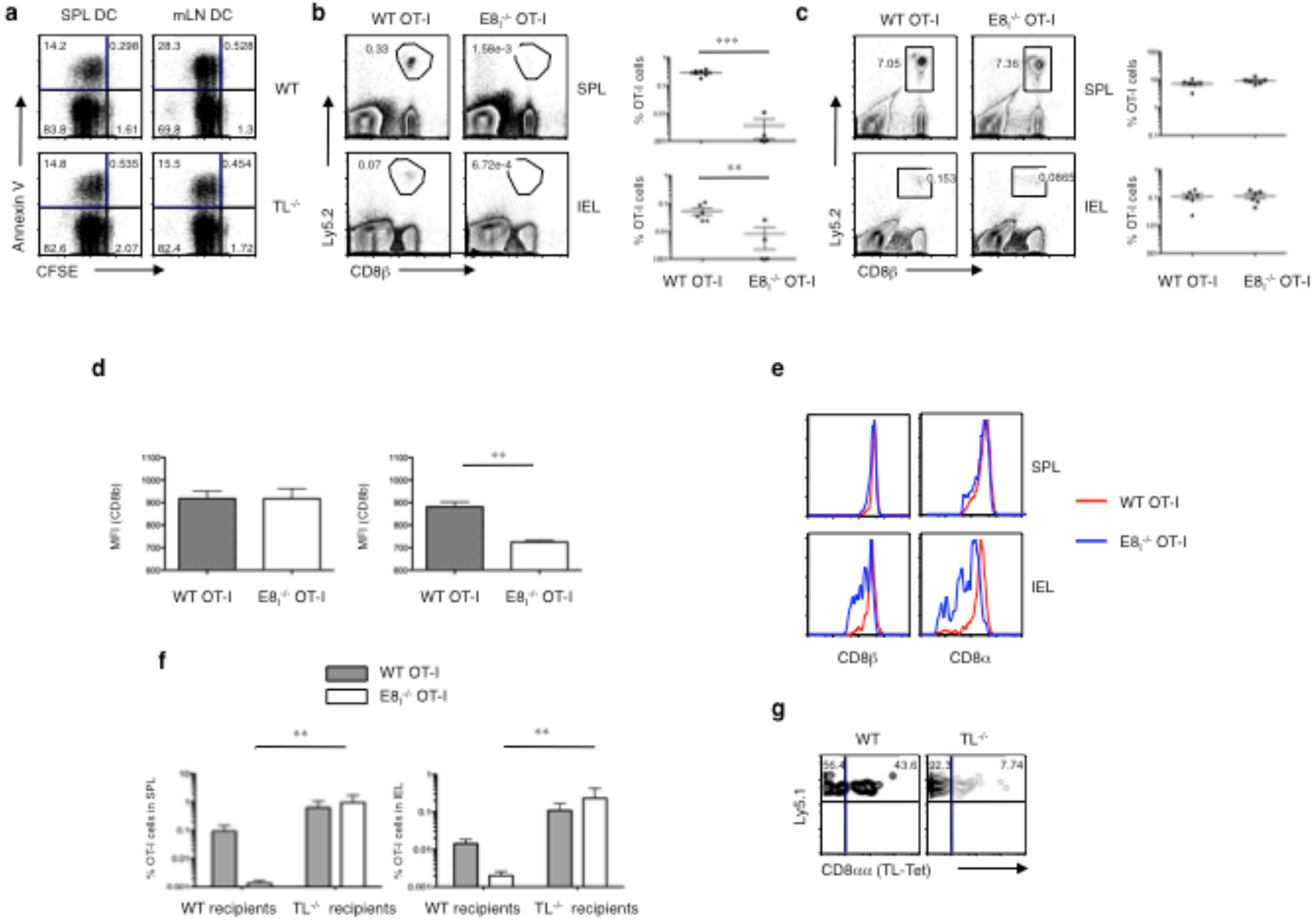

a
SPL DC
mLN DC
WT
TL-/-
Annexin V
CFSE
b
WT OT-I
E8I-/- OT-I
SPL
IEL
Ly5.2
CD8β
% OT-I cells
WT OT-I
E8I-/- OT-I
c
WT OT-I
E8I-/- OT-I
SPL
IEL
Ly5.2
CD8β
% OT-I cells
WT OT-I
E8I-/- OT-I
d
MFI (CD8b)
WT OT-I
E8I-/- OT-I
e
SPL
IEL
WT OT-I
E8I-/- OT-I
CD8β
CD8α
f
WT OT-I
E8I-/- OT-I
% OT-I cells in SPL
% OT-I cells in IEL
WT recipients
TL-/- recipients
g
WT
TL-/-
Ly5.1
CD8αα (TL-Tet)

