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Justine D. Mintern; ... et. al

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Thymic Selection and Peripheral Activation of CD8 T Cells by the Same Class I MHC/Peptide Complex[1]

Justine D. Mintern, Madelon M. Maurice,[2] Hidde L. Ploegh,[3] and Eckart Schott

Thymic selection is controlled by the interaction between TCR and MHC/peptide. Strength and quality of the signal determine whether thymocytes are selected or deleted. The factors that contribute to this signal remain poorly defined. Here we show that fetal thymic organ cultures (FTOCs) derived from OT-I transgenic mice (the OT-I TCR is restricted by K^b-SIINFEKL) on a $K^bD^{b-/-}$ background support positive selection, but only when provided with soluble H-2K^b-SIINFEKL complexes. Selection of CD8 T cells is independent of the valency of the ligand or its capability to coengage CD8 molecules. Both CD8$\alpha\alpha$ and CD8$\alpha\beta$ T cells are selected by H-2K^b-SIINFEKL, but only CD8$\alpha\beta$ cells are capable of releasing IFN-γ in response to the same ligand. The $\alpha_4\beta_7$ integrin is up-regulated on postselection thymocytes from FTOCs. After adoptive transfer, FTOC-derived OT-I CD8 T cells divide in response to the agonist peptide SIINFEKL. These results establish that CD8 T cells responsive to their nominal peptide-Ag can be generated in FTOC supplemented with soluble MHC class I molecules equipped with the same peptide. ***The Journal of Immunology*****, 2004, 172: 699–708.**

The generation of T cells in the thymus is a tightly controlled process that establishes a diverse T cell repertoire responsive to foreign pathogens. To avoid eliciting T cell responses against self-Ag, potentially self-reactive clones are deleted (negative selection) at the double positive (DP)[4] stage of thymocyte development. Selection is accomplished by the interaction of the newly expressed TCR on $CD4^+CD8^+$ DP thymocytes with the MHC/self-peptide complex on thymic epithelial cells (1, 2). The strength of this interaction determines the outcome of selection, such that strong interactions lead to deletion and intermediate strength interactions induce positive selection (reviewed in Ref. 3). In addition, adhesion molecules, costimulatory molecules, and cytokines contribute to the overall complexity of the signal (reviewed in Refs. 4 and 5).

Most experimental systems used to investigate T cell selection use mice transgenic for a TCR whose peptide ligands have been characterized in terms of specificity and affinity. Such ligands have traditionally been described as agonistic, partial-agonistic, antagonistic, or inert, depending on their capability to stimulate peripheral T cells (reviewed in Ref. 6). Although agonistic ligands generally provide the highest affinity for the TCR, exceptions to this rule have been reported (7). By applying the same categories of peptides to models of thymic selection, the capability of inducing positive selection has been attributed to partial-agonists and antagonists (8), whereas agonists induced deletion (9). These findings have helped shape the paradigm that selecting ligands differ from those recognized by the corresponding T cell in the periphery (10).

It is becoming increasingly apparent that there are exceptions to this model. Selection as a result of high-affinity interactions in the thymus can yield CD8 (11–14) and CD4 T cells (15–17), although controversy exists as to whether such selected cells are functional (13, 18) or are effectively silenced through the interaction with agonist-ligand (9). Specialized subsets of CD8 T cells (CD8$\alpha\alpha$) (13, 19, 20) and CD4 T cells (CD4 CD25 high regulatory cells) (21) may also be selected by agonist ligand in the thymus. For CD8$\alpha\alpha$ T cells generated in vivo, both cytolytic (13) and regulatory (22) functions have been described.

To investigate the role of the selecting ligand in thymocyte development, fetal thymic organ culture (FTOC) models have been widely used. Although FTOC provides a convenient means to investigate the effect of ligands added to the culture, the small number of cells usually generated has thus far prevented their functional characterization. To examine selection of CD8 T cells, TCR transgenic mice are bred onto a class I MHC-deficient background, commonly the β_2-microglobulin $(\beta_2m)^{-/-}$ or $TAP^{-/-}$ mouse. Selection is then investigated by culturing fetal thymic lobes in the presence of exogenous peptide (and β_2m) to restore class I MHC expression on the surface of thymic epithelial cells, which are essential for CD8 T cell selection. Both the $\beta_2m^{-/-}$ and the $TAP^{-/-}$ model suffer from expression at the cell surface of low levels of endogenous class I MHC molecules of ill-defined conformation that may contribute to selection (23–26). In addition, the precise number of selecting ligands on thymic epithelial cells restored by addition of synthetic peptide cannot be determined accurately. Finally, provision of peptide restores expression of class I MHC on all cell types present, including thymocytes themselves, with unknown consequences. To circumvent these limitations, H-2$K^bD^{b-/-}$ mice (27) were used here for FTOC studies. Such mice express no class Ia MHC molecules at all, but remain capable of expressing class Ib (nonclassical class I) MHC molecules. Therefore, addition of synthetic peptide cannot stabilize class I MHC expression on H-2$K^bD^{b-/-}$ cells and an exogenous source of class I MHC molecules must be provided. Here

Department of Pathology, Harvard Medical School, Boston, MA 02115

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[2] Current address: Hubrecht Laboratory, Uppsalalaan 8, 3584CT Utrecht, The Netherlands.

[3] Address correspondence and reprint requests to Dr. Hidde L. Ploegh, Department of Pathology, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115. E-mail address: ploegh@hms.harvard.edu

[4] Abbreviations used in this paper: DP, double positive; FTOC, fetal thymus organ culture; β_2m, β_2-microglobulin; RAG, recombination-activating gene; cγc, common γ-chain; LN, lymph node; IEL, intraepithelial lymphocyte; CD62L, CD62 ligand; SP, single positive; DN, double negative; ILN, inguinal LN; MLN, mesenteric LN; PP, Peyer's patch.

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we used recombinant H-2K^b molecules to reconstitute FTOC with selecting ligands. H-2K^{b}D$^{b-/-}$ mice were crossed onto the OT-I TCR-transgenic, recombination-activating gene (RAG)-deficient background. Such mice do not support selection of CD8 OT-I T cells. The OT-I TCR (Vα2Vβ5) is specific for the OVA-derived peptide SIINFEKL presented in the context of H-2K^b (8).

Here we examined the selection requirements for OT-I RAG$^{-/-}$ K^{b}D$^{b-/-}$ thymocytes in a model that allows control over the selection conditions by addition of soluble H2-K^b molecules. We find that agonist-loaded H-2K^b molecules induce positive selection of CD8 T cells that can respond to stimulation in vitro with the same ligand by producing IFN-γ. The MHC-I/peptide-complex is thus sufficient to induce selection, even when provided outside the context of the surface of a thymic epithelial cell. Both CD8$\alpha\alpha$ and CD8$\alpha\beta$ cells are generated, with the proportion of each population dependent on the dose of selecting ligand. Such selected cells home to the intestine upon adoptive transfer and are readily activated in vivo by the same MHC/peptide-ligand used for their selection. Our findings are consistent with the differential avidity model of thymic selection but argue against differentiation between agonist and antagonist ligands as determinants of negative and positive selection.

Materials and Methods

Mice

All mice were bred and maintained under pathogen-free conditions at the Harvard Medical School animal facility (Boston, MA). OT-I RAG$^{-/-}$ mice and OT-I K^{b}D$^{b-/-}$ mice have been described (28). For the experiments performed here, OT-I K^{b}D$^{b-/-}$ mice were bred onto the RAG$^{-/-}$ background. C57BL/6 common γ chain (cγc)$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). OT-I β_2m$^{-/-}$ mice were generated by breeding OT-I mice to β_2m$^{-/-}$ mice (The Jackson Laboratory).

