Published in final edited form as:
*J Immunol*. 2013 August 15; 191(4): 1704–1715. doi:10.4049/jimmunol.1300417.

# Cellular and Molecular Requirements for the Selection of *in vitro*-Generated CD8 T Cells Reveals a Role for Notch[1]

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## Abstract

Differentiation of CD8 single-positive (SP) T-cells is predicated by the ability of lymphocyte progenitors to integrate multiple signaling cues provided by the thymic microenvironment. In the thymus and the OP9-DL1 system for T-cell development, Notch signals are required for progenitors to commit to the T-cell lineage, and necessary for their progression to the $CD4^+CD8^+$ (DP) stage of T-cell development. However, it remains unclear whether Notch is a prerequisite for the differentiation of DP cells to CD8 SP stage of development. Here, we demonstrate that Notch receptor-ligand interactions allow for efficient differentiation and selection of conventional CD8 T-cells from bone marrow (BM)-derived hematopoietic stem cells (HSCs). However, BM-HSCs isolated from *Itk*$^{-/-}$ *Rlk*$^{-/-}$ mice gave rise to T-cells with decreased IFNγ production, while gained the ability to produce IL-17. We further reveal that positive- and negative-selection *in vitro* are constrained by peptide-MHC (pMHC) class I expressed on the OP9 cells. Finally, using an MHC class I-restricted T-cell receptor (TCR) transgenic model, we show that the commitment of DP precursors to the CD8 T-cell lineage is dependent on Notch signaling. Our findings further establish the requirement for Notch receptor-ligand interactions throughout T-cell differentiation, including the final step of CD8 SP selection.

## INTRODUCTION

T cells develop in the thymus after colonization by blood-borne bone marrow (BM)[3]-derived progenitors, wherein they undergo a highly regulated processes of differentiation, proliferation, and lineage commitment to generate a pool of naïve effector and regulatory T cells (1–8). Notably, the most prevalent population in the thymus is comprised of $CD4^+$ $CD8^+$ (double positive, DP) thymocytes. These cells express randomly rearranged αβ-T cell receptors (TCRs), which interact with different cell types within the thymus to produce multiple functionally distinct cell lineages. The lineage commitment of DP thymocytes

[1]This work was supported by funds from the Canadian Institutes of Health Research (CIHR, MOP-12927 and MOP-119538). DZ was supported by a Scholarship from the Lady Tata Memorial Fund; and, JCZP is a recipient of Canada Research Chair in Developmental Immunology.

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[3]Abbreviations used in this paper: BM, bone marrow; Dll, delta-like; DN, double negative; DP, double positive; HSC, hematopoietic stem cell; pMHC, peptide-MHC; RCN, relative cell number; SP, single positive; tg, transgenic; FTOC, Fetal Thymic Organ Cultures.

entails qualitatively and quantitatively distinct signals, which are initiated and constrained by the duration of interactions between αβ-TCRs expressed on $CD4^+$ $CD8^+$ thymocytes and ligands, presented by class-I or class-II major histocompatibility complex (MHC-I or MHC-II, respectively) molecules, expressed on thymic epithelial cells (TECs) or hematopoietic cells.

The best-described interactions of DP thymocytes involve engagements of the TCR complex and both CD4 and CD8 co-receptors with self-peptides presented by classical MHC-II or MHC-I expressed by TECs, respectively. Typically, low affinity TCR-self-peptide/MHC-II (pMHC-II) or pMHC-I interactions allow for positive selection and differentiation of DP thymocytes into mature conventional MHC-II-restricted CD4 and MHC-I-restricted CD8 T cells. In contrast, 'insufficient' or 'excessive' affinity of the TCR for pMHC leads to cell death of DP thymocytes by "neglect" and "negative selection", respectively (9–15).

The positive selection outcome is further refined by the strength and/or duration of TCR signaling, whereby stronger and/or longer signals direct DP thymocytes to adopt a CD4 cell fate, while weaker and/or shorter interrupted signals promote CD8 T cell development (15, 16). Correspondingly, the TCR-signaling induced upon TCR/pMHC ligation influences signal-transduction pathways ($p56^{lck}$, Ras, Raf, Cn, MAPK and Erk) involved in positive selection and thus regulate the expression of key factors implicated in CD4 or CD8 lineage outcome (17–23).

Although conventional CD4 and CD8 T cells arise from the same DP precursor and utilize the same TCR-induced signaling pathways, recent studies identified a requirement for TEC kinases, Itk (interleukin-2 (IL2)-inducible T-cell kinase) and Rlk (resting lymhocyte kinase), as independent signaling pathways implicated in the development of conventional CD8 T cells (24–27). Mice deficient in Itk or Itk and Rlk failed to develop conventional CD8 T cells, and instead supported the development of CD8 T cells that have an innate-like phenotype ($CD44^{hi}$, $CD122^{hi}$, IL-15-dependant), and resemble T cells selected by non-classical MHC-Ib molecules. Later findings revealed that these non-conventional CD8 T cells from $Itk^{-/-}$ or $Itk^{-/-}$ $Rlk^{-/-}$ deficient mice are selected by classical MHC-I molecules expressed on hematopoietic cells in the thymus (27–30). Additional non-conventional lineages selected by classical pMHC-II expressing hematopoietic cells comprise of natural Forkhead box P3 $(FoxP3)^+$ $CD4^+$ $CD25^+$ regulatory T ($T_{reg}$), and innate-like $CD4^+$ T cells, as seen in mice expressing exogenous MHC-II activator transcription factor (CIITA) in thymocytes and in humans expressing endogenous MHC-II on immature thymocytes (31–33). Conversely, other non-conventional lineages that originate from DP precursors and acquire innate-like characteristics include cells with TCRs specific for ligands presented by non-classical MHC-Ib molecules expressed on hematopoietic cells in the thymus. These include, natural killer T (NKT) cells selected by glycolipids presented by CD1d molecules expressed on DP thymocytes, and mucosal-associated invariant T cells (MAIT) selected by H2-M3 (Histocompatibility 2, H2, M region locus 3), Qa-1 (H2-T23) and MR1 (MHC-I-related) molecules expressed on hematopoietic cells in the thymus (28, 34–39).

Although the molecular mechanisms and signaling pathways involved in differentiation of these non-conventional T cells have not been fully characterized, it is increasingly recognized that the signaling lymphocyte activation molecule (SLAM)-associated protein (SAP) is required for the development of innate T-cell lineages (24, 30, 40–45). This notion is supported by evidence from SAP-deficient mice, which fail to develop innate-NKT and -CD8 T lymphocytes (43, 44); and from $SAP^{-/-}$ $Itk^{-/-}$ mice, which prevent the development of innate-like CD8 T cells (30). Of note, the SLAM family of receptors expressed by DP thymocytes initiates signaling through homotypic interactions, and thus acts as a self-ligand. These findings imply that lineage commitment at the DP stage of T cell development is cell

to cell contact-dependent commencing with specific TCR-induced signaling pathways and ending with gene expression patterns associated with distinct lineage outcomes.

In addition, early studies suggested that the process of positive selection and CD4/CD8 lineage commitment was linked to the induction of Notch signaling (46). Since Notch is known to function in binary cell fate decisions, it was posited that this signaling pathway might also regulate positive selection and CD4/CD8 lineage commitment. However, a number of studies using gain-of- and loss-of-function approaches produced conflicting conclusions. The first evidence based on the over-expression of activated form of Notch-IC in mice, suggested that Notch regulates development of the CD8 T cell lineage (47). This finding was supported by studies performed using Notch-1 antisense and blocking antibodies in a two-step re-aggregation culture system, which demonstrated that Notch signaling was involved in CD8 but not in CD4 T cell development after lineage commitment was already established (17). However, a later finding failed to support a role for Notch in the CD8 lineage choice, suggesting that Notch-1 signaling promotes the maturation of both CD4 and CD8 single positive (SP) thymocytes (48). These results were further challenged by the conditional deletion of Notch-1 in immature DN3 cells, which revealed no role for Notch 1 in CD4/CD8 commitment, thymocyte survival or maturation of DPs, but did not exclude functional redundancy for this pathway, as DP thymocytes may express other Notch receptors (49, 50). For this reason, two studies were performed to eliminate all Notch signaling. The first study demonstrated that conditional deletion of RBP-Jκ had no effect on CD4/CD8 T cell fate (51), while the second study demonstrated that conditional deletion of *Presenilin* 1 and 2 genes, resulted in the reduction of CD4 lineage, as an indirect result of impaired TCR signaling (52). Thus, the functional importance of Notch/ligand interactions in CD4/CD8 lineage commitment remains unclear.