Figure 4

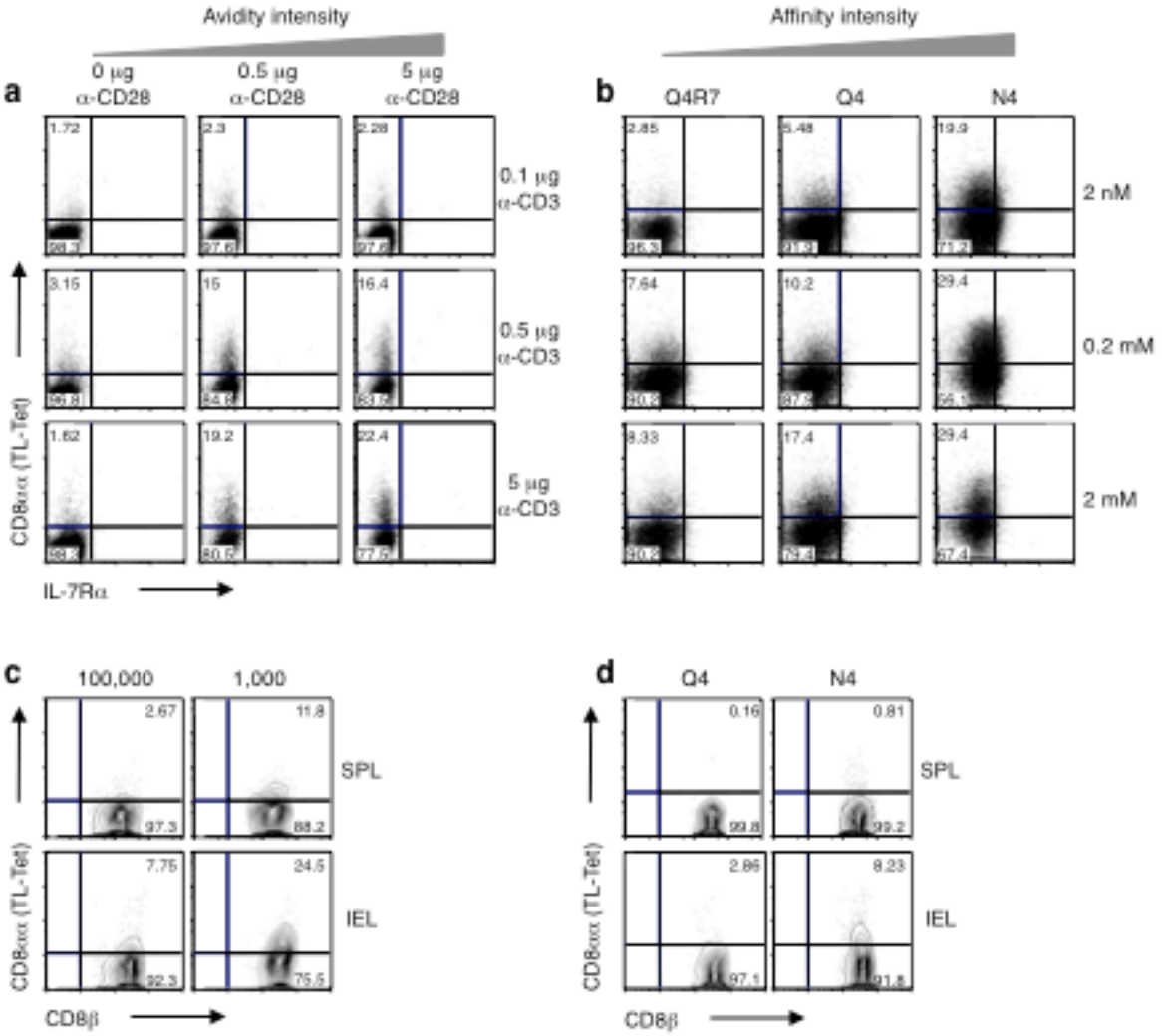

Avidity intensity
a
0 μg α-CD28
0.5 μg α-CD28
5 μg α-CD28
0.1 μg α-CD3
0.5 μg α-CD3
5 μg α-CD3
CD8αα (TL-Tet)
IL-7Rα
1.72
2.3
2.28
3.15
15
16.4
1.62
19.2
22.4
Affinity intensity
b
Q4R7
Q4
N4
2 nM
0.2 mM
2 mM
2.85
5.48
19.9
7.64
10.2
29.4
8.33
17.4
29.4
c
100,000
1,000
SPL
IEL
CD8β
2.67
11.8
7.75
24.5
d
Q4
N4
0.16
0.81
2.86
8.23