Recombinant H-2K^b molecules

K^b monomers were prepared as described (29). In brief, recombinant K^b and β_2m were refolded in the presence of the peptides SIINFEKL, SIIRFEKL, EIINFEKL, or SSYSYSSL (polyS). Refolded K^b/β_2m/peptide complexes were purified by size exclusion chromatography on a Superdex 200 column (Pharmacia, Peapack, NJ). Monomers were obtained at this step. To obtain K^b tetramers, monomers were biotinylated by using recombinant BirA enzyme and purified by size exclusion chromatography. The α3-domain mutant (E223K) of K^b was generated by site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA), replacing glutamic acid at position 223 with lysine.

K^b molecules were mixed in a molar ratio of 4:1 with streptavidin or PE-conjugated streptavidin (Molecular Probes, Eugene, OR). The tetramerized molecules were purified again by size exclusion chromatography (28).

FTOC

Thymic lobes were excised from fetuses at gestational day 16 (the detection of the vaginal plug is defined as day 1). The lobes were placed on polycarbonate membranes (Costar, Cambridge, MA) in DMEM supplemented with 10% FCS, penicillin, streptomycin, 2 mM L-glutamine, and 10 μM 2-ME. Recombinant H-2K^b molecules were added to the culture at the indicated concentrations. Medium was exchanged every 48 h. For in vitro activation experiments, lobes were allowed to rest in medium containing no H-2K^b molecules for 12 h before activation. After 7 days in culture, the thymic lobes were harvested and thymocytes were extruded mechanically by pressing the tissue through nylon mesh.

Cell preparation and flow cytometry

Cells were harvested from thymus, spleen, and lymph nodes (LNs). Intestinal epithelial lymphocytes (IELs) were prepared as described (30), with minor modifications. Briefly, the small intestine was removed and cleaned by flushing with PBS/5% FCS, and Peyer's patches were carefully excised. The intestine was then opened longitudinally and cut it into 0.5-cm pieces. IELs were isolated by chemical and mechanical disruption of the epithelial layer in extraction buffer (PBS, 5% FCS, 1 mM DTT, 1 mM EDTA) for 45 min at 37°C. Cells were collected and isolated on a discontinuous 40/70% Percoll gradient (Amersham Pharmacia Biotech, Piscataway, NJ) at 600 $\times$ *g* for 20 min. Cells at the 40/70% layer were collected and washed in FACS staining buffer. Cells were stained for FACS analysis in ice-cold FACS staining buffer (PBS, 0.5% BSA, and 0.02% sodium azide) with combinations of the following Abs: CyChrome-anti-CD8α, PE-anti-CD8α, FITC-anti-CD8β, PE-anti-CD4, FITC-anti-CD4, PE-anti-CD69, PE-anti-CD25, PE-anti-CD122, FITC-anti-CD5, FITC-anti-CD24, PE-anti-$\alpha_4\beta_7$, PE-anti-CD62 ligand (CD62L), CyChrome-anti-CD44, PE-anti-LFA1, PE-anti-CD3, and PE-anti-Vα2. All Abs were purchased from eBioscience (San Diego, CA) or BD Biosciences (Mountain View, CA).

For intracellular IFN-γ staining, cells were first stained for surface markers, followed by fixation in 0.5% paraformaldehyde and permeabilization in 0.5% saponin (Sigma-Aldrich, St. Louis, MO). Staining was then performed with PE-conjugated anti-IFN-γ Ab (BD Biosciences) in buffer containing 0.5% saponin on ice. Cells were washed in 0.5% saponin twice before resuspension in FACS buffer and analysis.

For tetramer staining, cells were incubated with PE-conjugated K^b(SIINFEKL) tetramer on ice for 30 min.

In vitro activation assay

A total of 5×10^5 spleen cells isolated from B6 mice was incubated with SIINFEKL peptide at the concentrations indicated for 2 h at 37°C. After washing four times to remove free peptide, 1×10^5 OT-I RAG$^{-/-}$ LN cells or FTOC-selected cells were added in the presence of 10 U/ml mIL-2 (Roche) for 24 h. In some experiments, CD4$^-$CD8$^-$ thymocytes were removed by positive selection of thymocytes using anti-CD8α-coated magnetic beads (Miltenyi Biotec, Auburn, CA), followed by separation on LS columns (Miltenyi Biotec). Experiments were conducted in duplicate in 96-well plates. Six hours before termination of the incubation, 100 μl of medium was removed and substituted with medium containing brefeldin A (Sigma-Aldrich) at a final concentration of 5 μg/ml. At 24 h, cells were analyzed by flow cytometry.

Supernatants were analyzed by enzyme-linked immunosorbent assay for mouse IFN-γ (eBioscience) according to the manufacturer's instructions.

Adoptive transfer and in vivo activation assay

FTOC-selected or LN cells were harvested as described above. In some experiments, positive selection of CD8α or CD8β positive cells was conducted by magnetic cell sorting using anti-CD8α magnetic beads (Miltenyi Biotec) or biotinylated anti-CD8β Ab (BD Biosciences), followed by retrieval using magnetic streptavidin beads (Miltenyi Biotec). Cells were then separated on LS columns (Miltenyi Biotec).

Labeling with CFSE was performed as described (31). Briefly, 10^7/ml LN cells from OT-I RAG$^{-/-}$ animals or FTOC-selected CD8 T cells were incubated in 15 μM CFSE (Molecular Probes) in RPMI 1640 for 10 min at 37°C. Cells were washed twice in DMEM supplemented with 10% FCS, counted, and resuspended in HEPES-buffered saline solution.

Mice used as recipients in transfer experiments were anesthetized using avertin (Sigma-Aldrich), and cell suspensions were transferred i.v. For homing experiments, mice were sacrificed 72 h after transfer. For activation experiments, recipient mice were challenged with 20 μg of peptide in PBS i.p. 24 h after transfer and were sacrificed another 48 h later.

C57/BL6J mice used as hosts were depleted of NK cells by i.p. administration of 25 μg/mouse anti-NK1.1 Ab (ebioscience), 1 wk before and at the day of the transfer.

Results

OT-I CD8 T cells are positively selected by agonist-loaded H-2K^b molecules in K^{b}D$^{b-/-}$ FTOC

Fetal thymic lobes were obtained from OT-I RAG$^{-/-}$ K^{b}D$^{b-/-}$ mice and cultured in the presence of increasing concentrations of tetrameric recombinant H-2K^b molecules loaded with the agonist peptide SIINFEKL (K^{b}4-SIINFEKL). After 7 days of in vitro culture, cellular content was examined by flow cytometry. When no recombinant class I MHC molecules were included in the FTOC, the majority of cells retrieved were CD4$^+$CD8$^+$ DP thymocytes. This observation reflects the known requirement for class I MHC to allow DP thymocytes to mature into CD8 single positive (SP) cells. A sizable number ($\sim$30% of total cells recovered) of CD8 SP cells was also present. This population has been described previously in the 2C TCR transgenic model and is believed to be generated by selection on nonclassical class I MHC molecules (32) because they are absent from FTOC prepared from 2C β_2m$^{-/-}$ thymi and, as we show below, from OT-I β_2m$^{-/-}$ FTOC. Upon

addition of K^b4-SIINFEKL, the number of OT-I CD8 T cells increased. At the highest concentrations of K^b4-SIINFEKL added, ~80% of cells recovered were CD8 SP (Fig. 1*A*) with an increase in the number of CD8 SP cells of twofold to threefold above cultures not supplemented with K^b4-SIINFEKL (Fig. 1*B*). Approximately 3×10^5 CD8 SP cells were recovered per thymic lobe. We then examined the expression of CD8$\alpha\alpha$ vs CD8$\alpha\beta$ coreceptors (Fig. 1, *A* and *C*). In the absence of added H-2K^b molecules, or at lower concentrations of K^b4-SIINFEKL, culture conditions favored the production of CD8$\alpha\beta$-expressing CD8 SP cells. With increasing concentrations of K^b4-SIINFEKL, the number of CD8$\alpha\beta$ OT-I CD8 T cells also increased. By raising the concentration of the selecting ligand even further, CD8$\alpha\alpha$ OT-I CD8 T cells were generated, such that at the highest ligand concentration, CD8$\alpha\alpha$ cells outnumbered CD8$\alpha\beta$ cells (Fig. 1*C*).