Given the prominent role of cell-cell interactions in lineage commitment of DP thymocytes, we investigated the differentiation of DP cells to the CD8 SP stage of T cell development in OP9-DL1 cocultures initiated with BM-derived HSCs (53). This approach simplifies the potential cellular interactions involved in lineage commitment, and facilitates the use of HSCs from TCR-transgenic, as well as, gene-deficient mice to interrogate the role of specific signaling pathways. Here, we demonstrate that heterotypic interactions between DP cells and OP9 cells are required for the efficient selection of conventional CD8 T cells, which do not involve SAP function, but are affected by the absence of Itk and Rlk function. Moreover, Notch receptor-ligand interactions between DPs and OP9 cells, respectively, play a critical role in the survival and differentiation of CD8 T cells. Taken together, this work demonstrates for the first time that OP9-DL1 cells provide a unique microenvironment for conferring pMHC-I-restricted TCR-specific signals to immature DP cells to enable effective positive and negative selection, and revealing a requirement for Notch signaling in CD8 lineage commitment and differentiation *in vitro*.

## MATERIALS AND METHODS

### Mice

C57BL/6 (B6), C3H and H2D$^{b}$K$^{b}$ mice were purchased from either Jackson or Taconic Laboratory. RAG-2-deficient mice (54) were bred and maintained in our animal facility. SAP-deficient, Itk/Rlk-deficient, P14-TCR-tg and TGB-TCR-tg mice have been previously described (30, 55, 56). TGB-TCR-tg mice (C3H-background, H2$^{k}$) were backcrossed to B6 mice to obtain TGB-B6 (H2$^{b}$) F4 generation that were further backcrossed to Rag2$^{-/-}$ (H2$^{b}$). Mice were used between 6 and 8 weeks of age, and were maintained in Sunnybrook Health Science Centre animal facility under specific pathogen-free conditions. All animal procedures were reviewed and approved by the Sunnybrook Health Science Centre Animal Care Committee.

## Cell Lines

OP9 cells without (GFP-only) or with expression of the Notch ligand, Dll1, were generated from the OP9 bone marrow stromal cell line and maintained as previously described (57). These are referred to as OP9-CTLR or OP9-DL1 cells respectively. The L929 ($H2^k$) mouse fibroblast cell line and human embryonic kidney 293 cell line were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and maintained as indicated by the supplier. For the generation of shTAP1-M and shTAP-L OP9-DL1 cell lines, different levels of MHC class I expression by OP9-DL1 cells were obtained using shRNA constructs targeting *Tap-1* transcripts purchased from OriGene with the indicated modifications. Specifically, shRNA sequences against *Tap-1* (1451,1453) were subcloned using *EcoRI* and *XhoI* sites from pRS shRNA into pMIR retrovial vector containing an IRES-RFP cassette (a gift of Dr. Vignali, St. Jude Children's Research Hospital, Memphis, TN). Calcium phosphate co-transfections of 293 fibroblasts with Gag-Pol-Env-plasmid- and pMIR-shRNA–constructs were performed. Viral supernatants were collected and used to transduce retrovirus packing cell line GP&E.86, and were further sorted for high expression of RFP and supernatants containing viral particles from these cells were used for transductions of OP9-DL1 cells. Retrovirally transduced OP9-DL1 cells were further sorted for high expression of RFP and low expression of MHC-I, and are referred throughout the text as shTAP1-M (medium) and shTAP1-L (low). The expression of MHC-I was verified by flow cytometry and compared to the expression of MHC-I by non-transduced OP9-DL1 cells. OP9-DL1-$H2D^b$ cells were generated by transfecting OP9-DL1 cells with a plasmid encoding $D^b$ and the puromycin resistance gene. Following transfection, the cells were selected for puromycin resistance and $H2D^{b+}$ OP9-DL1 cells were isolated by flow cytometric cell sorting.

## Bone Marrow and Tissue Cell Isolation

Bone marrow (BM) cells were harvested from the femur and tibia of 6–8 weeks old mice. The dissected long bones were crushed using a Pyrex glass stopper in α-MEM (Gibco) supplemented with 20% FBS (HyClone) and 2.2 g/ml $NaHCO_3$, referred to as OP9-media. Lineage negative ($Lin^-$) bone marrow samples were enriched by immunomagnetic depletion of cells expressing CD45R, CD19, CD3, NK1.1, TER119, CD11b and Gr-1, followed by flow cytometric cell sorting of $CD117^+$ $Sca\text{-}1^{hi}$ expressing cells, referred as the hematopoietic stem cell (HSC) enriched fraction. Thymus and spleen were isolated from 4- to 8- weeks old mice. Single-cell suspensions were disaggregated through a 40 μm nylon cell strainer with a syringe plunger and were washed further with OP9-media. Spleens were depleted of RBCs with Ammonium Chloride Lysing Reagent (PharMingen) as recommended by the supplier. $Lin^-$ DN3 and DN4 T cell subsets were enriched by immunomagnetic depletion of cells expressing CD3, TCRβ (GL3), CD4 and CD8, followed by flow cytometric cell sorting of $CD44^-CD25^+$ and $CD44^-CD25^-$ expressing cells referred to as DN3 and DN4 T cell subsets, respectively. Prior to the cell sorting OP9-DL1 cocultures were passaged through 40 μm nylon cell strainer.

## Flow Cytometry

Biotin-, FITC-, PE-, PE-Cy5-, PE-Cy7-, APC-, APC-Cy7- mAbs were purchased from BD Biosciences or e-Boisciences. The following conjugated antibodies were used: anti-B220 (RA3-6B2), anti-CD3e (145-2C11), anti-CD4 (GK1.5, L3T4), anti-CD5 (Ly-1), anti-CD8a (LY-2, Lyt-2, 53–6.7), anti-CD11a (M17/4, 2D7), anti-CD11b (M1/70), anti-CD16/CD32 (2.4G2), anti-CD19 (1D3), anti-CD24 (M1/69), anti-CD25 (7D4), anti-CD27 (LG.7F9), anti-CD28 (37.51), anti-CD44 (IM7), anti-CD45 (30-F11), anti-CD45.2 (104), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD117 (2B8), anti-CD122 (TM-1), anti-CD244 (eBio244F4), anti-TCRβ (H57-597), anti-GR-1 (RB6-8C5), NK1.1 (PK136), Sca-1 (E13-161.7), TER119 (TER-119) and anti-$H2K^bD^b$ (28-8-6). An appropriate isotype control

antibodies were matched to the specific primary antibody and used where indicated. Cells were stained by standard staining techniques and analyzed on a FACSCalibur or LSRII flow cytometers (BD Biosciences). Data Files were analyzed with Flow-Jo (Tree Star). Dead cells were excluded from all data by forward- and side- scatter and propidium iodide (PI) or 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes). AnnexinV-FTIC staining was performed following the suppliers recommended procedure (BD Pharmigen), and the percentage of cell death was calculated by adding percentages of Annexin $V^+$, Annexin $V^+$ $PI^+$, and $PI^+$ cells.

For intracellular staining, the cells were surface-stained with the appropriate mAbs followed by intracellular (ic) staining using fixation solution and permeabilization buffer as indicated by the supplier (e-Bioscience). Cell sorting was performed with a FACSDiVa or a FACSAria cell sorters (BD Biosciences). Purity was typically greater than 98% for all populations as determined by post-sort analysis.

## PCR and QRT-PCR

Genotyping of TGB-TCR-Tg mice crossed to B6 and $Rag2^{-/-}$ background was preformed using published primers (58, 59). For quantitative real-time reverse-transcriptase-PCR (QRT-PCR), RNA was obtained from sorted CD8 $TCR\beta^{hi}$ T cells isolated from thymus, spleen or BM-HSC/OP9-DL1 cocultures using TRIzol Reagent (Invitrogen). Total RNA was treated with Quanti Tect Reverse Transcription cDNA synthesis kit (Qiagen). QRT-PCR reaction was carried out with SYBR Green (Invitrogen) and analysis was performed using ABI $Prism^R$ 7500 Sequence Detection System. Calculations were completed using relative quantification method, where the samples were normalized to β-actin expression. Following primer sequences for Real Time RT-PCR were purchased from Invitrogen: SAP forward 5′-ACACATATCGAGTGTCCCAGAC-3′; SAP reverse 5′-CCGGAAAAATCTTTTATGTACTCC-3′; Runx1 forward 5′-CTCGGCAGAACTGAGAAATG-3′; Runx1 reverse 5′-GACGGTGATGGTCAGAGTGA-3′; Runx3 forward 5′-GGTTCAACGACCTTCGATTC-3′; Runx3 reverse 5′-GGTCCATCCACAGTGACCTT-3′; ThPok forward 5′-CACCTTCCACCTCTCTCTGG-3′; ThPok reverse 5′-GGTGTCCCTTCCTCTTCCTC-3′; β-actin forward 5′-ATGGTGGGAATGGGTCAGAA-3′; β-actin reverse 5′-TCTCCATGTCGTCCCAGTTG-3′.