Figure 5

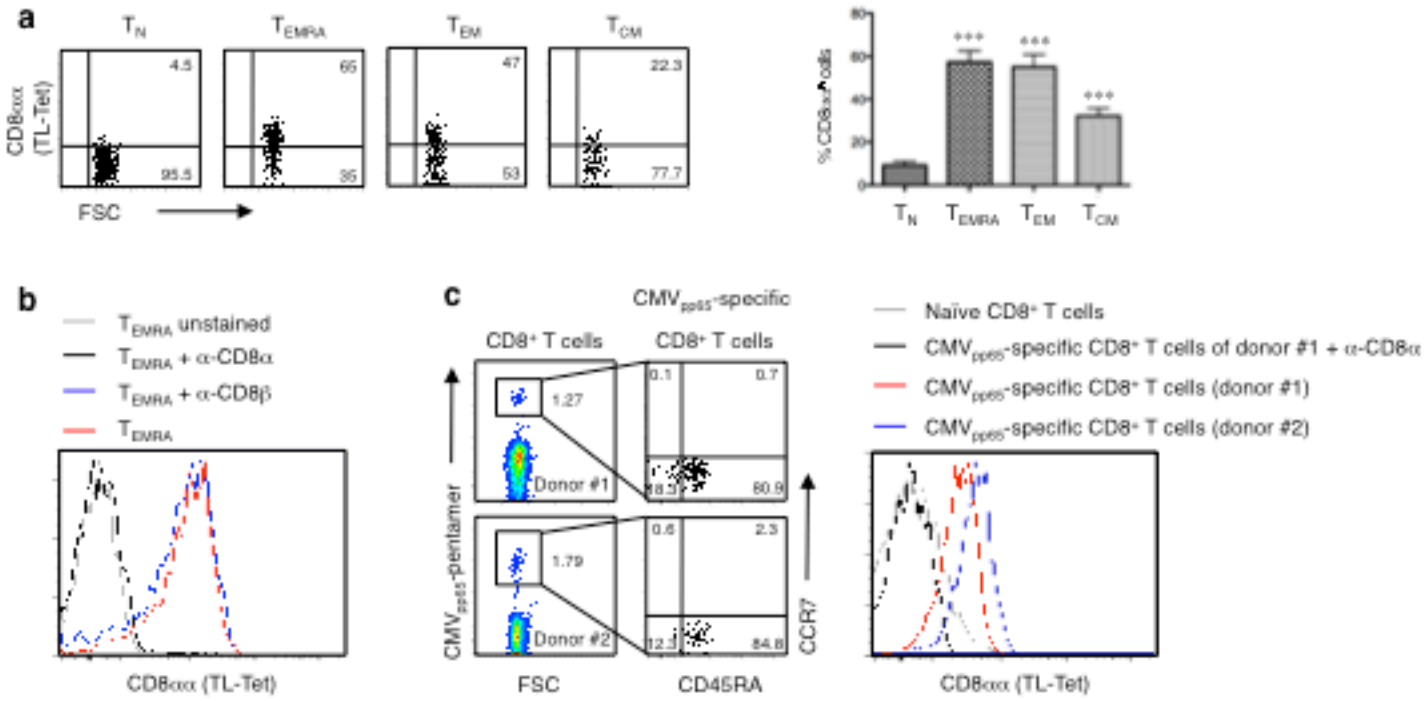

a
T_N
T_{EMRA}
T_{EM}
T_{CM}
CD8αα (TL-Tet)
FSC
4.5
95.5
65
35
47
53
22.3
77.7
% CD8αα+ cells
***
b
T_{EMRA} unstained
T_{EMRA} + α-CD8α
T_{EMRA} + α-CD8β
T_{EMRA}
CD8αα (TL-Tet)
c
CMV_{pp65}-specific
CD8+ T cells
CD8+ T cells
1.27
Donor #1
0.1
0.7
88.5
80.9
1.79
Donor #2
0.6
2.3
12.3
84.8
CMV_{pp65}-pentamer
FSC
CD45RA
CCR7
Naïve CD8+ T cells
CMV_{pp65}-specific CD8+ T cells of donor #1 + α-CD8α
CMV_{pp65}-specific CD8+ T cells (donor #1)
CMV_{pp65}-specific CD8+ T cells (donor #2)
CD8αα (TL-Tet)