The generation of CD8 SP cells requires inclusion of K^b4-SIINFEKL. When FTOCs were supplemented with H-2K^b molecules loaded with partial agonist peptides EIINFEKL or SIIRFEKL (Fig. 1*D*), no positive selection of OT-I CD8 T cells was observed at the concentrations of ligand tested.

Naive CD8 T cells require both engagement of the CD8 coreceptor (33) and multivalent stimulation for optimal activation (28) when using recombinant H-2K^b molecules as a stimulus in vitro. To examine whether the same requirement also applied to positive selection of OT-I CD8 T cells by soluble H-2K^b molecules, we performed FTOC in the presence of monomeric ligand or in the presence of K^b4-SIINFEKL unable to bind CD8 (K^b4 E223K) (Fig. 1*E*). For both conditions, positive selection was observed, although maximal selection required slightly higher concentrations of K^b4 E223K or the monomeric ligand than wild-type K^b4-SIINFEKL.

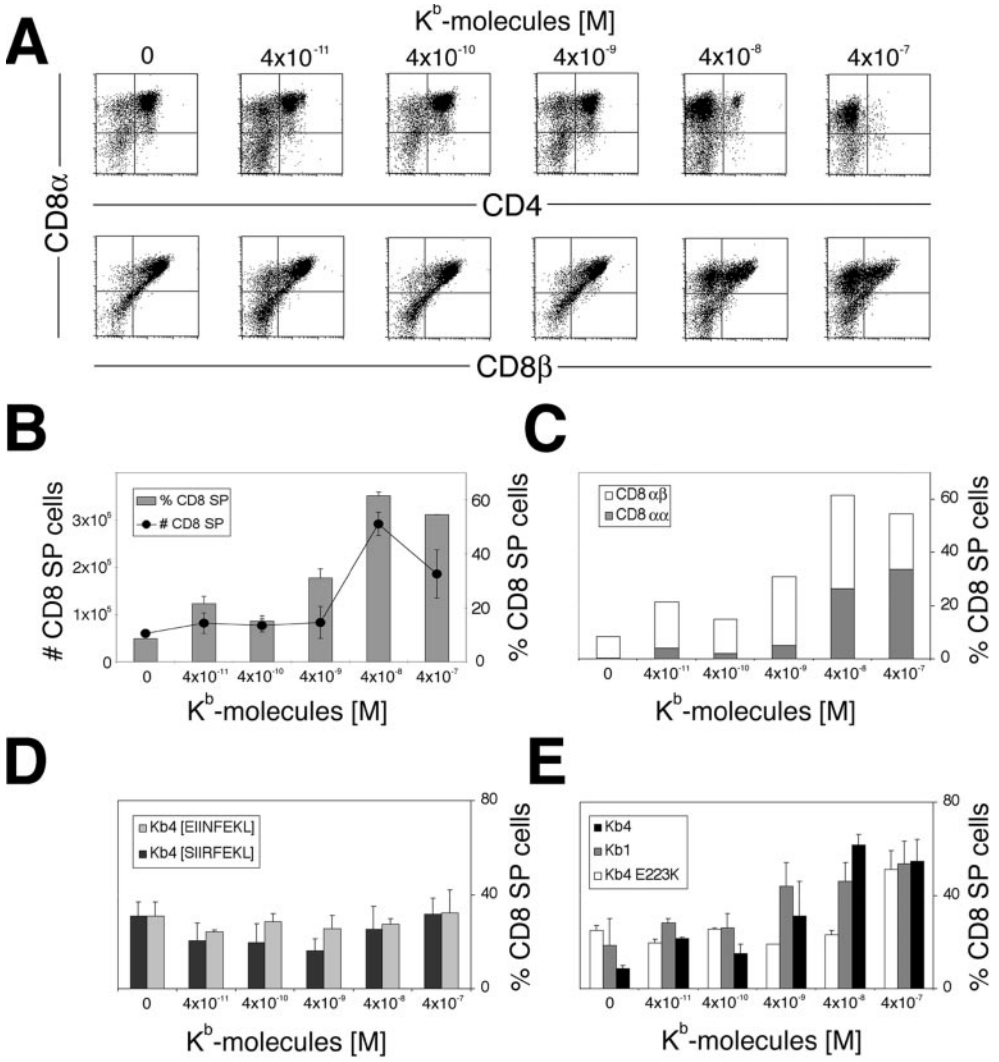

FIGURE 1. CD8 T cells are positively selected by soluble H-2K^b molecules loaded with SIINFEKL peptide in FTOC. *A*, Thymic lobes were excised from OT-I $K^bD^{b-/-}$ $RAG^{-/-}$ embryos at gestational day 16 and were cultured in vitro with H-2K^b tetramers loaded with the peptide SIINFEKL in the indicated concentrations. Lobes were harvested after 7 days in culture and were analyzed by flow cytometry after staining for CD4, CD8α, and CD8β. *B*, Quantitation of the data in *A*. Lobes were harvested and total cell numbers were determined in addition to flow cytometry. Data are presented as mean ± SEM of duplicate samples and represent data from two independent experiments. *C*, Quantitation of the data in *A*. Data are presented as mean of duplicate samples and represent data from two independent experiments. *D*, Thymic lobes were excised from OT-I $K^bD^{b-/-}$ $RAG^{-/-}$ embryos at gestational day 16 and cultured in vitro with H-2K^b tetramer loaded with partial agonist peptides EIINFEKL or SIIRFEKL at the indicated concentrations. Lobes were harvested after 7 days in culture and were analyzed by flow cytometry after staining for CD4 and CD8. Data are presented as mean ± SEM of duplicate experiments and represent two independent experiments. *E*, Thymic lobes were excised from OT-I $K^bD^{b-/-}$ $RAG^{-/-}$ embryos at gestational day 16 and were cultured in vitro with H-2K^b tetramer (Kb4), monomer (Kb1), or H-2K^b tetramer unable to bind CD8 due to a mutation in its α3 domain (Kb4 E223K) loaded with the peptide SIINFEKL at the indicated concentrations. Lobes were harvested after 7 days in culture and were analyzed by flow cytometry after staining for CD4 and CD8. Data are presented as mean ± SEM of duplicate experiments and represent two independent experiments.

Addition of high-affinity ligands to an existing set of self-peptides should induce negative rather than positive selection. We examined whether this was true for soluble K^b4-SIINFEKL. Indeed, inclusion of K^b4-SIINFEKL into FTOC from OT-I $RAG^{-/-}$ $K^bD^{b+/+}$ animals induced negative selection, whereas inclusion of class I MHC tetramers loaded with an irrelevant peptide did not (Fig. 2). Because FTOCs from $K^bD^{b-/-}$ animals contain a population of CD8 SP T cells in the absence of selecting ligand (Fig. 1*A*), it was necessary to establish that the CD8 SP T cells generated by addition of K^b4-SIINFEKL resulted from positive selection rather than expansion of the preexisting population. To that end, K^b4-SIINFEKL was added to FTOC from OT-I $\beta_2m^{-/-}$ animals, in which few SP CD8 cells are observed in the absence of selecting ligand. The generation of SP CD8 thymocytes by inclusion of K^b4-SIINFEKL (Fig. 2), accompanied by an increase in SP CD8 cell numbers (data not shown), demonstrates that the SP CD8 thymocytes are generated by positive selection mediated by K^b4-SIINFEKL.