## T cell stimulation and ELISA

Coculture derived and ex-vivo isolated cells from thymus or spleen were sorted for CD8 $TCR\beta^{hi}$ expression and cultured in triplicates with plate-bound anti-CD3 (10 g/ml) and anti-CD28 (5 g/ml) mAb in 200ul of OP9-media supplemented with 5 ng/ml of IL-7 and 10 U/ml of IL-2 (PeproTech). The cytokine levels were determined by using a commercially available DuoSet Elisa Development System kit (R&D Systems). In all assays, sample concentrations were calculated from a standard curve trendline equation using OD450nm readings for a given standard concentration. OD450nm raw values from triplicate wells were subtracted from OD450nm readings obtained from triplicate wells of negative controls (media). To obtain final concentration, these values were multiplied by the dilution factor. Results were plotted using one grouping variable scatter plot (Prism).

## Synthetic Peptides

Synthetic peptides were generated by Advanced Automated Peptide Technologies (AAPPTEC) and were purified with HPLC to ≥ 98 % purity and verified by Mass Spectrometry. The peptides were dissolved in PBS at a concentration of 10 mg/ml and further diluted in culture medium before use. $H2K^k$-restricted peptides used in these

experiments corresponded to agonist SV40 Tag 560–568 SEFLLEKERI peptide, and to an irrelevant β-actin HETTFNSI peptide, referred to as S and H peptides, respectively.

### CTL assay

To generate cytotoxic effector cells, CD8 T cells derived from the spleens of TGB-B6 mice and from HSC/OP9-DL1 cocultures, initiated with BM-derived HSCs isolated from $Rag2^{-/-}$ TGB-B6 mice, were sorted and cultured for 5 days with irradiated L929 ($H2^k$) stimulator cells pulsed with $10^{-12}$ M of the S peptide in the presence of 50 U/ml of rhIL-2 (PeproTech). Target cells, L929 ($H2^k$) were pulsed with $10^{-14}$ M of either S or H peptide, and labeled with $^{51}Cr$ for 2 h at 37°C. The effector and target cells were added to 96-well round bottom plate at the indicated (Effector : Target, E:T) ratios. After 4h, the released $^{51}Cr$ in the supernatants was harvested, dried overnight and counted by a Packard TopCount NXT Microplate Scintillation and Luminasance Counter (Perkin Elmer). The specific percentage of lysis was calculated as the ratio of experimental release less spontaneous release over total release less spontaneous release. The data presented are means of duplicates.

## RESULTS

### OP9-DL1 cells permit selection of functionally mature CD8 T cells from MHC-I deficient hematopoietic progenitor cells

The thymus contributes to the continuous generation of naïve T cells from incoming BM-derived progenitors. The maintenance of T cell numbers in the thymus can be readily observed, with a constant DP to CD8 SP cell ratio of ~25 to 1 (88% to 3%) (Fig. 1A). In contrast, T cell generation from BM-derived HSC/OP9-DL1 cocultures can attain a ratio of ~2 to 1 (52% DP to 24% CD8 SP). To assess the process of selection in OP9-DL1 cocultures, we evaluated the differentiation potential of BM HSCs isolated from MHC-I ($H2D^b$ $H2K^b$ double knock-out, dko) mice. We consistently found that similar numbers of *in vitro*-derived CD8 T cells were generated from cocultures initiated with MHC-I dko- or wild-type (WT)-BM HSCs (Fig. 1A and 1B). Furthermore, high levels of expression of TCRβ were detected on equal proportion of CD8 cells from both cocultures, suggesting that *in vitro* derived CD8 cells attained a mature phenotype (Fig. 1A). This was further supported by the detection of differentiation and maturation cell-surface markers (CD5, CD28, Q2a), and lack of the expression of innate-like surface markers (CD44, CD122 and CD244) from both cocultures on $TCR\beta^+$ CD8SP cells (Fig. 1C). Of note, while as expected, MHC-I dko-derived cells lacked MHC-I expression, WT-derived and thymus $TCR\beta^+$ CD8SP cells showed high levels of MHC-I expression. Although, T cell maturation is associated with the down-regulation of CD24 (HSA) cell-surface marker *in vivo*, the levels of CD24 remained high on *in vitro*-derived CD8 T cells (53). Nevertheless, the maturational profile depicted in Figure 1C coincides with the acquisition of mature conventional phenotype by *in vitro*-derived $TCR\beta^+$ CD8 T cells obtained from cocultures initiated with either MHC-I dko- or WT-BM-derived HSCs. Moreover, the functional analyses, such as induction of activation markers (CD11a, CD25, CD44 and CD62L) and intracellular expression of Granzyme B following anti-CD3/CD28-mediated stimulation, were analogous between *in vitro*-derived and *ex vivo* CD8 T cells (Fig. 1D and 1E). Taken together, the differentiation outcome of CD8 $TCR\beta^+$ T cells generated *in vitro* can be attributed to MHC-I expressed by the OP9 cells.

### Development of conventional CD8 T cells is supported by OP9-DL1 cells

To address whether conventional or innate-like CD8 cells are generated *in vitro*, we examined the differentiation of BM-HSCs isolated from SAP-deficient or WT (B6) mice in OP9-DL1 cocultures. Flow cytometry analysis of cocultures revealed that $TCR\beta^+$ CD8 cells develop equally well in both, $SAP^{-/-}$ and WT cocultures (Fig. 2A). The notion that *in vitro*

generated CD8 TCRβ$^+$ cells from both cocultures acquired a conventional phenotype was verified as these cells did not express CD44 and CD122, while β7-integrin was expressed (Fig. 2B). These observations imply that OP9 stromal cells similar to thymic stromal cells allow for the generation of conventional CD8 T cells.

We confirmed the absence of SAP expression by *in vitro*-derived CD8 TCRβ$^+$ T cells generated from SAP$^{-/-}$ BM HSCs, in comparison to the WT (data not shown). Moreover, similar expression levels of the transcription factors Runx1 and Runx3, and lack of expression of ThPok, from *in vitro*-derived SAP$^{-/-}$ and WT CD8 cells were detected (data not shown). Additionally, following TCR-stimulation, a similar increase in proliferation, induction of activation markers (CD11a, CD25, CD44, CD69 and CD62L), generation of IFN-γ and Gzm-B were observed between *in vitro*-derived SAP$^{-/-}$ and WT CD8 cells (Fig. 2C–E and data not shown).

We further investigated whether *in vitro*-generated CD8 TCRβ$^+$ cells, like conventional T cells, required Itk/Rtk signals for their differentiation. To this end, BM-HSCs isolated from Itk/Rlk-deficient or WT (B6) mice were cultured with OP9-DL1 cells. Surprisingly, the differentiation of TCRβ$^+$ CD8SP cells was similar in cocultures initiated with BM-HSCs isolated from Itk/Rlk-deficient or WT mice (Fig. 2F). Additionally, Itk$^{-/-}$Rlk$^{-/-}$ and WT *in vitro*-derived CD8 T cells were CD44$^-$ CD122$^-$ β7 integrin$^+$ (Fig. 2G), indicating that they do not possess an innate-like phenotype. However, functional assessment of *in vitro*-derived Itk$^{-/-}$Rlk$^{-/-}$ in comparison to the WT CD8 T cells revealed that Itk$^{-/-}$Rlk$^{-/-}$ CD8 T cells have a diminished ability to secrete IFN-γ (Fig. 2H) but a similar ability to express GzmB (Fig. 2J). In striking contrast to WT CD8 T cells, Itk$^{-/-}$Rlk$^{-/-}$ CD8 T cells produced IL-17 following anti-CD3/CD28-mediated stimulation (Fig. 2I). Thus, these data imply that in the absence of Itk$^{-/-}$Rlk$^{-/-}$ an unusual and distinct subset of conventional IL-17-producing CD8 TCRβ$^+$ T cells are generated *in vitro*.

Together, these results demonstrate that the OP9-DL1 cells provide an inductive environment not only for T-lineage differentiation from hematopoietic progenitors, but also for the efficient selection of functionally-mature conventional CD8 TCRβ$^+$ T cells.

