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Comparative and Functional Evaluation of In Vitro Generated to Ex Vivo CD8 T Cells

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The generation of the cytotoxic CD8 T cell response is dependent on the functional outcomes imposed by the intrathymic constraints of differentiation and self-tolerance. Although thymic function can be partly replicated in vitro using OP9-DL1 cell cultures to yield CD8+ TCR-bearing cells from hematopoietic progenitor cells, a comprehensive and functional assessment of entirely in vitro generated CD8 T cells derived from bone marrow hematopoietic stem cells has not been established and remains controversial. In this study, we demonstrate that a phenotypic, molecular, and functional signature of in vitro derived CD8 T cells is akin to that of ex vivo CD8 T cells, although several significant differences were also observed. Transfer of in vitro derived CD8 T cells into syngeneic and immunodeficient host mice showed no graft-versus-host response, whereas a robust homeostatic proliferation was observed, respectively. These findings, along with a diverse and broad TCR repertoire expressed by the in vitro derived CD8 T cells, allowed for the successful generation of Ag-specific T cells to be obtained from an entirely in vitro generated CD8 T cell pool. These findings support the use of Ag-specific in vitro derived effector CD8 T cells for immune reconstitution approaches, which would be amenable to further tailoring for their use against viral infections or malignancies. The Journal of Immunology, 2012, 189: 3411–3420.

T cells are generated in the thymus after colonization from the blood by bone marrow (BM)-derived progenitors (1–6). In the thymus, T cell development can be characterized as a series of distinct subsets based on the expression of cell surface proteins. The earliest T cell progenitors, double-negative (DN) cells, lack expression of the TCR, CD4, or CD8. Subsequent signaling through the rearranged pre-TCR and growth factor receptors induces cell proliferation and differentiation into double-positive (DP) cells that express both CD4 and CD8 coreceptors, as well as the mature αβ-TCR (7–9). Further differentiation is initiated by interaction of the TCR with the self-peptides presented by MHC proteins, MHCII or MHCI, to generate mature CD4 and CD8 T cells, respectively (10). The generation of mature CD4 and CD8 T cells is thought to be achieved through a neglect, select, eliminate process that is partly dependent on the strength of signal induced by the self-peptide MHC (pMHC) complex. Taken together, these processes determine not only survival, but also the appropriate lineage specification of αβ T cells in the periphery, which in general correlates with their phenotypic and functional specialization into MHCII-restricted CD4 Th cells or into MHCI-restricted CD8 CTLs (11–19).

Moreover, the development of mature, functional, and self-restricted T cells, expressing a diverse Ag-specific repertoire, which is essential for an efficient immune response, is achieved through signals induced via reciprocal interactions between T cell precursors and multiple soluble as well as membrane-bound factors provided by cortical and medullary thymic stromal cells. Among these signals, Notch–ligand interactions are compulsory for T lymphopoiesis (20–25).

With regard to T lymphopoiesis, Notch signaling has been implicated in B versus T, and αδ versus γδ T cell lineage commitment, differentiation through early DN stages of thymocyte development, progression from DN to DP stages, CD4 Th1 versus Th2 cell specification, as well as regulation of cytotoxic effector function in CD8 T cells (22, 26–37). Similarly, ectopic expression of the Notch ligand Delta-like 1 by mouse OP9 BM cells supports efficient T lymphopoiesis from mouse fetal liver (FL) progenitors, adult BM-derived hematopoietic stem cells (HSCs), embryonic stem cells, and cord blood-derived CD34+ into mature TCR αβ+ CD8+ T cells (22, 29, 38). However, the functional assessment of entirely in vitro generated CD8+ T cells remains ambiguous and not fully characterized.