The cell surface phenotype of agonist-selected OT-I CD8 T cells resembles that of naive OT-I CD8 LN cells

The cell surface phenotype of agonist-selected OT-I CD8 T cells was examined by flow cytometry. First, the positively selected cells were compared with those recovered from nonselecting FTOC conditions (Fig. 3*A*). CD24, a marker lost during maturation of SP thymocytes, was down-regulated in response to the selecting stimulus. The phenotypic changes in expression of CD24 upon selection were confined to the CD8$\alpha\beta$ population retrieved from FTOC, whereas the CD8$\alpha\alpha$ cells generated were $CD24^{low}$ even in the absence of selecting ligand (data not shown). Conversely, CD5, a marker up-regulated in vivo by cells undergoing positive selection (34) was up-regulated in FTOC supplemented with K^b4-SIINFEKL.

The cell surface phenotype of FTOC-derived, agonist-selected OT-I CD8 T cells was compared with OT-I CD8 T cells harvested from peripheral LNs of OT-I $RAG^{-/-}$ mice (Fig. 3*B*). CD24 was down-regulated on FTOC-derived SP CD8 T cells, compared with preselection thymocytes, but it had not receded to the level expressed by OT-I $RAG^{-/-}$ LN cells, which lack CD24 altogether. Similarly, CD5 expression on FTOC-derived, agonist-selected CD8 T cells was slightly lower than that on peripheral OT-I $RAG^{-/-}$ LN cells. The FTOC-derived, agonist-selected OT-I CD8 T cells expressed comparable levels of the coreceptor CD8, whereas CD3 and TCR (Vα2) were expressed at slightly lower levels than seen on OT-I $RAG^{-/-}$ LN cells. Agonist-selected OT-I CD8 T cells did not express early activation markers CD69, CD25 (IL-2Rα), CD122 (IL-2Rβ), or CD44. Analysis of adhesion/homing receptors showed that the FTOC-derived, agonist-selected OT-I CD8 T cells were $CD62L^{low}$ and $\alpha_4\beta_7^{high}$, whereas LN OT-I CD8 T cells are $CD62L^{high}$ and $\alpha_4\beta_7^{low}$.

We conclude that the OT-I CD8 T cells selected by agonist in FTOC display a phenotype resembling that of naive LN cells, although the levels of CD24 and CD5 suggest that these cells are slightly less mature. The agonist-selected cells displayed a naive phenotype and showed an unusual pattern of adhesion/homing receptors.

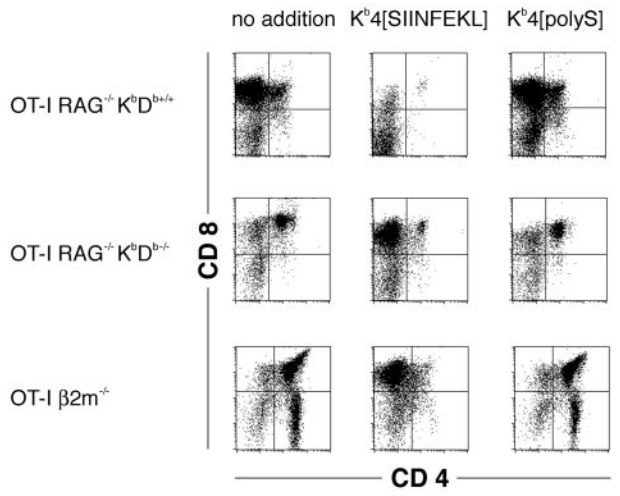

FIGURE 2. Soluble H-2K^b molecules induce positive selection of CD8 thymocytes in OT-I $K^bD^{b-/-}$ and OT-I $\beta_2m^{-/-}$ FTOC, but negative selection in $K^bD^{b+/+}$ FTOC. Thymic lobes were excised from OT-I $RAG^{-/-}$ $K^bD^{b+/+}$ (*top row*), OT-I $RAG^{-/-}$ $K^bD^{b-/-}$ (*middle row*), or OT-I $\beta_2m^{-/-}$ (*bottom row*) embryos at gestational day 16 and were cultured in vitro without selecting ligand, supplemented with H-2K^b tetramers loaded with the agonist peptide SIINFEKL (*middle*), or supplemented with H-2K^b tetramers loaded with the irrelevant peptide SSYSYSSL (polyS; *right*) at 1×10^{-8} M H-2K^b tetramer (4×10^{-8} M K^b molecules). Lobes were harvested after 7 days in culture and were analyzed by flow cytometry after staining for CD4 and CD8.

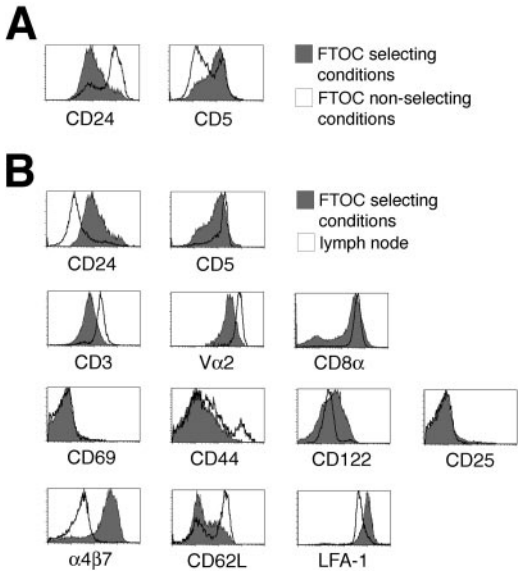

FIGURE 3. Agonist-selected CD8 T cells display a mature and naive phenotype. *A*, Thymic lobes were excised from OT-I $K^bD^{b-/-}$ $RAG^{-/-}$ embryos at gestational day 16 and were cultured in vitro in the presence (selecting conditions) or absence (non-selecting conditions) of 1×10^{-8} M H-2K^b tetramer (4×10^{-8} M K^b molecules) loaded with the peptide SIINFEKL. Lobes were harvested after 7 days in culture and analyzed by flow cytometry after staining for CD4, CD8, CD24, and CD5. *B*, Thymic lobes were excised from OT-I $K^bD^{b-/-}$ $RAG^{-/-}$ embryos at gestational day 16 and were cultured in vitro in the presence of 1×10^{-8} M H-2K^b tetramer (4×10^{-8} M K^b molecules) loaded with the peptide SIINFEKL (FTOC selecting conditions). OT-I LN cells were isolated from OT-I $RAG^{-/-}$ animals (lymph node). Cells were stained with the indicated Abs before flow cytometry. Data presented are gated on CD8 SP cells.

FTOC-derived OT-I CD8 T cells express high levels of the integrin $\alpha_4\beta_7$

The profile of homing and adhesion molecules revealed an interesting characteristic of FTOC-derived OT-I CD8 T cells: the FTOC-derived cells expressed high levels of $\alpha_4\beta_7$ integrin. Expression of $\alpha_4\beta_7$ integrin is detected also on a low percentage of cells present in FTOC under nonselecting conditions (Fig. 4*A*). Further investigation of $\alpha_4\beta_7$ integrin expression in response to