## Positive selection of MHC-I-restricted TCR-Tg CD8 T cells in OP9-DL1 cocultures

To directly demonstrate that the CD8 T cell selection is mediated by OP9 cells, we examined the differentiation potential of BM-HSCs isolated from MHC-I (K$^k$)-restricted Vα5/Vβ8 TCR-transgenic (TGB-tg) mouse. TGB TCR-tg recognizes the SV40 Large T antigen in the context of H2$^k$, which is the MHC-I haplotype expressed by OP9 cells (Fig. 4A). To ensure that the selection of TGB-tg cells is dependent on the selecting MHC-I expressed by OP9 cells, RAG2$^{-/-}$ TGB-tg mice carrying the non-selecting H2$^b$ (B6) haplotype were used. BM-HSCs were isolated from these mice to initiate cocultures with OP9-DL1 cells. Of note, RAG2$^{-/-}$ TGB-TCR-tg mice fail to develop CD8 T cells (Suppl. Fig. 1A). Figure 3A shows the efficient differentiation and selection of CD8 T cells (25%) initiated with BM-HSCs isolated from Rag2$^{-/-}$TGB-tg mice in OP9-DL1 cocultures. Moreover, high expression levels of TCRβ and TCR-Vβ8 were detected on *in vitro*-derived CD8 T-cells, with a profile that is analogous to the one observed from the thymus of TGB-tg (C3H H2$^k$) mice. These data strongly suggest that OP9-DL1 cells can provide an efficient environment for the differentiation and selection of TCR-MHC-I-restricted CD8 T cells. Additionally, a similar array of cell-surface markers (CD5, CD27, CD28 and MHC-I H2$^b$) were detected on *in vitro*-derived CD8 SP cells generated from Rag2$^{-/-}$ TGB-tg BM-derived HSC/OP9-DL1 cocultures as that seen on thymocytes (Fig. 3B). These data demonstrate that OP9 cells, expressing H2$^k$ MHC-I, provide a positive selecting environment for CD8 SP TCRβ$^+$ T cells. Furthermore, higher levels of expression of β7-integrin and low (almost undetectable) levels of expression of CD44, CD122, and CD244, were observed on *in vitro*-

generated CD8 cells and comparable to *ex vivo* CD8 cells (Fig. 3B). However, expression of CD24 remained high on *in vitro*-derived CD8 cells and differed from the low expression observed on *ex vivo* CD8 thymocytes (Fig. 3B). While CD24 is typically used to distinguish mature from immature thymocytes, we find that its down-regulation is not a prerequisite for acquisition of functional maturity (53). This is supported by Figure 3C, whereby similar induction of target cell-specific lysis of L929 ($H2^k$) by cells pulsed with an agonist peptide (S-peptide) was seen by *in vitro*, as well as *ex vivo* TGB-tg CD8 T cells, suggesting that *in vitro*-derived CD8 TCR$\beta^+$ T cells have fully matured to become effector cells (Fig. 3C). Together, these data confirm that TCR-MHC-I specific T cells undergo efficient positive selection in OP9-DL1 cocultures.

## MHC-I expression on OP9 cells constraints positive selection of CD8 T cells

To further evaluate whether MHC-I expressed by OP9 cells is critical for the selection of CD8 SP cells, we used small hairpin RNA (shRNA) targeting of the mouse transporter 1, ATP-binding cassette, subfamily B (MDR/TAP) or *Tap1* gene. Two shRNA constructs, referred to as shTAP1-M and shTAP1-L, were utilized for further investigation. Cell surface staining of shTAP1-M and shTAP1-L cells showed partial, but noticeable inhibition of MHC-I expression on OP9 cells (Fig. 4A). To evaluate whether the decrease in MHC-I expression would have an effect on the process of positive selection, DN4 ($CD4^-CD8^-CD44^-CD25^-$) $RAG2^{-/-}$ TGB-tg thymocytes were cocultured with OP9-DL1, shTAP1-M and shTAP1-L cells. While total cell numbers obtained from these cocultures were similar (Fig. 4C), a clear increase in the DP/SP ratio and decrease in the numbers of CD8 SP T cells generated on shTAP1-M and shTAP1-L were observed, which suggested a block in positive selection (Fig. 4D). We detected a two-fold increase in percentages and numbers of DP T cells, followed by a two-fold decrease in numbers of CD8 SP TCR$\beta^+$ T cells generated in shTAP1-M and shTAP1-L in comparison to OP9-DL1 cocultures (Fig. 4B–D). These results imply that expression of MHC-I by OP9 cells is necessary for the positive selection of CD8 SP cells (Fig. 4B–D). Moreover, while surface expression of the transgenic TCR was identical between CD8SP cells generated from shTAP1-M, shTAP1-L and OP9-DL1 cocultures; noticeably lower levels of CD5 surface expression were detected on CD8SP cells generated from shTAP1-M and shTAP1-L in comparison to OP9-DL1 cocultures (Fig. 4B), likely due to reduced TCR signals derived from the lower MHC-I levels (60). Thus, the decrease in the differentiation and maturation of TCR$\beta^+$ CD8 SP from DP precursors appears to be directly related to the levels of MHC-I expressed by OP9 stromal cells.

Additionally, we used BM-HSCs expressing the P14 TCR-tg, which requires $H2D^b$ for positive selection (55), in OP9-DL1 cocultures and noted that TCR$\beta^+$ CD8 SP cells were not readily generated, as compared to OP9-DL1 cells ectopically expressing $D^b$ (OP9-DL1-$H2D^b$; Suppl. Fig.2A) that showed a clear and robust generation of TCR$\beta^+$ CD8 T cells (Suppl. Fig. 2B). These results, together with the knockdown approach, further establish that expression of the selecting MHCI haplotype on the OP9 cells mediates CD8 SP selection.

## Agonist peptide-induced negative selection of developing thymocytes *in vitro*

We have demonstrated that the process of positive selection by developing T cells can be mediated by TCR-pMHC-I interactions within OP9-DL1 cocultures, however it is still unknown whether OP9 cells can mediate the process of negative selection. To address this question, we looked at the selection potential of MHC-I restricted $Rag2^{-/-}$ TGB-tg DN3/DN4 T cell subsets in OP9-DL1 cocultures, without or with addition of irrelevant (H)- or agonist (S)-peptide at day 5 (Fig. 5A). Analysis of CD4, CD8, TCRβ, and Vβ8 expression demonstrated a similar differentiation and selection potential of CD8 SP cells in the absence or presence of the irrelevant (H) peptide (Fig. 5A). In marked contrast, lower concentrations

(up to $10^{-17}$ M) of agonist peptide (S peptide) did not affect process of positive selection; while higher concentrations ($10^{-16}$ M and above) of the agonist peptide appeared to induce negative selection (Fig. 5A). Moreover, cocultures without or with irrelevant (H) peptide attained comparable numbers of total and CD8 SP TCR$\beta^+$ T cells (Fig. 5B, C). Conversely, decreases in total and CD8 SP cell numbers were detected in cocultures treated with agonist (S) peptide at concentrations of $10^{-16}$ M and higher (Fig. 5B, C, and data not shown). To further evaluate whether the cells from cocultures treated with agonist (S) peptide die by apoptosis, we measured cell surface expression of Annexin V (AnnV) and Propidium Iodide (PI). Figure 5D clearly demonstrates that the cells in cocultures without or with irrelevant (H) peptide remain mostly AnnV$^-$ PI$^-$. In contrast, cells from cocultures treated with agonist (S) peptide at the concentrations of $10^{-15}$ and above undergo apoptosis with approximately 4-fold increase in the percentage of AnnV$^+$ PI$^+$ cells (Fig. 5D, and data not shown). Supplemental Figure 3 shows that at high agonist peptide concentrations, the remaining CD8 and emergent DN cells, expressed similarly high levels of TCRβ on the cell surface. These findings are consistent with recent data from the Singer laboratory showing that the DN phenotype represents an alternative outcome to clonal deletion of thymocytes undergoing negative selection (61). Collectively, these data indicate that the signals generated by engagement of MHC-I-restricted TCR with the agonist peptide presented by MHC-I expressed by OP9 stromal cells are sufficient to effectively induce apoptosis of DP thymocytes and that the selection potency is dependent on the concentration of agonist peptide used in cocultures.

## Requirement for Notch signaling in CD8 T cell lineage commitment

In addition to peptide-mediated MHC-I-restricted positive and negative selection by OP9 cells, we examined the possible involvement of Notch signaling in the positive selection of CD8 T cells. We cultured Rag2$^{-/-}$ B6 TGB-TCR-tg DP cells (Suppl. Fig. 1B) with OP9 cells that lack expression of Notch ligands (OP9-CTLR) or with OP9-DL1 cells. Flow cytometry analysis revealed that only OP9-DL1 cells supported the differentiation of CD8 TCR$\beta^+$ T cells from DP T cells (Fig. 6A). While similar total cell numbers as well as percentage of cell death (measured by AnnV and PI staining) were observed between OP9-CTLR and OP9-DL1 cocultures, a substantial increase in CD8 T cell numbers was detected only in OP9-DL1 cocultures (Fig. 6B, C). These results imply that Notch signaling can influence the positive selection of CD8 T cells from DP T cell precursors. This finding was further assessed by treatment of TGB-TCR tg DP T cells/OP9-DL1 cocultures with γ-secretase inhibitor (GSI) at different concentrations (Fig. 6E). Of note, 1 μM concentration of GSI inhibitor induces a complete block in Notch signaling. We observed that GSI concentration of 0.6 μM and above blocked the selection of CD8 TCR$\beta^+$ T cells in these cocultures (Fig. 6E). Conversely, 0.3 μM concentration induced partial block in selection of CD8 T cells from TGB-TCR tg DP T cells in OP9-DL1 cocultures in comparison to GSI untreated cocultures. Correspondingly, CD8 T cell numbers increased with decreased GSI concentrations used in OP9-DL1 cocultures (Fig. 6F). These results indicate that Notch signaling is a prerequisite for effective positive selection of MHC-I specific TCR tg CD8 T cells from DP T cells in OP9-DL1 cocultures.