Figure 6

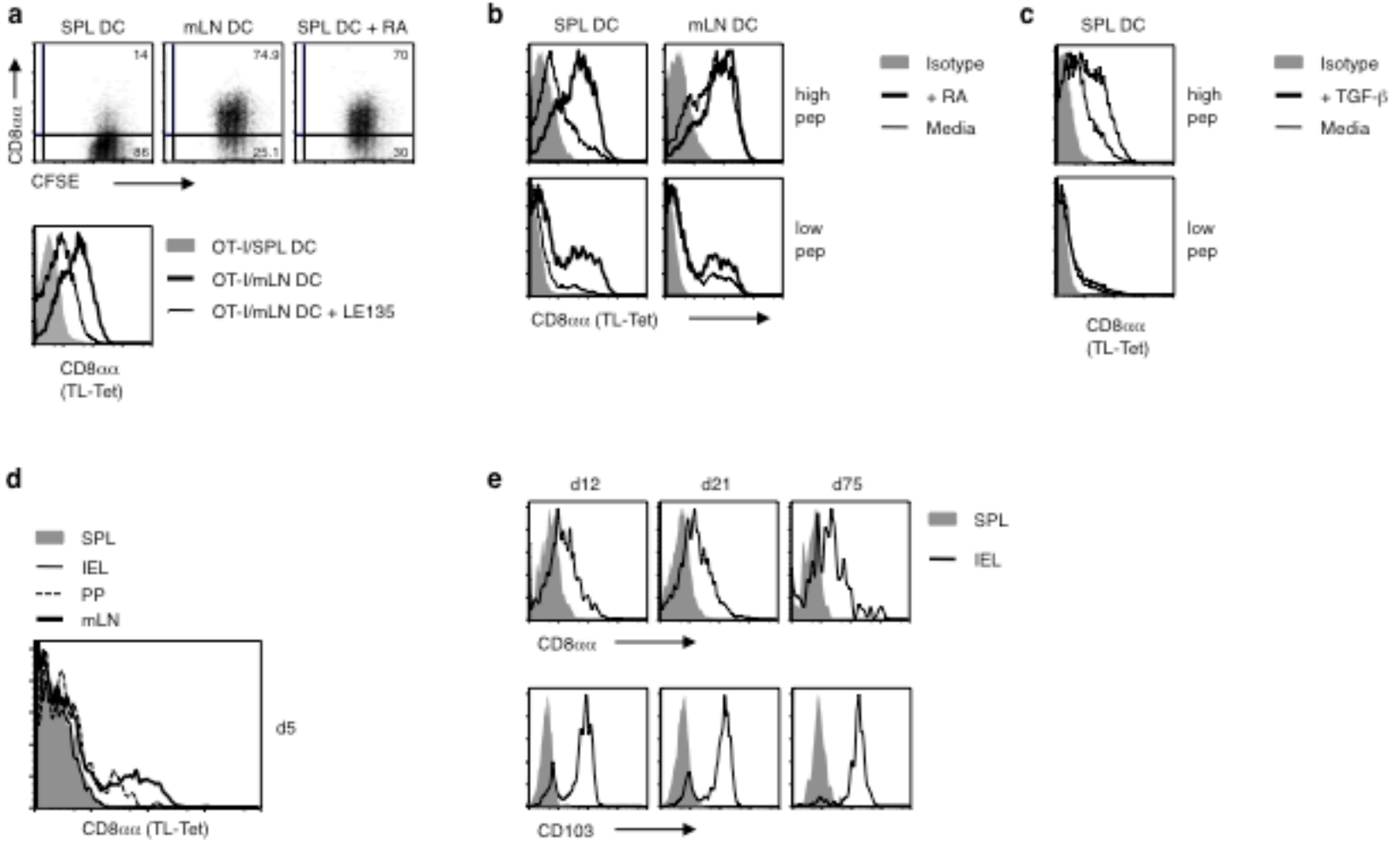
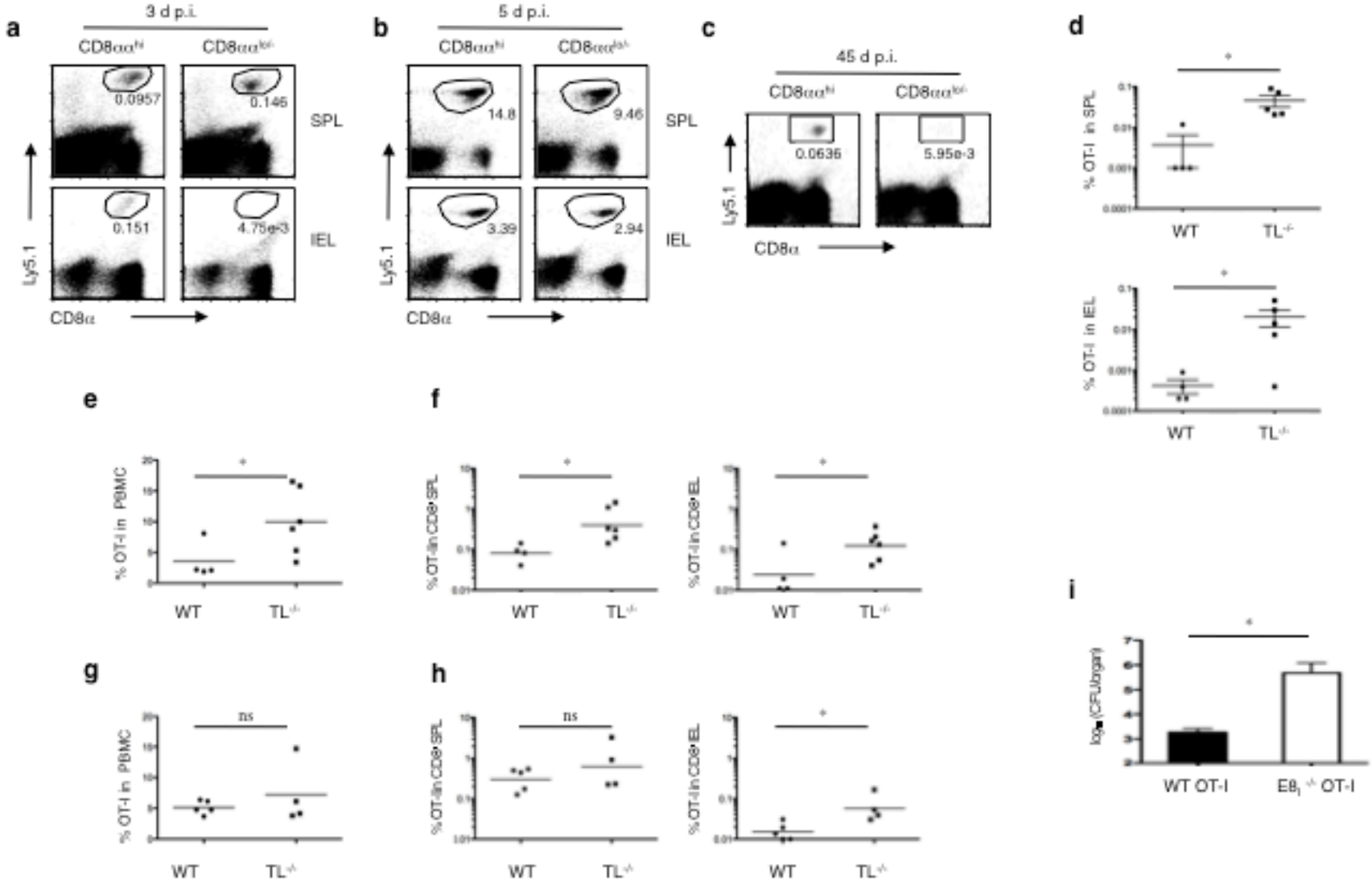