In this study, we demonstrate that αβ+ CD8 T cell development proceeds normally in HSC/OP9-DL1 cocultures, leading to their differentiation into functionally mature cells. The maturation status of in vitro generated CD8 T cells showed similarities to that of ex vivo CD8 T cells as determined by the surface expression of differentiation markers, with some significant differences also noted. At the molecular level, in vitro derived CD8 T cells portrayed predominantly a normal CD8-biased gene expression pro-
file, albeit Notch-regulated genes showed elevated expression. In addition, in vitro stimulation assays confirmed that these CD8 T cells are functionally mature cells that can proliferate and express CD8-specific activation markers. Correspondingly, we found that these cells mature into effector cells as defined by their capacity to produce antimicrobial cytokines and cytolytic effector molecules. We also demonstrate that CD8 T cells generated in HSC/OP9-DL1 cocultures are broadly tolerant to self-pMHC Ags, as assayed by in vitro and in vivo responses to allogeneic or syngeneic APCs. In addition, we observed augmented lymphopoeisis-driven proliferation and expansion of in vitro derived CD8 T cells when transferred into RAG2−/− host mice. In addition, we revealed the presence of a diverse and polyclonal TCR repertoire in coculture-derived CD8 T cells. Finally, we show that in vitro derived CD8 T cells generated an enhanced primary Ag-specific response against tumor-associated Ag, tyrosinase-related protein-2 (TRP-2), and lymphocytic choriomeningitis virus (LCMV) Ag g33 in vitro. Taken together, our results demonstrate that the use of a Notch ligand-expressing stromal cell line allows for the efficient generation of Ag-specific, diverse, functionally competent, and self-tolerant CD8 T cells from adult BM-derived HSCs.

Materials and Methods

Mice

CD1, BALB/c, C57BL/6 (B6), and B6-Ly5.1 congenic mice were purchased from The Jackson Laboratory. RAG2-deficient mice were bred and maintained in our 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-DL1 cells were generated from the OP9 BM stromal cell line and maintained, as previously described (22).

Flow cytometry

Biotin, FITC, PE, PE-Cy5, PE-Cy7, allophycocyanin, and allophycocyanin-Cy7 mAbs were purchased from BD Biosciences or eBioscience, except for the anti-perforin mAb that was purchased from Kamyia Biomedical. The following conjugated Abs were used: anti-B220 (RA3-6B2), anti-CD3e (145-2C11), anti-CD4 (GL1.1, L3T4), anti-CD5 (Ly-1), anti-CD8α (LY-2, Ly-2, 53-67), anti-CD11a (M1/70), anti-CD11b (M1/70), anti-CD16/ CD32 (2.4G2), anti-CD19 (1D3), anti-CD25 (7D4), anti-CD27 (LG.7F9), anti-CD28 (37.51), anti-CD44 (IM7), anti-CD45 (30-F11), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD54 (3E.2), anti-CD62L (MEL-14), anti-CD69 (1H.2F3), CD90.2 (53-2.1), anti-CD117 (2B8), anti-CD122 (TM-β1), anti-CD244 (eBio244FA), anti-TCRβ (H57-597), anti-GR-1 (RB6-8C5), NK1.1 (PK136), Sca-1 (E13-161.7), TER119, and anti-H2Kdβ (28-8-6). Cells were stained by standard staining techniques and analyzed on a FACScalibur or LSRII flow cytometers (BD Biosciences). Data files were analyzed with FlowJo (Tree Star). Dead cells were excluded from all data by forward and side scatter and propidium iodide (PI) (Molecular Probes). Cell sorting was performed with FACSdiva or a FACSaria cell sorter (BD Biosciences). Purity was typically >98% for all populations, as determined by postsort analysis.

Intracellular staining

Coculture-derived and sorted CD8 TCRβhigh T cells (5 × 10⁴) were stimulated with plate-bound anti-CD3e (10 μg/ml) and anti-CD28 (5 μg/ml) mAb in 200 μl OP9 media supplemented with 1 ng/ml IL-7 and 10 U/ml IL-2 (PeproTech). Cells were stained with appropriate Abs and analyzed by flow cytometry at the indicated time points. The average mean fluorescence intensity (MFI) of each sample was plotted against the time point(s) from the analysis. For CFSE labeling, sorted CD8 TCRβhigh T cells (5 × 10⁴) were labeled with 0.5 μM CFSE, as recommended by the supplier (Molecular Probes), and the number of cell divisions after stimulation was measured by flow cytometry and calculated by FlowJo software using the same constrains for all samples. Proliferation indexes were calculated using the same parameters.