## Agonist peptide induces clonal deletion but not positive selection of the transgenic TCR in the absence of Notch signaling

To examine the influence of agonist peptide engagement during thymocyte selection in the absence of Notch signaling, Rag2$^{-/-}$ B6 TGB-TCR tg DP cells were cultured with OP9-CLTR cells in the presence of a broad range of concentrations of agonist (S) peptide (Fig. 7A). Although addition of agonist (S) peptide revealed concentration-dependent clonal deletion of TGB-TCR-tg DP T cells in OP9-CTLR cocultures, as seen in OP9-DL1 cocultures (Fig. 6), we did not observe the positive selection of CD8 SP cells (Fig. 7A).

Correspondingly, a substantial decrease in cellularity was observed with addition of increasing concentrations of agonist (S) peptide to TGB-TCR tg DP T cells/OP9-CTLR cocultures (Fig. 7B). Thus, these results show that in the absence of Notch signaling a failure to see efficient CD8 SP positive selection is not rescued by increasing the binding avidity of TCR to pMHC complex, rather this leads to a negative selection outcome. These data further imply that in addition to TCR-pMHC interactions, Notch receptor-ligand interactions are required for positive selection of CD8 TCR$\beta^+$ T cells.

## DISSCUSION

OP9 cells expressing Delta-like Notch ligand(s) have been extensively used to identify critical requirements of Notch signaling during early T cell differentiation (62). However, several lines of evidence indicated that CD8 SP T cells can be generated *in vitro* from BM-derived HSCs, mouse embryonic stem cells (ESCs), and human postnatal thymus- or cord blood -CD34$^+$ HSCs (53, 57, 63–66). While a recent study identified that functionally competent conventional- and non-conventional-CD8 T cells can be attained from CD34$^+$ human progenitors (67, 68), in the present study we demonstrate for the first time that the process of positive and negative selection of CD8 SP TCR$\beta^+$ T cells from mouse progenitors is dependent on pMHC-I expressed by OP9-DL1 cells.

Our results reveal that BM-derived HSCs when cultured with OP9-DL1 cells differentiate into CD8 SP T cells with high efficiency. Because a diverse repertoire of functionally competent CD8 SP cells can be attained from BM-derived HSCs in OP9-DL1 cocultures (53), we sought to understand how these cells are selected. Initially, we asked whether OP9 cells could serve the same function as cTECs to support positive selection of CD8 T cells. Since genetic studies have identified SAP as a requirement for the selection on hematopoietic cells of innate-like CD4 T cells that drive the differentiation of nonconventional CD8 T cells (40), we took advantage of the SAP$^{-/-}$ BM-derived HSCs to verify whether selection is mediated by pMHC expressed on OP9 cells or hematopoietic cells within these cocultures, as has been proposed to occur with human HSC *in vitro* (67). The efficient development of functionally competent CD8 SP cells, which lack the expression of innate-like cell surface proteins (CD122, CD44), were readily obtained from the SAP$^{-/-}$ BM-HSC/OP9-DL1 cocultures, providing evidence that interactions between developing T cells and OP9 cells govern the process of positive selection of conventional CD8 T cells *in vitro*. In contrast to the finding observed with human progenitors (67), our data establish that OP9 cells can act in an analogous fashion to TECs and provide quantitatively and qualitatively similar signals to DP cells to promote selection of conventional CD8 T lineage *in vitro* from DP precursors.

In addition to the selection of SAP$^{-/-}$ BM HSC into conventional CD8 T cells, Itk$^{-/-}$ Rlk$^{-/-}$ BM-HSC failed to differentiate into innate CD8 T cells in OP9-DL1 cocultures, and instead they developed into a distinct subset of IL-17-producing conventional CD8 T cells. Consistent with their signaling deficiency, in vitro-generated Itk$^{-/-}$ Rlk$^{-/-}$ CD8 T cells displayed lower levels of IFNγ production, but did not produce IL-4 (data not shown), further supporting the notion that these were not innate-like T cells. Thus, OP9 cells do not appear to support selection of innate CD8 T cells with characteristic activated or memory phenotype, which could be partially explained by the absence of cytokines, IL-15 and/or IL-4 produced by *Plzf* expressing NKT or γδ cells, required for the maturation of innate CD8 T cells (69, 70). Additionally, the generation of IL-17-producing CD8 SPs in this context may be due to constant Notch receptor-ligand interactions, which have been shown to regulate expression of RORγt (*Rorc*) (71), and data not shown, that would in turn influence the differentiation of IL-17$^+$ CD8 (Tc17) cells (72).

Although we observed the efficient selection of functionally competent CD8 T cells from OP9-DL1 cocultures initiated with BM-HSCs isolated from MHC-I (H2-K/D) deficient mice, this does not exclude the possibility that expression of MHC class-Ib molecules (eg. CD1d) by developing thymocytes could directly or indirectly induce the selection of CD8 SP cells with innate-like characteristics. However, the lack of innate-like cell surface markers on CD8 SPs, as well as the selection of CD8 cells from the $SAP^{-/-}$ BM-HSCs/OP9-DL1 cocultures, argues against this possibility.

The notion that MHC-I molecules expressed on OP9 cells mediate the selection of conventional CD8 T cells was further addressed by using progenitors from the TGB TCR-tg mice, which were $RAG2^{-/-}$ and from a B6 ($H2^{b}$) non-selecting background. Thus, these cells could only be selected by pMHC-I ($H2^{k}$) present on the OP9 cells. Since functionally competent TCR-tg CD8 SP cells were obtained, it suggests that the endogenous pMHC-I expressed on OP9 cells is of adequate affinity to support their selection. In addition, lowering the expression of MHC-I resulted in a clear reduction in the generation of CD8 SP cells, suggesting that quantitative difference in MHC-I expression by OP9-DL1 cells plays a critical role in the process of selection of CD8 T cells *in vitro*, which was further highlighted by changes in the levels of CD5 expressed on these cells. These data were additionally supported by the lack of effective selection of P14 TCR-tg CD8 T cells when using non-selecting OP9-DL1 ($H2^{k}$) cells. Together, these findings confirm that positive selection of DP cells into mature CD8 SP T cells is dependent on their interaction with pMHC-I molecules expressed by OP9 cells. Consequently, analogous to the selection process in the thymus, differentiation into mature conventional CD8 SP T cells *in vitro* requires heterotypic interactions between developing T cells and OP9 cells.

We further assessed whether using the known agonist peptide for the TGB TCR-tg could induce negative selection. This was achieved by adding the agonist peptide to OP9-DL1 cocultures at a two-fold lower concentration that is otherwise sufficient to activate TGB TCR-tg CD8 splenocytes (58). A clear threshold between positive and negative selection was defined, where lower concentrations of the agonist peptide promoted or allowed for positive selection while higher concentrations of the same peptide induced negative selection. Although molecules involved in the signaling cascade associated with the induction of negative selection were not examined, these data suggest that the strength of specific signals delivered through the TCR upon peptide/MHC recognition governs the cell fate decision similarly to what is seen within the thymus. These results support the notion that different concentrations of an agonist peptide can play a determining role in directing positive versus negative selection of TCR-specific DP precursors in OP9-DL1 cocultures, similar to what is seen using FTOCs (73, 74). Furthermore, these data support the avidity model of selection, which proposes that the quantity of a given peptide influences the fate of the TCR-specific thymocytes (21, 55, 75). Therefore, peptide-induced selection of DP T cells in vitro appears to have the same effect as that observed in the thymus.

So far, similar to *in vivo* studies, we have demonstrated that the nature of lympho-stromal interactions between DP T cells and pMHC-I expressed by OP9 cells allow for the processes of positive and negative selection of conventional CD8 SP T cells *in vitro*. While TCR/pMHC interactions and resulting signaling events are the essence of positive selection and thus functional immunity, we also revisited the involvement of Notch signaling in the selection of CD8 SP cells. In particular, we investigated the transition of DP cells isolated from $Rag2^{-/-}$ TGB mice cultured with OP9-CTLR or OP9-DL1 cells. We found that Notch signaling is required for the selection of CD8 SP T cells *in vitro*. This finding is in line with an early *in vivo* study that suggested that Notch signaling regulates CD8 T cell development (52, 76–78). In addition to the previous reports addressing the role of Notch receptor/ligand interactions in positive selection and CD4 or CD8 lineage commitment, we now demonstrate

by using an MHC-I-restricted TCR-tg that Notch signaling promotes the CD8 lineage fate decision. Although the molecular targets of Notch signaling that converge with TCR signaling necessary for selection of CD8 T cells remain unknown, by using MHC-I-restricted TCR-tg and Notch-ligand deficient OP9 cells we further demonstrate that Notch signaling is indispensable for the process of positive selection of CD8 T cells.