a
SPL DC
mLN DC
SPL DC + RA
14
74.9
70
86
25.1
30
CD8αα
CFSE
OT-I/SPL DC
OT-I/mLN DC
OT-I/mLN DC + LE135
CD8αα
(TL-Tet)
b
SPL DC
mLN DC
high pep
low pep
Isotype
+ RA
Media
CD8αα (TL-Tet)
c
SPL DC
high pep
low pep
Isotype
+ TGF-β
Media
CD8αα
(TL-Tet)
d
SPL
IEL
PP
mLN
d5
CD8αα (TL-Tet)
e
d12
d21
d75
SPL
IEL
CD8αα
CD103

Figure 7


a
3 d p.i.
CD8αα^hi
CD8αα^lo/-
0.0957
0.146
0.151
4.75e-3
SPL
IEL
Ly5.1
CD8α
b
5 d p.i.
CD8αα^hi
CD8αα^lo/-
14.8
9.46
3.39
2.94
SPL
IEL
Ly5.1
CD8α
c
45 d p.i.
CD8αα^hi
CD8αα^lo/-
0.0636
5.95e-3
Ly5.1
CD8α
d
% OT-I in SPL
% OT-I in IEL
WT
TL-/-
e
% OT-I in PBMC
WT
TL-/-
f
% OT-I in CD8+ SPL
% OT-I in CD8+ IEL
WT
TL-/-
g
% OT-I in PBMC
ns
WT
TL-/-
h
% OT-I in CD8+ SPL
% OT-I in CD8+ IEL
ns
WT
TL-/-
i
log10 (CFU/organ)
WT OT-I
E8I-/- OT-I