Enzyme-linked immunosorbent assay

Sorted CD8 TCRβhigh T cells (5 × 10⁴) were cultured in triplicates with plate-bound anti-CD3e (10 μg/ml) and anti-CD28 (5 μg/ml) mAb in 200 μl OP9 media supplemented with 1 ng/ml IL-7 and 10 U/ml IL-2 (PeproTech). Cells were stained with appropriate Abs and analyzed by flow cytometry at the indicated time points. The average mean fluorescence intensity (MFI) of each sample was plotted against the time point(s) from the analysis. For CFSE labeling, sorted CD8 TCRβhigh T cells (5 × 10⁴) were labeled with 0.5 μM CFSE, as recommended by the supplier (Molecular Probes), and the number of cell divisions after stimulation was measured by flow cytometry and calculated by FlowJo software using the same constrains for all samples. Proliferation indexes were calculated using the same parameters.
Mixed lymphocyte reaction

Sorted CD8 TCRβb6+ cells (5 × 10^5) were cultured with γ-irradiated (25 Gy) splenocytes (5 × 10^5) as stimulators in each well, in triplicates, of a 96-well round-bottom plate. The cultures were incubated in OP9 media for 48–72 h in a humidified 37°C incubator in an atmosphere of 5% CO2 in air. A total of 1 μl in 10 μl [^3H]Tdr was added to each well, in a volume of 200 μl for the final 18 h of culture. Cells were harvested using a multiple well harvester, and [^3H]Tdr incorporation was determined in liquid scintillation counter. The mean incorporation values of [^3H]Tdr in DNA were plotted using a spreadsheet program (Prism).

Adoptive cell transfer

Cotransfer-determined and sorted CD8 TCRβb6+ T cells were labeled with 10 μM CFSE, as described above. The labeled cells (1–2 × 10^9) were washed extensively in PBS and then injected in 300 μl PBS per mouse (B6, B6- Ly5.1, CD1, or RAG2^-/-) via the lateral tail vein. Three and/or 7 days after injection, LN and spleen cells were isolated, and single-cell suspensions were stained with specific mAbs for analysis by flow cytometry.

TCR spectratyping analysis

Spectratyping was performed using the PCR primers and methodology published by Pannetier et al. (41), with some modifications. Briefly, 25 ng cDNA from coculture-derived and sorted CD8 TCRβb6+ T cells was subjected to 35 cycles of elongation for each of the 24 Vβ-chains as follows: 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C. After the final cycle, the mixture was heated for 5 min at 72°C. A total of 5 μl of the primary PCR product was subjected to additional 25 cycles of PCR amplification. The 6-FAM-labeled εβ primer was used in the second round of amplification. Electrophoresis was performed on a 3730 XL DNA sequencer (Applied Biosystems), and CDR3 length and fluorescence intensity of PCR products were determined with GeneMapper software (Applied Biosystems).

Generation of Ag-specific CD8 T cells and pMHC-tetramer staining

CD11c^+ DCs (5 × 10^5) were infected with 100 PFU/cell with an adenovirus expressing the TRP-2 or LCMV-associated Ag gp33 (provided by J. Bramson, McMaster University, Hamilton, ON, Canada), and cultured with 0.5–1 × 10^5 coculture-derived and sorted CD8 TCRβb6+ T cells every 7 days. CD8^+ TCRβb6+ T cells were washed and replated with adenovirus expressing the TRP-2- or gp33-infected DCs every 7 days, as indicated. To detect the TRP-2-specific T cells, cells were stained with allophycocyanin-coupled H-2Kα5/VSVYDFVWV (human TRP-2 [hTRP-2] 180–188) or gp33-41 H-2Kα/KA VYNFATC as well as H2Kβ/SINFEKL (ova 257–264) or (gp276–286) SGEVNPNGYGCL/H2Dβ as a negative control, according to the manufacturer’s protocol (Proimmune).