The apparent requirement for Notch-ligand interactions for the generation of CD8 T cells in vitro may be related to the reported role of Notch signaling affecting the metabolic and trophic state of developing thymocytes (79, 80). We recently extended this to show that Notch signaling, via a HES1-mediated repression of PTEN indirectly affects the PI3K pathway (81). Increased PI3K signaling capacity would likely affect the ability of selecting CD8 T cells to respond to IL-7R signals, which were shown to be instrumental in CD8 lineage commitment and differentiation (82). Thus, it is likely that Notch influences key signals downstream of the IL-7R, which would affect whether CD8 T cells undergo positive selection, such that in the absence of Notch signals, a decrease in PI3K-derived signals would dramatically impact the generation/differentiation of CD8 SPs.

Taken together, an important implication of our data is that OP9-DL1 cells not only support molecular events associated with T cell lineage commitment and early T cell differentiation (eg. β-selection process) from BM-derived HSCs (83), but also provide an inductive environment for positive and negative selection of functionally mature conventional CD8 SP cells. Furthermore, using MHC-I-restricted TCR-tg DP thymocytes, we demonstrate the involvement of Notch signaling in the process of CD8 lineage commitment. Given that the OP9-DL1 culture system can recapitulate the complex process of positive and negative selection of conventional CD8 SP cells seen in the thymus, this system may prove instrumental in future studies aimed at characterizing molecular events involved in conventional versus innate CD8 lineage commitment. Moreover, given the peptide-mediated selection of a self-tolerant diverse repertoire of CD8 SP cells generated in vitro (53), this system could elicit a considerable amount of interest over the possibility of generation of specialized T cells that may serve to enhance cellular immunity *in vivo*.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We gratefully acknowledge G. Knowles, A. Khandani and C. Macintosh for their expert assistance in cell sorting, and the D. Martin and M. Wright at the Comparative Research facility for their technical assistance. We would like to thank Dr. Laurent Sabbagh for assistance in the generation of the OP9-DL1-H2D$^{b}$ cells.

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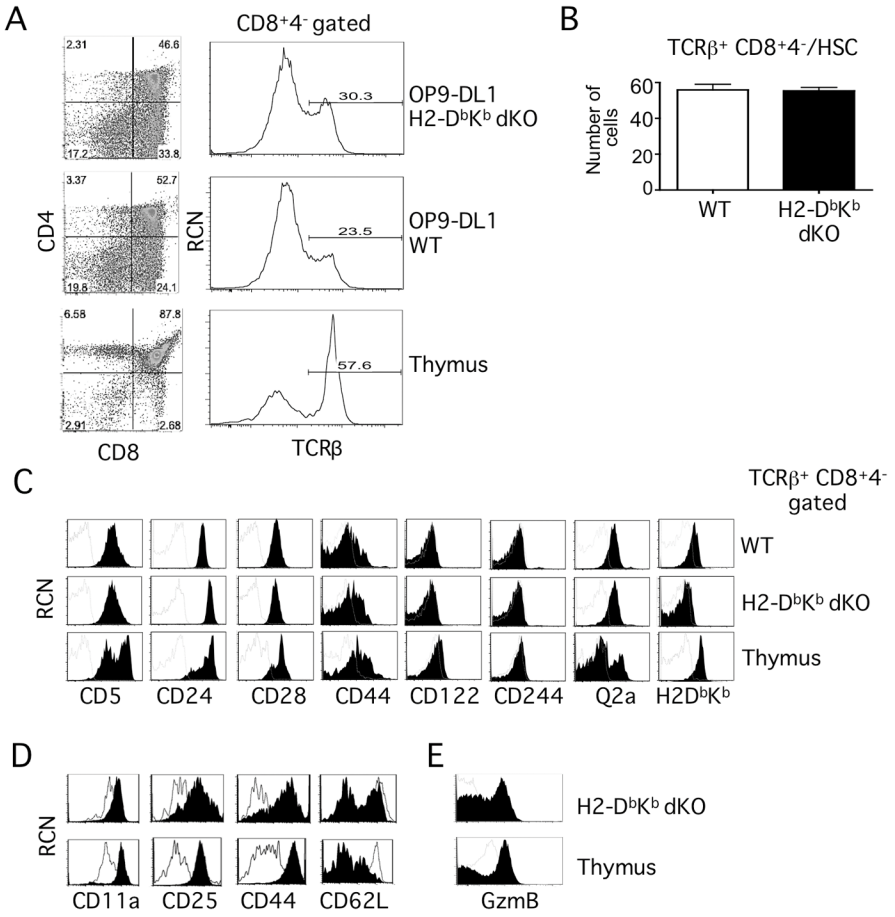


**Figure 1. Selection of functionally mature CD8 T cells is mediated by MHC class I expressed by OP9 cells**

**A.** Representative plot of CD4 and CD8 expression from day 35 cocultures initiated with $Lin^{-}CD117^{+}Sca1^{+}$ HSCs isolated from BM of 6–8 weeks old $H2D^{b}K^{b}$ dko or B6 (WT) mouse. These were compared to CD4 and CD8 expression by thymocytes isolated from 5–6 weeks old B6 mouse. TCR expression was measured on gated CD8 T cells by flow cytometry. RCN represents relative cell numbers. **B.** Bar graph shows the number of CD8 $TCR\beta^{+}$ T cells generated from a single BM-HSC at the start of the coculture, as shown in A. Error bars shown are the SD obtained from triplicate cocultures. **C.** Flow cytometry analysis of expression of specified markers CD5, CD24, CD28, CD44, CD122, CD244, Q2a and $H2D^{b}K^{b}$ (black histograms) or isotype controls (gray histograms) on $CD8^{+}$ cells generated from d35 cocultures initiated with BM-derived HSCs isolated from 6–8 weeks old $H2D^{b}K^{b}$ dko or B6 (WT) mouse. These were compared to the expression of the same markers on thymocytes isolated from 5–6 weeks old mouse. For **D.** and **E.** $CD8^{+}$ $TCR\beta^{hi}$ T cells were purified by flow cytometry from d35 cocultures initiated with BM-derived HSCs isolated from $H2D^{b}K^{b}$ dko or thymuses of 5–6 weeks old B6 mice. **D.** Flow cytometry analysis of activation markers CD11a, CD25, CD44 and CD62L (black histograms) on $CD8^{+}$ T cells was compared to isotype controls (gray histograms) after stimulation with anti CD3/CD28 mAbs. **E.** Flow cytometry analysis of Gzm-B production before and after stimulation with anti CD3/CD28 mAbs on CD8 T cells purified from d35 coculture and thymus. Results are representative of at least 5 independent experiments.