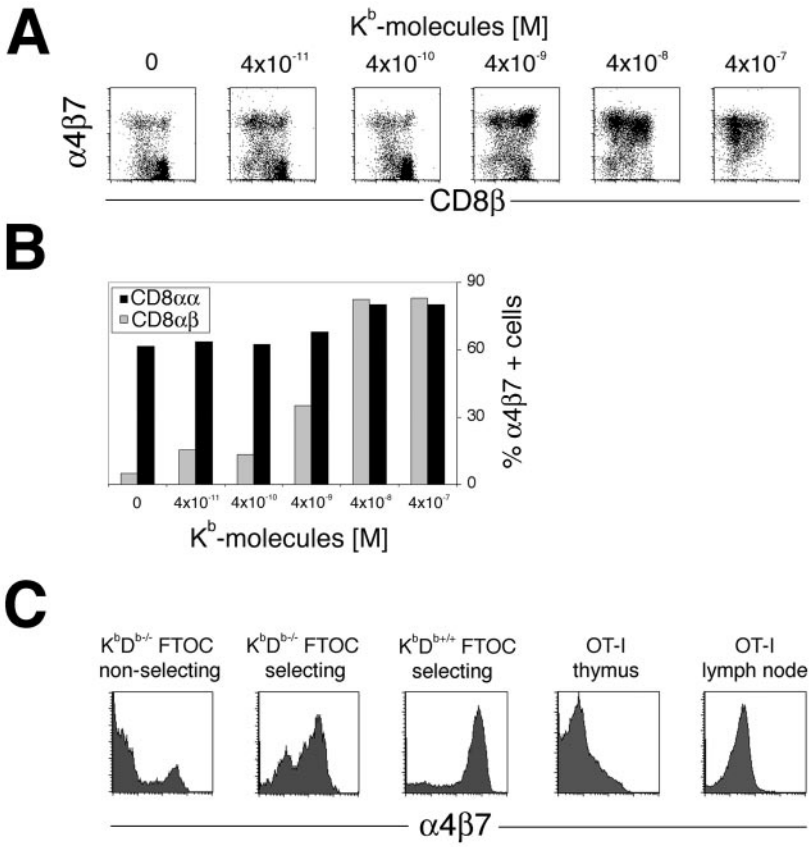

FIGURE 4. $\alpha_4\beta_7$ integrin is up-regulated in response to positive selection in FTOC. *A*, Thymic lobes were excised from OT-I $K^bD^{b-/-}$ $RAG^{-/-}$ embryos at gestational day 16 and were cultured in vitro with H-$2K^b$ tetramer loaded with the peptide SIINFEKL at the indicated concentrations. Lobes were harvested after 7 days in culture and were analyzed by flow cytometry after staining for CD4, CD8α, CD8β, and $\alpha_4\beta_7$ integrin. Data presented are gated on CD8α^+ cells. *B*, Quantitation of the data in *A*. Data are presented as mean of duplicate samples and represent data from two independent experiments. *C*, Cells were harvested from OT-I $K^bD^{b-/-}$ FTOC cultures without addition of tetramer (non-selecting) or supplemented with 1×10^{-8} M H-$2K^b$ tetramer loaded with SIINFEKL (selecting) or from OT-I $K^bD^{b+/+}$ FTOC cultures without addition of tetramer. Cells were also harvested from thymus and LN of adult OT-I $RAG^{-/-}$ mice. Cells were stained for $\alpha_4\beta_7$ integrin. Data presented are gated on CD8α^+ cells.

increasing concentrations of K^b4-SIINFEKL showed that $\alpha_4\beta_7$ integrin expression was induced by increasing concentrations of the selecting ligand (Fig. 4*A*). Expression of $\alpha_4\beta_7$ integrin was consistently high on CD8$\alpha\alpha$ OT-I cells at all concentrations of ligand. In contrast, CD8$\alpha\beta$ cells that arise as a result of exposure to increasing concentrations of selecting ligand show increased expression of $\alpha_4\beta_7$ integrin (Fig. 4*B*). Expression of $\alpha_4\beta_7$ integrin was analyzed in FTOC from $K^bD^{b+/+}$ OT-I animals, where positive selection results from interactions of varied affinities. High levels of $\alpha_4\beta_7$ integrin expression were seen on thymocytes from OT-I $K^bD^{b+/+}$ FTOC, whereas thymocytes from adult OT-I $K^bD^{b+/+}$ animals mostly showed low levels of $\alpha_4\beta_7$ integrin. In comparison, OT-I LN cells showed low to intermediate levels of $\alpha_4\beta_7$ expression (Fig. 4*C*). Therefore, selection in FTOC promotes the expression of $\alpha_4\beta_7$ integrin on postselection thymocytes independent of the selecting ligands themselves.

Agonist-selected OT-I CD8 T cells are capable of generating IFN-γ in response to the antigenic peptide SIINFEKL in vitro

Previous reports suggest that selection of OT-I T cells by their agonist ligand in FTOC renders the cells anergic to further stimulation with agonist (9). To assess the functional properties of the K^b4-SIINFEKL-selected OT-I CD8 T cells, cells were harvested following an overnight resting period of the intact thymic lobe in medium without ligand. The cells were recovered and cultured in the presence of irradiated H-2^b splenocytes loaded with SIINFEKL peptide. After 24 h, production of IFN-γ was assessed by ELISA. The agonist-selected OT-I CD8 T cells were capable of generating IFN-γ in response to the Ag-specific stimulus. The dose-response, as determined by ELISA, was similar to that generated by LN OT-I CD8 T cells (Fig. 5*A*). To exclude a contribution to IFN-γ-production by $CD4^-CD8^-$ double negative (DN) cells that may contain $\gamma\delta$ lineage cells, a CD8-enriched population was obtained by positive sorting using anti-CD8α magnetic beads. Intracellular staining for IFN-γ was then performed in conjunction with surface staining for CD8α and CD8β. Cells that responded to the antigenic stimulus by production of IFN-γ were almost exclusively CD8$\alpha\beta$ T cells and showed a response quantitatively similar to that obtained from LN cells (Fig. 5*B*). CD8$\alpha\alpha$ cells produced very little IFN-γ, and only in response to high doses of Ag. We conclude that agonist-selected OT-I CD8$\alpha\beta$ T cells are not anergic and can produce the effector cytokine IFN-γ by responding to the same agonist ligand that induced positive selection. In contrast, CD8$\alpha\alpha$ cells did not produce significant amounts of IFN-γ.

Agonist-selected OT-I CD8 T cells adoptively transferred into recipients preferentially home to Peyer's patches and intestinal epithelium in vivo

Given that positive selection of OT-I CD8 T cells in the K^bD^b-deficient FTOC model yielded a high number of cells, it was possible to examine their functional properties in vivo. To our knowledge, functional analysis of FTOC-derived T cells has not been previously performed. Before undertaking such experiments, the in vivo homing pattern of the K^b4-SIINFEKL selected OT-I CD8 T cells needed to be established, particularly in view of the high expression of $\alpha_4\beta_7$ integrin. The $\alpha_4\beta_7$ integrin binds to mucosal addressin cell adhesion molecule-1 (mucosal vascular addressin) expressed by venules in mucosal-associated lymphoid tissue and is implicated in the homing of lymphocytes to the intestinal mucosa (35). OT-I CD8 T cells were harvested either from peripheral LNs of OT-I $RAG^{-/-}$ mice or from FTOC by selection on K^b4-SIINFEKL. These cells were then adoptively transferred into nontransgenic C57/BL6 mice. The recipient mice were depleted of NK cells to prevent elimination of the transferred cells due to their lack