Statistics

The data are presented as mean ± SEM. To determine statistical significance, a two-tailed Student t test was used for comparison between two experimental groups, using Prism software. Statistical significance was determined as p < 0.05 (*p < 0.05, **p < 0.01).

Results

Generation of mature conventional CD8 T cells from BM-HSC/OP9-DL1 cocultures

We have previously demonstrated that OP9-DL1 cells support T lymphopoiesis from fetal liver (FL)-derived HSCs, resulting in the generation of a small number of mature CD8 T cells (22). Although we later showed that adult BM-derived HSC/OP9-DL1 cocultures could also give rise to CD8 T cells, a detailed characterization and comprehensive analysis of the generation of these T cells were not done and remained undefined whether functional T cells could be generated from adult hematopoietic progenitors. In this study, we sought to characterize the generation of mature functional CD8 T cells from Lin^+ Scal^+ CD117^+ BM-derived HSCs cultured with OP9-DL1 cells.

Flow cytometric analysis of the BM-HSC/OP9-DL1 cultures performed at different time intervals revealed an apparently synchronous phenotypic progression through the various stages of T cell development, commencing with the earliest CD4^-CD8^- double-negative (DN) from days 5 to 23, through CD4^+CD8^- DP maturation stages, followed by CD4^-CD8^+ double-positive (DP) at 25 days, and finally CD4^+CD8^+ double-negative (DN) at 35 days of culture. This progression was confirmed using TCR spectratyping analysis, which showed a well-defined and ordered appearance of different DN subsets, characterized by CD44 and CD25 expression, with DN2 (CD4^-CD25^-) cells appearing first (day 5), then DN3 (CD4^-CD25^+) cells (day 8), and finally DN4 (CD44^-CD25^-), or pre- DP, cells within the first 2 wk of coculture. Cellular expansion obtained after 35–38 d of culture from BM-HSCs differentiating into T-lineage cells, and CD8 TCRβb6+ T cells ranged from 2 to 3 × 10^4-fold and 1 to 2 × 10^5-fold, respectively.

In addition, by day 35, CD8^+ SP cells appeared to have attained a mature phenotype, as 10–30% of these cells showed high levels of TCRβ expression (Fig. 1A, 1B). Further analysis of these in vitro generated CD8 TCRβ^+ SP T cells showed that they expressed a similar pattern of differentiation and maturation cell surface markers to that of ex vivo thymus CD8^+ T cell, including similar levels of CD27, CD28, Qa2, and MHCI expression (Fig. 1C). In vitro derived CD8^+ SP T cells upregulated CD5 expression to lower levels than that of CD8 cells from the thymus or spleen (Fig. 1C, lower panel). Although the expression of CD24 (heat-stable Ag) remained high on in vitro generated CD8 T cells as compared with ex vivo CD8 SP cells, the clear upregulation of a broad set of differentiation cell surface markers (CD5, CD27, CD28, Qa2, MHCI) coincided with a mature CD8 T cell profile (Fig. 1C). Of note, in vitro derived CD8 T cells lacked surface expression of CD44, CD122, and CD244 (Fig. 1D), suggesting that in vitro derived CD8 T cells are likely to be akin to conventional, rather than innate-like CD8 T cells (42–45). Taken together, these findings indicate that OP9-DL1 cells can support the efficient differentiation of mature conventional CD8 T cells from BM-HSCs in vitro.