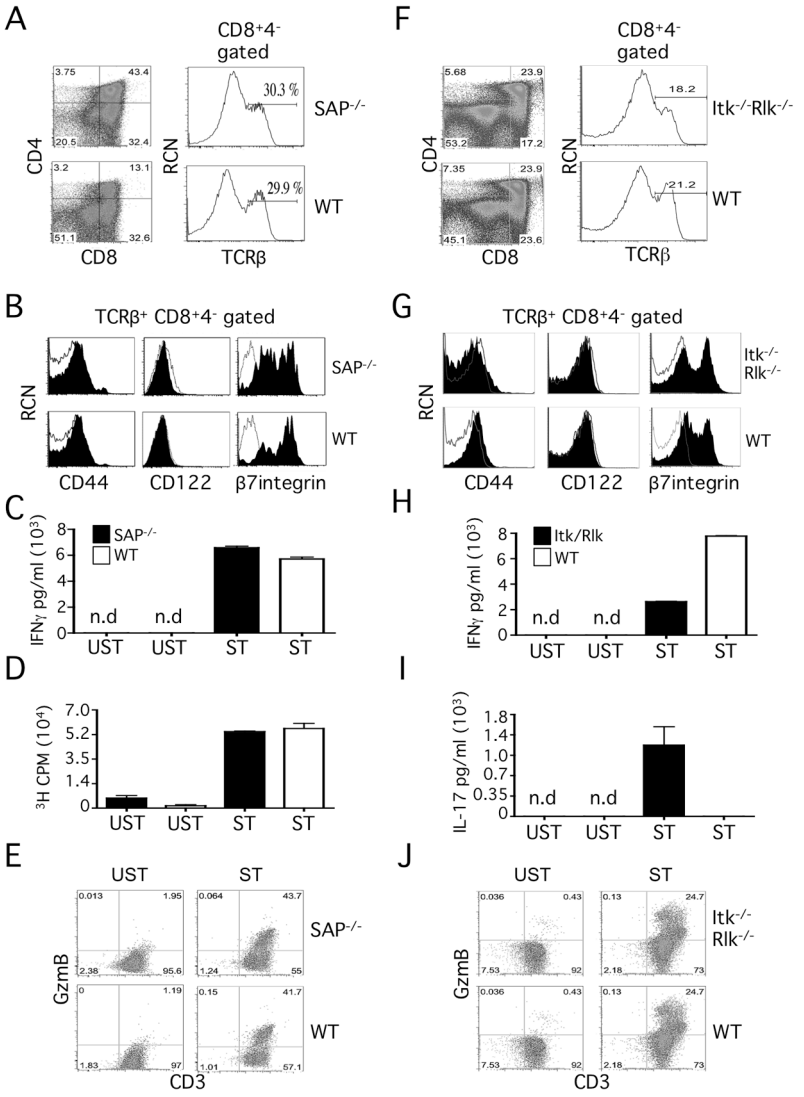


**Figure 2. Functionally mature conventional CD8 T cells are generated from BM-HSC/OP9-DL1 co-cultures**

$Lin^{-}CD117^{+}Sca1^{+}$ HSCs were isolated from BM of 6–8 weeks old $SAP^{-/-}$, $Itk^{-/-}Rlk^{-/-}$, or B6 (WT) mouse. **A, F)** Flow cytometry analysis of CD4 and CD8 expression on total T cells generated from BM-derived HSCs isolated from $SAP^{-/-}$, $Itk^{-/-}Rlk^{-/-}$, and WT (B6) mice in OP9-DL1 cocultures on day 35. Histogram plots represent TCR expression on gated CD8 T cell population. **B, G)** Flow cytometry analysis of CD44, CD122 and β7-integrin expression on gated CD8 T cell population is depicted by black- and corresponding isotype control Abs by gray -lines from indicated cocultures. **C, H)** IFN-γ production, detected by ELISA, from unstimulated vs. anti-CD3/CD28 stimulated WT (B6) or $SAP^{-/-}$ and $Itk^{-/-}Rlk^{-/-}$ culture-derived $CD8^{+}$ $TCR\beta^{hi}$ cells is represented by white or black bars, respectively. **D.** $^{3}H$-thymidine incorporation was measured in culture derived $SAP^{-/-}$ or WT $CD8^{+}TCR\beta^{hi}$ cells with (black bars) or without (white bars) plate-bound anti-CD3/CD28-mediated stimulation. Data represent average values and error bars represent standard deviations. **I.** IL17 production was measured by ELISA from culture-derived $Itk^{-/-}Rlk^{-/-}$ or WT CD8 T cells, without (white bars) or with (black bars) TCR-mediated stimulation by anti-CD3/CD28 antibody engagement. **E, J)** Flow cytometry analysis of Gzm-B production by anti-CD3/

CD28 stimulated vs. unstimulated culture-derived $CD8^+$ $TCR\beta^{hi}$ cells generated from indicated cocultures. Results shown are representative of three independent experiments performed in triplicates.

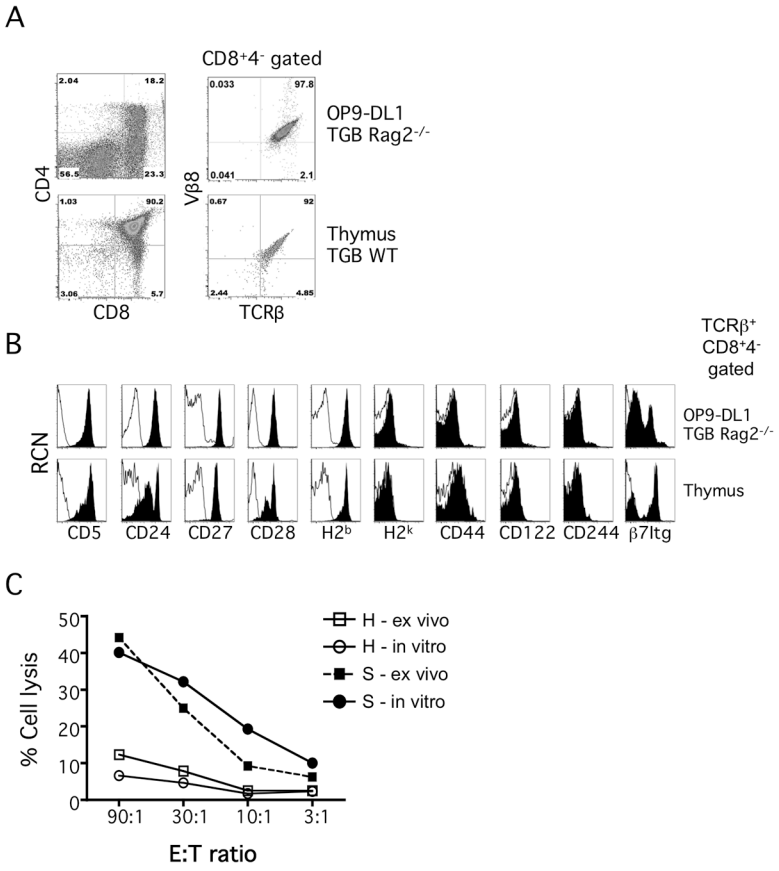


**Figure 3. MHC class I expression by OP9 stromal cells is required for selection of functionally mature CD8 T cells**

**A.** Representative FACS plots of CD4, CD8, TCRβ and Vβ8 expression from d12 OP9-DL1 cocultures initiated with BM-derived HSCs isolated from TGBxRag2$^{-/-}$ mice in comparison to 4–6 weeks old thymus isolated from TGBxC3H mice. **B.** FACS analysis of maturational markers CD5, CD24, CD27, CD28, H2$^{b}$, H2$^{k}$, CD44, CD122, CD244, β7Itg (black histograms) and isotype controls (gray histograms) gated on CD8$^{+}$CD4$^{-}$ population from d12 cocultures initiated with BM-derived HSCs from Rag2$^{-/-}$TGB mice on OP9-DL1 cells in contrast to 4–6 weeks old thymus isolated from TGBxC3H mice. **C.** CTL assay by effector in vitro- or ex vivo-derived CD8 T cells (E) against target L929 cells (T) pulsed with agonist (S) or irrelevant (H) peptide. Chromium release was measured at different ratios of effector (E) to target (T) cells. Results are representative of at least five independent experiments.

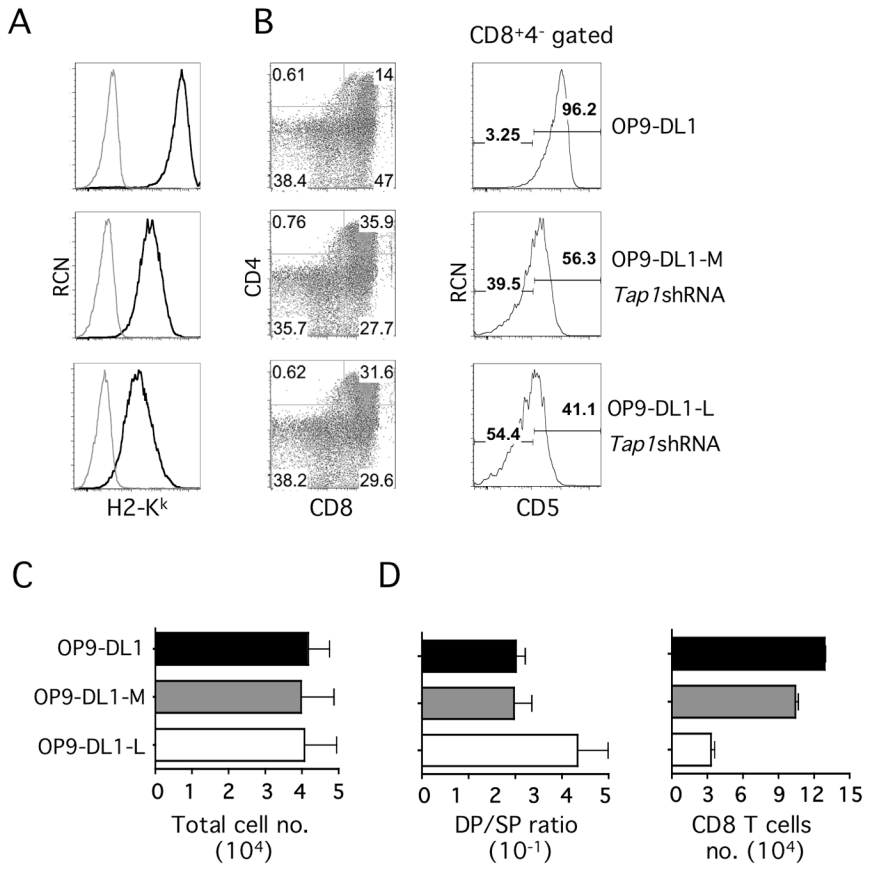


**Figure 4. MHC class I dependent selection of MHC class I restricted CD8 T cells**

**A.** Flow cytometry analysis of MHC class I expression on OP9-DL1, OP9-DL1-M *Tap1*shRNA and OP9-DL1-L *Tap1*shRNA. Black lines represent endogenous $H2^k$, while gray lines represent non-specific $H2D^bH2K^b$ (mock control) expression of MHC class I. **B.** FACS analysis of CD4 and CD8 expression on total cells, and CD5 expression on $CD8^+$ $CD4^-$ gated cells initiated with TGB-TCR-Tg DN3/DN4 subsets from OP9-DL1-M *Tap1*shRNA and OP9-DL1-L *Tap1*shRNA at day 3. **C, D)** Total cell numbers, DP/SP ratio and CD8 T cell numbers were measured by flow cytometry from aforementioned cocultures, respectively. Results are representative of three independent experiments performed in triplicates.