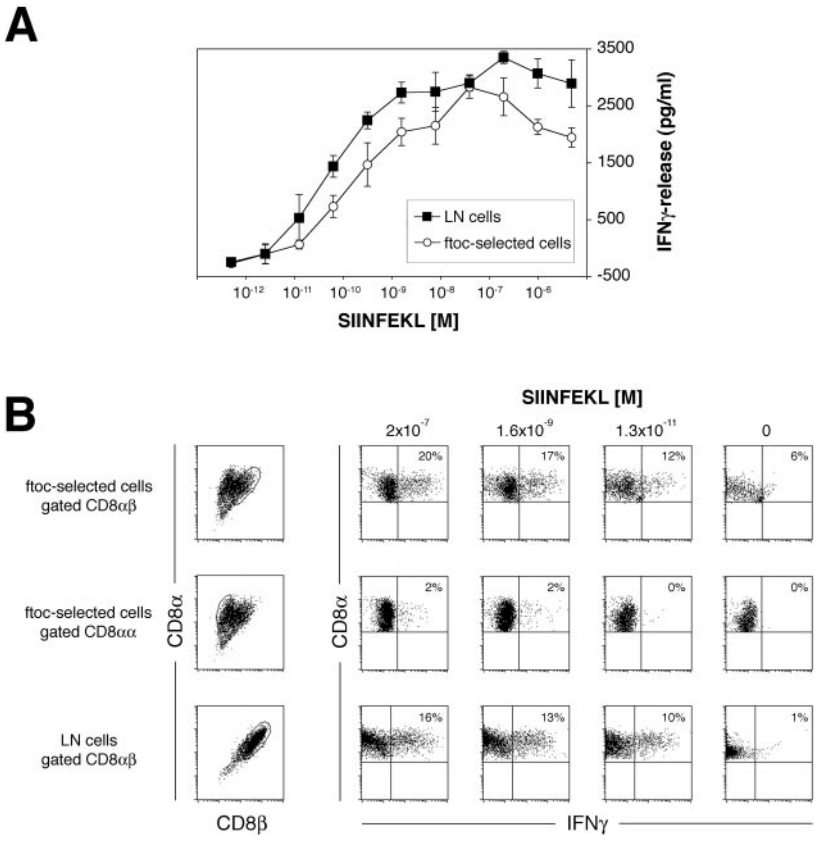

FIGURE 5. Agonist-selected CD8$\alpha\beta$ T cells produce the effector cytokine IFN-γ in response to Ag. *A*, Thymic lobes were excised from OT-I $K^bD^{b-/-}$ $RAG^{-/-}$ embryos at gestational day 16 and cultured in vitro in the presence of 1×10^{-8} M H-$2K^b$ tetramer (4×10^{-8} M K^b molecules) loaded with the peptide SIINFEKL for 7 days. Fresh medium without selecting ligands was provided 12 h before harvesting the cells. LN cells were harvested from OT-I $RAG^{-/-}$ mice. Cells were incubated with H-2^b splenocytes coated with SIINFEKL at the indicated concentrations for 18 h. Supernatants were collected and analyzed by ELISA. Data are presented as mean ± SEM of duplicate experiments and represent data from two independent experiments. *B*, Cells were treated as in *A*, except that Brefeldin A was added to cultures for the last 6 h at a final concentration of 5 μg/ml. Cells were harvested after 24 h and were analyzed by flow cytometry after staining with Abs against CD8α and CD8β and intracellular staining with Ab against IFN-γ.

of class I MHC expression. We have observed that activated class I MHC-deficient cells are highly susceptible to NK cell-mediated elimination in vivo (our unpublished observations). Three days after transfer, spleen, inguinal LNs (ILNs), mesenteric LNs (MLNs), Peyer's patches (PPs), and IELs of recipient mice were harvested and examined by flow cytometry (Fig. 6). The transferred transgenic CD8 T cells were distinguished from endogenous nontransgenic CD8 T cells by staining with fluorescent K^b-SIINFEKL tetramer to identify T cells that express the OT-I TCR. As expected from the reciprocal patterns of $\alpha_4\beta_7$ and CD62L expression (36, 37), the transferred LN cells obtained from OT-I $RAG^{-/-}$ mice were recovered from spleen, MLN, ILN, and PP.

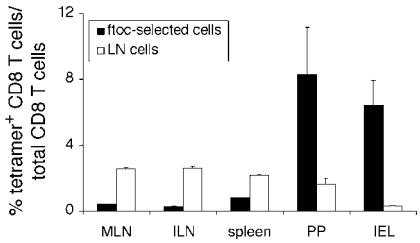

FIGURE 6. FTOC-derived CD8 T cells home to gut-associated lymphoid tissues upon adoptive transfer. *A*, Thymic lobes were excised from OT-I $K^bD^{b-/-}$ $RAG^{-/-}$ embryos at gestational day 16 and were cultured in vitro in the presence of 1×10^{-8} M H-$2K^b$ tetramer (4×10^{-8} M K^b molecules) loaded with the peptide SIINFEKL. LN cells were harvested from OT-I $RAG^{-/-}$ mice. A total of 2×10^6 cells were transferred i.v. into NK-depleted B6 hosts. Recipient mice were sacrificed 3 days after transfer and MLNs and ILNs, as well as spleen and PPs, were isolated from recipient mice. IELs were isolated from the intestine. Cells were analyzed by flow cytometry after staining with anti-CD8α Ab and PE-conjugated K^b(SIINFEKL) tetramer. Data are presented as mean ± SEM of duplicate mice and represent data from two independent experiments.

Very few OT-I LN cells were recovered from IEL preparations. In contrast, OT-I CD8 T cells obtained from $K^bD^{b-/-}$ FTOC were not recovered from spleen, ILN, or MLN in significant numbers. As predicted by the expression of the $\alpha_4\beta_7$ integrin, agonist-selected OT-I CD8 T cells were selectively recovered from PP and intestinal epithelium. These results show that CD8 T cells generated in FTOC survive in vivo and display homing properties consistent with the pattern of $\alpha_4\beta_7$ integrin expression.

Agonist-selected OT-I CD8 T cells divide in response to the antigenic peptide SIINFEKL in vivo

We then examined the ability of agonist-selected OT-I CD8 T cells to divide in response to administration of SIINFEKL peptide in vivo. To that end, FTOC-derived or LN OT-I CD8 T cells were labeled with CFSE, and 2×10^6 cells were transferred into NK-depleted, nontransgenic C57BL/6 recipients. The ability of the cells to proliferate in response to i.p. administration of SIINFEKL peptide was assessed by measuring progressive dilution of CFSE (Fig. 7). Spleen, ILN, MLN, PP, and IEL were harvested and examined by flow cytometry for the presence of CFSE-labeled cells. In mice that received OT-I LN cells and no Ag, CFSE-labeled cells were recovered from spleen (Fig. 7*A*), ILN (data not shown), and MLN (data not shown). In response to the administration of SIINFEKL, dividing OT-I cells were observed in the spleen (Fig. 7*A*) as well as in LN (data not shown). In addition, some dividing OT-I cells were recovered from PP, but not intestinal epithelium (data not shown). In mice that had received FTOC-selected OT-I cells and no Ag, we recovered the nondivided CFSE-labeled cells exclusively from IEL (Fig. 7*A*) and PP (data not shown). The presence of an undivided CD8 T cell population indicates that the K^b4-SIINFEKL-selected OT-I CD8 T cells do not possess intrinsic

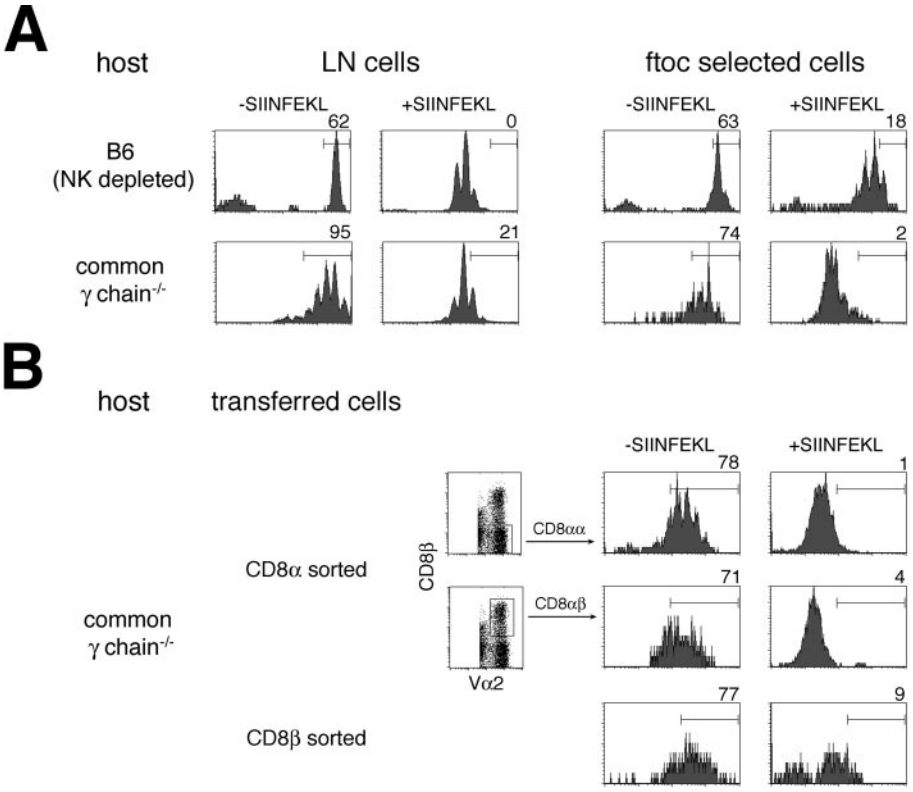

FIGURE 7. Agonist-selected CD8 T cells proliferate in response to SIINFEKL peptide after adoptive transfer. *A*, Thymic lobes were excised from OT-I $K^bD^{b-/-}$ $RAG^{-/-}$ embryos at gestational day 16 and were cultured in vitro in the presence of 1×10^{-8} M H-2K^b tetramer (4×10^{-8} M K^b molecules) loaded with the peptide SIINFEKL for 7 days. LN cells were harvested from OT-I $RAG^{-/-}$ mice. A total of 2×10^6 CFSE-labeled cells were transferred i.v. into NK-depleted B6 hosts or $RAG^{-/-}$ $c\gamma c^{-/-}$ hosts. Mice were injected with Ag (+SIINFEKL) or saline (−SIINFEKL) after 24 h. Recipient mice were sacrificed 2 days after priming. Spleen (of mice that had received LN cells) and IEL (of mice that had received FTOC-selected cells) were analyzed by flow cytometry after staining with anti-CD8α Ab and PE-conjugated K^b(SIINFEKL) tetramer. Data are gated on $CD8^+$tetramer$^+$ cells. The percentage of cells that did not respond to Ag is denoted. *B*, FTOC-selected cells were generated as in *A*. Before transfer into $RAG^{-/-}$ $c\gamma c^{-/-}$ hosts, cells were sorted for CD8α or CD8β by magnetic cell sorting. Sorted CFSE-labeled populations were then transferred. Hosts were challenged with SIINFEKL peptide as in *A*. Cells retrieved from host animals were stained with anti-Vα2 and anti-CD8β Abs, and CFSE profiles are shown for $V\alpha2^+CD8b^-$ and $V\alpha2^+CD8b^+$ cells in the two *upper panels*. In the *lower panel*, cells were stained with anti-CD8α Ab and K^b(SIINFEKL) tetramer. CFSE profiles are shown for $CD8\alpha^+$tetramer$^+$ cells. The percentage of cells that did not respond to Ag is denoted.

proliferative capacity in the absence of exogenous Ag. Agonist-selected, FTOC-derived OT-I CD8 T cells were capable of responding to SIINFEKL in vivo, with actively dividing cells recovered from both the intestinal epithelium (Fig. 7*A*) and PP (data not shown). Dividing cells were absent from spleen, ILN, or MLN, suggesting that there is little recirculation of responding cells to these secondary lymphoid tissues at this time point. The kinetics of the response of agonist-selected FTOC-derived OT-I CD8 T cells showed a delay compared with that of OT-I LN cells. This finding indicates either an intrinsic slower proliferative response of FTOC-selected cells, or it may be due to a lag in the time required for the SIINFEKL Ag to access the sites where the T cells reside. Regardless, the agonist-selected OT-I CD8 T cells were capable of dividing in response to their agonist ligand in vivo, a further indication that such agonist-selected cells cannot be considered anergic.

To facilitate analysis of the in vivo proliferative response of the transferred T cells, $c\gamma c^{-/-}$ mice were used because these animals lack NK cells altogether. The CFSE profiles obtained from $c\gamma c^{-/-}$ mice used as hosts for FTOC-selected or LN-derived cells were similar to those seen in C57/BL6J mice, with the notable difference that homeostatic proliferation was induced due to the lack of B and T cells in these lymphopenic hosts. This proliferation results in considerable CFSE dilution, even in the absence of an antigenic stimulus. However, the Ag-dependent response was clearly distinguishable from T cell expansion in the lymphopenic host. To establish that cells proliferating in response to Ag do not represent DN cells, FTOC-derived cells were enriched for $CD8^+$ cells by positive selection on magnetic CD8α beads. Flow cytometry showed no remaining DN cells in the transferred population (data not shown). The procedure of selection had no effect on the proliferative response of OT-I LN cells (data not shown), indicating that positive selection using CD8α beads did not alter the capacity to respond to Ag. Because anti-CD8β Abs interfere with tetramer-staining (38), cells retrieved from adoptively transferred hosts were stained with anti-Vα2 and anti-CD8β Abs and CFSE profiles were obtained (Fig. 7*B*). Gating on $CD8\beta^-$ (CD8$\alpha\alpha$) and $CD8\beta^+$ (CD8$\alpha\beta$) cells showed that both populations were capable of proliferating in response to SIINFEKL. Because it could not be excluded that the population that responded initially was CD8$\alpha\alpha$ and that CD8β was up-regulated in response to the antigenic stimulus, CD8$\alpha\beta$ cells generated in FTOC were enriched by positive selection using biotinylated anti-CD8β Ab and streptavidin-coated magnetic beads. Analysis of mice into which purified CD8$\alpha\beta$ cells derived from FTOC had been transferred showed a proliferative response of this population to Ag, establishing that CD8$\alpha\beta$ cells generated in FTOC by selection on agonist ligand retain the ability to respond to their nominal Ag in vivo.

Discussion

Here we report the generation of functional CD8 T cells in an FTOC system derived from H-2K^{b}D^b-deficient mice. The number of cells generated was sufficient to allow in vivo analysis of their functional properties. Our model has advantages over the commonly used models derived from TAP$^{-/-}$ or β_2m$^{-/-}$ mice. FTOC from TAP$^{-/-}$ or β_2m$^{-/-}$ mice are supplemented with a source of synthetic peptide and recombinant β_2m to achieve reconstitution of class I MHC expression on the surface of the cells involved in selection. However, even in the absence of added peptide or β_2m, sizable numbers of class I MHC reach the cell surface. The extent to which such molecules are folded at least partially and can contribute to T cell selection is not known. Furthermore, the extent of reconstitution achieved by addition of peptide and β_2m is difficult to gauge. Addition of recombinant class I MHC molecules, at known concentration, of known valency, and with defined peptide content was used here to restore positive selection of CD8 T cells.

Even in the absence of added recombinant class I MHC molecules, CD8 SP T cells are generated in K^{b}D$^{b-/-}$ FTOC. These cells resemble the CD8 T cells observed in the 2C TCR transgenic model, crossed onto the K^{b}D^b-deficient background (32). Because for both 2C and OT-I their occurrence is abolished in FTOC from β_2m-deficient mice, cross-reactivity of these TCRs with nonclassical class I MHC molecules may be responsible for their selection. Cross-reactivity is an inherent feature of the TCR, and it is the unavoidable consequence of the need for T cells to be selected in the thymus by self-Ag yet to react with foreign Ag in the periphery. CD8 T cells generated in 2C K^{b}D$^{b-/-}$ animals have been shown to display cytotoxicity toward the relevant Ag-coated target cells (32). The CD8$\alpha\beta$ cells we observed in FTOC in the presence of selecting ligand are clearly distinct from the cells generated in the absence of soluble class I MHC molecules, as judged from up-regulation of $\alpha_4\beta_7$ integrin upon selection and their generation in OT-I β_2m$^{-/-}$ FTOC when the selecting ligand is included.