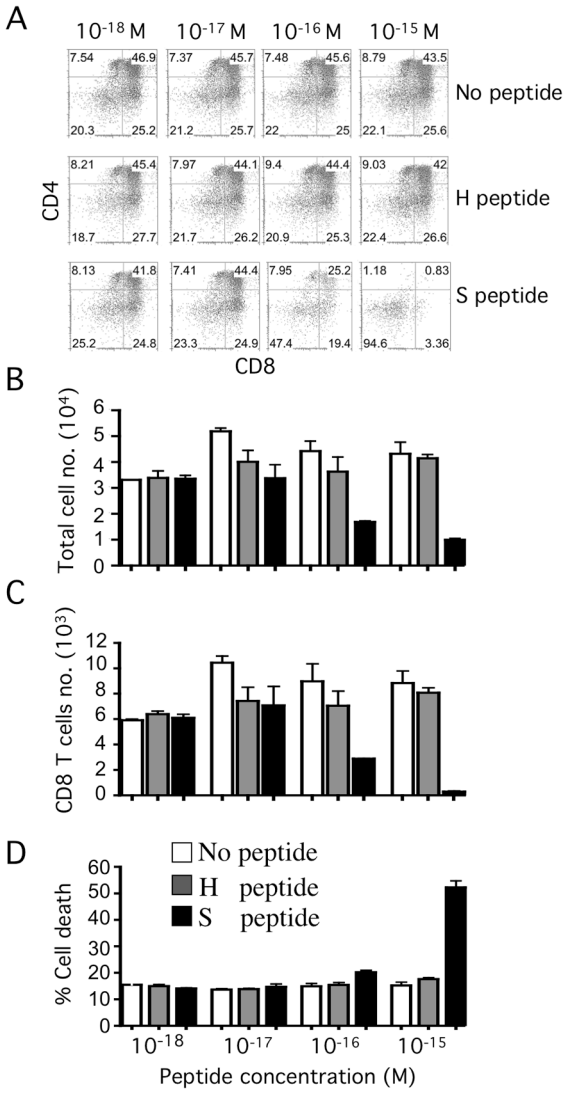


**Figure 5. Peptide-induced negative selection of T cells in OP9-DL1 cocultures**

**A.** Flow cytometry analysis of CD4 and CD8 expression from cocultures initiated with DN3/DN4 FACS purified thymocytes isolated from RAG2$^{-/-}$ TGB mice, untreated or treated with indicated concentrations of irrelevant (H) or agonist (S) peptide. **B, C, D.** Total cell numbers **(B),** numbers of CD8 T cells **(C)** and % of AnnV$^{+}$PI$^{-}$, AnnV$^{+}$PI$^{+}$, and AnnV$^{+}$PI$^{-}$ cells **(D)** were calculated from cocultures described in **A** in the absence (white bar) or presence of irrelevant (H, gray bar) or agonist (S, black bar) peptide. Results are representative of at least five independent experiments performed in triplicates.

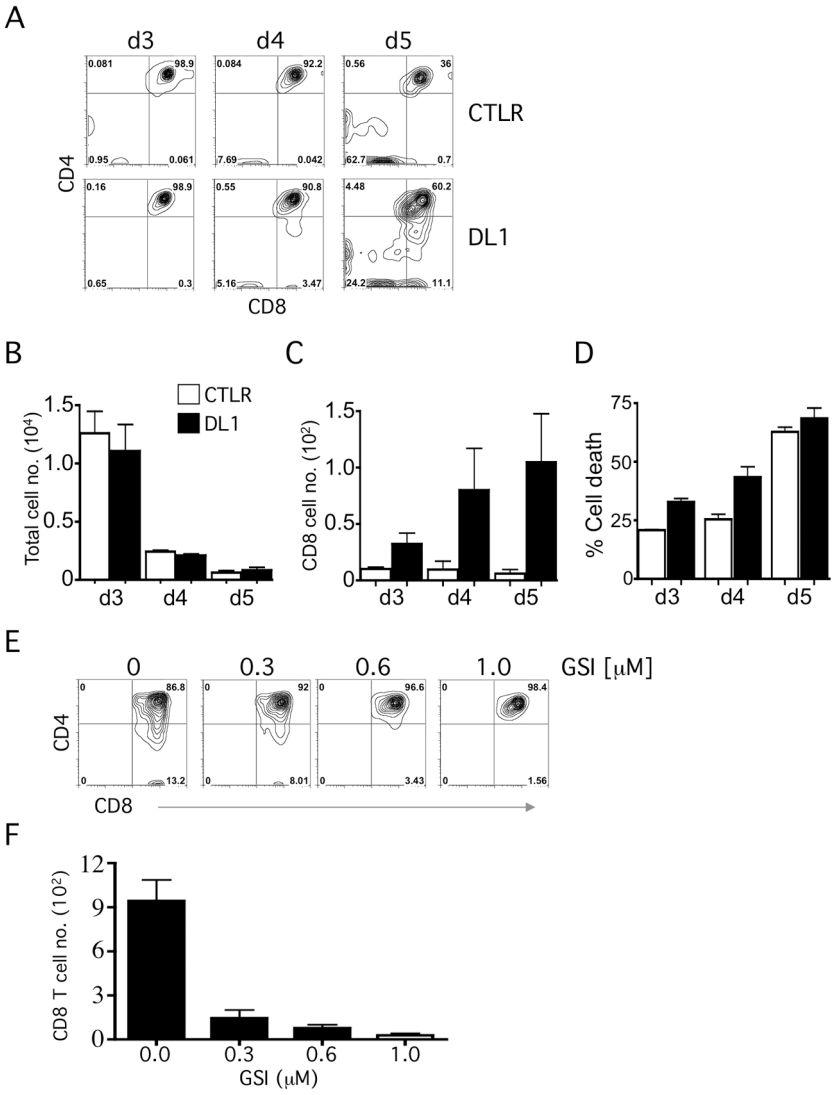


**Figure 6. Notch signaling is required for positive selection of CD8 T cells**

**A.** Flow cytometry analysis of CD4 and CD8 expression from day 3, 4, and 5 cocultures initiated with TGB-TCR-tg DP T cells plated with CTLR or DL1 OP9 cells. **B.** Total cell numbers **C.** CD8 T cell numbers and **D.** % of cell death were measured by flow cytometry on day 3, 4 and 5 from the above-mentioned cocultures. **E.** FACS analysis of cocultures from day 4 initiated with RAG2$^{-/-}$TGB-tg DP T cells plated with OP9-DL1 cells in the absence or presence of different concentrations of GSI. **F.** Numbers of CD8 T cells obtained from cocultures described in 7E. Results are representative of three independent experiments performed in triplicates.

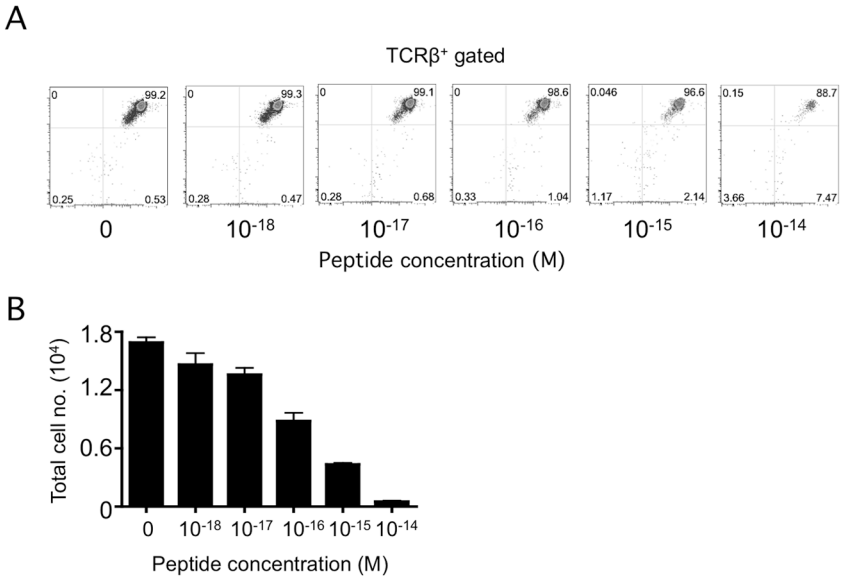


**Figure 7. Agonist peptide induces negative selection but not positive selection in the absence of Notch signaling**

**A.** Flow cytometry analysis of CD4 and CD8 expression after gating for TCR$\beta^+$ cells from day 4 cocultures initiated with TGB-TCR-tg DP T cells plated with OP9-CTLR cells in the presence of a wide range of agonist (S) peptide, as indicated. **B.** Total cell numbers were measured by flow cytometry on day 4 from the above-mentioned cocultures. Results are representative of at least three independent experiments performed in triplicates.