Current models of positive selection predict that self-Ags need to be presented by cortical thymic epithelial cells to induce positive selection (1, 2). Likewise, costimulatory molecules and cytokines are involved in the process (4, 5). We demonstrate here that in the H-2K^{b}D$^{b-/-}$ FTOC system, to generate functional CD8 T cells the selecting stimulus can be reduced to the provision of the class I MHC molecule itself, presented in soluble form. We find that agonist peptide, but not partial agonist or antagonist peptide, can mediate selection of CD8 T cells. It is important to note that the results obtained with FTOC from K^{b}D^b-deficient mice are quite different from those seen in FTOC prepared from K^{b}D^b-positive OT-I TCR transgenic mice. When K^{b}4-SIINFEKL complexes are introduced into class I MHC-positive, OT-I TCR transgenic FTOC, we observe deletion, in agreement with expectations (9). Clearly the density of ligand available to T cells in the course of selection is a key determinant of the outcome of the selection process. In a class I MHC-positive thymic lobe, all class I MHC complexes present will be occupied with self-peptides distinct from the nominal Ag, yet with a range of affinities for the OT-I TCR. Introduction of the K^{b}4-SIINFEKL-complex will simply add a set of high-affinity interactions to the total number of interactions registered by developing T cells and lead to deletion. In the class I MHC-deficient FTOC, the only class I MHC molecules present are those introduced in soluble form, and all of these molecules carry the SIINFEKL peptide ligand, yet they are unlikely to attain the same local density as class I MHC molecules expressed on normal thymic epithelial cells. Generation of CD8 T cells by agonist-ligand is in contrast with published data for the OT-I model that used β_2m$^{-/-}$ mice (9). It is possible that endogenous peptide/MHC complexes present in β_2m-deficient FTOC raise the avidity of the selecting signal above a threshold that will lead to deletion rather than positive selection.

Neither the valency of the class I MHC ligand added nor its ability to bind to the CD8 coreceptor had a major effect on the success of generating CD8 T cells in K^{b}D$^{b-/-}$ FTOC. This is in contrast with our observations on activation of naive class I MHC-deficient CD8 T cells. For activation, K^{b}D$^{b-/-}$ CD8 T cells require multivalent ligation and an intact CD8 binding site on the class I MHC molecule with which they interact (28, 33). Our data are consistent with the notion that the TCR is promiscuous during the selection process, whereas activation of mature T cells requires a more specific signal.

Even though the recombinant K^b molecules are added in soluble form, we do not know their physical disposition in the FTOC itself. Cell adhesion molecules and costimulatory molecules may provide for multivalent interactions between the developing T cells and APCs, with multiple class I MHC molecules inserting themselves in these interactions as if they, too, were engaged in multivalent interactions. Formally, aggregation of monomers in the course of FTOC cannot be ruled out, although no evidence for aggregation was seen in experiments exploring T cell activation requirements (28). The lack of CD8 (39–41) as well as the mutation of its α3 domain (42, 43) compromise positive and negative selection. Agonist-ligands that induce depletion at high concentrations allow positive selection in the absence of CD8 (44, 45), indicating that the coreceptor function of CD8 is dispensable when TCR-peptide/MHC interactions are of high affinity. Given the high affinity interaction between TCR and peptide/MHC in our system, it is conceivable that the coreceptor CD8 is no longer required.

The CD8 T cells generated by inclusion of K^{b}4-SIINFEKL in FTOC are functional, as gauged from their ability to produce IFN-γ when confronted with SIINFEKL-loaded APCs in vitro and as shown by induction of proliferation in response to SIINFEKL-peptide in vivo. Although high-affinity agonist-selected CD8 T cells generated in FTOC were anergic in other models (9, 46), we observed similar levels of IFN-γ production when comparing FTOC-generated CD8 T cells and naive CD8 T cells obtained from OT-I LNs. Our conditions of FTOC allow the production of sufficiently large numbers of CD8 T cells to undertake their transfer in vivo to assess the functional properties of these in vitro generated cells. After transfer of CFSE-labeled cells in vivo, the FTOC-generated cells are responsive to Ag and proliferate, as assessed by dilution of the CFSE label. To our knowledge, the in vivo functionality of T cells generated in FTOC has not been previously demonstrated.

We observed that FTOC supplemented with soluble H-2K^b molecules supports the development of both CD8$\alpha\alpha$ and CD8$\alpha\beta$ cells. The generation of CD8$\alpha\alpha$ cells was favored in the presence of high concentrations of selecting ligand, confirming previous results (47). CD8$\alpha\alpha$ cells displayed constitutively high levels of integrin $\alpha_4\beta_7$, indicating that this subset of cells may be prone to home to gut-associated lymphoid tissues. In contrast, few CD8$\alpha\beta$ cells expressed integrin $\alpha_4\beta_7$ in the absence of agonist ligand, whereas its expression was strongly up-regulated by the addition of selecting ligand. By sorting the cells selected in FTOC, we demonstrate that the CD8$\alpha\beta$ subset that most closely resembles mature CD8 T cells, as well as CD8$\alpha\alpha$ subset, proliferates in response to Ag. However, only the CD8$\alpha\beta$ subset contributed significantly to the production of IFN-γ. Therefore, two different populations of CD8 T cells were selected by agonist-loaded H-2K^b molecules. First, we observed CD8$\alpha\beta$ cells that resemble mature peripheral CD8 T cells, up-regulate $\alpha_4\beta_7$ integrin upon selection, and acquire effector function. Second, we find CD8$\alpha\alpha$ cells that

constitutively express $\alpha_4\beta_7$ integrin, yet acquire only partial effector function.

The expression of $\alpha_4\beta_7$ integrin on the majority of SP CD8 T cells generated in FTOC is an obvious difference from their counterparts isolated from LNs. Up-regulation of $\alpha_4\beta_7$ expression accompanying CD8 T cell selection is also observed in OT-I FTOC (data not shown) and upon selection by nonclassical class I MHC molecules (32) and therefore can be mediated by stimuli other than the agonist ligands described here. Although thymocytes from adult animals express $\alpha4$ and $\beta7$, the levels of $\alpha_4\beta_7$ heterodimer are low. High levels of $\beta7$ are found only on the most mature subset of thymocytes that is about to exit the thymus, although it pairs with $\alpha4$ mostly on CD4 SP thymocytes (48). The FTOC system allows neither immigration of precursor cells nor the export of cells that have differentiated in situ. Cells that in vivo might have left the thymus obviously cannot do so in FTOC, and likely this forced residence is the reason why the surface marker profile of CD8 T cells from a normal thymus and in FTOC is different. It will be interesting to see whether forced emigration of T cells from FTOC, as might be brought about by inclusion of the appropriate chemokine(s) (49), would affect the expression of $\alpha_4\beta_7$ integrin. Alternatively, a bias toward generation of $\alpha_4\beta_7^{high}$ cells may be an intrinsic quality of the fetal organ, as has been demonstrated for the rat embryonic thymus (50).

In T cell selection experiments that make use of a TCR transgenic model, the generation of $\gamma\delta$-precursors driven into the $\alpha\beta$-lineage through the inappropriate signal of the transgenic TCR at an early stage of thymocyte development (51–53) has been a concern. Such cells have been reported to be DN or to express low levels of CD8α, which can be up-regulated upon encounter of Ag. DN thymocytes contribute significantly to CD8$\alpha\alpha$ IELs in TCR-transgenic animals (54). The CD8 T cells generated in our model displayed robust levels of CD8 indistinguishable from the CD8 levels of peripheral CD8 T cells. Also, significant numbers of CD8$\alpha\beta$ cells were generated, for which no connection to the $\gamma\delta$-lineage has been reported. Instead, they develop through the conventional pathway (55).

In summary, we describe thymic selection of CD8 T cells by a soluble H-2K^b/SIINFEKL complex to which they are also capable of responding as effector cells, both in vitro and in vivo. By exploiting K^{b}D$^{b-/-}$ FTOC, we show that soluble class I MHC molecules widely used for detection (56) or activation (28, 38) of peripheral Ag-specific T cells are valuable also for the investigation of selection requirements in the thymus. They allow facile introduction of mutations, precise variation of ligand specificity and density, and a distinction of the MHC/peptide-TCR interaction from other contributing factors.

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