In vitro generated CD8 T cells display a similar gene expression profile to ex vivo CD8 T cells

We further characterized in vitro generated CD8 T cells by comparing their gene expression profile with that of ex vivo thymic CD8 T cells, using the Agilent’s whole mouse genome oligo microarray comprised of 41,534 (60-mer) oligonucleotide probes, representing ~22,000 mouse genes (Milltenyi Biotech). The scatter plot shown in Fig. 2A provides an analysis of the differential expression patterns between in vitro derived and ex vivo CD8 T cells for ~41,000 individual probes, in which the data correlation of log ratio intensity 1 on the x-axis represents ex vivo derived and log ratio intensity 2 on the y-axis represents in vitro derived CD8 TCRβ^+ cells (accession GSE37669). Of note, given the overall similarity in phenotype between in vitro and ex vivo CD8 T cells (Fig. 1C), we found that most gene probes (>97%) were expressed at equivalent (~3-fold change) levels (blue). However, ∼1400 probes, or ∼3.5% of the total number analyzed, whose levels differed by 3-fold or greater, were either upregulated (red) or downregulated (green) by the in vitro generated CD8 T cells (Fig. 2A, Supplemental Table 1A). Given the distinctive feature of the inclusion of OP9 cells within these cocultures, and sort purities that may include 1–2% OP9 cells, a subtractive gene expression analysis between genes highly expressed by OP9 stromal cells and in vitro derived CD8 T cells was performed (Supplemental Table 1B). This analysis resulted in the reduction of the number of differentially expressed genes between in vitro and ex vivo CD8 cells to only ∼0.6%, which will be described further in depth separately.

In this study, we focused on the expression of several key transcription factors recognized to play critical roles in CD8 (Runx1 and Runx3) or CD4 (GATA3 and ThPok) lineage specification and commitment (Fig. 2A, bar graph) (46–50). A 4-fold
In vitro generated CD8 T cells are functionally equivalent to ex vivo thymus-derived cells

To evaluate the functional properties of CD8 T cells generated from BM-HSC/OP9-DL1 cocultures, we measured the cellular expansion, cell division, and effector function of CD8 TCRβ<sup>high</sup> cells in response to TCR/CD3 stimulation, by anti-CD3 and anti-CD28 Ab-mediated engagement (Fig. 3A–D). As expected, in vitro derived CD8 T cells underwent proliferation and cell division, as measured by [3H]thymidine uptake and CFSE dilution, in response to TCR stimulation (Fig. 3A, 3B). Additionally, flow cytometric analysis of stimulated in vitro generated CD8 T cells revealed a bona fide activation profile (Fig. 3C). In particular, the acquisition of an activated effector profile was marked by the increased expression of CD25 (IL-2Rα), CD44 (Pgp1), and CD11a (LFA-1), and decreased expression of CD62L (L-selectin) upon TCR stimulation (Fig. 3C). Importantly, activated in vitro generated CD8 SP T cells produced IFN-γ (Fig. 3D), and also expressed high levels of granzyme-B (Gzm-B) and perforin, key effector molecules that define the cytolytic capacity of a CD8 T cell (Fig. 3E). Of note, we consistently detected higher levels of Gzm-B and perforin production by in vitro generated CD8 T cells. Because in vitro CD8 T cells are exposed to continuous Notch signaling provided by the OP9-DL1 cells, these results are in line with recent reports suggesting a Notch-dependent induction of cytolytic molecules in CD8 T cells (34, 35). Thus, the effector profile signature observed upon anti-CD3/CD28–induced activation of in vitro generated CD8 T cells was similar to that of functionally mature ex vivo CD8 cells.
indicated genes, measured by QRT-PCR from sorted CD8+ TCR derived from day 35 HSC/OP9-DL1 cocultures or ex vivo thymocytes, are value of four independent hybridizations for the indicated genes to Agilent used to hybridize onto an Agilent GPL4134 microarray. Selected fold change (gene ratios; color coded as in the scatter plot) represents the mean value of four independent hybridizations for the indicated genes to Agilent whole mouse genome oligo microarrays. (B) RNA transcript levels for the indicated genes, measured by QRT-PCR from sorted CD8+ TCRβrep cells derived from day 35 HSC/OP9-DL1 cocultures or ex vivo thymocytes, are shown. Results of replicate samples were normalized to β-actin expression. These data are representative of at least five independent experiments.

**FIGURE 2.** Gene expression profile of in vitro derived CD8 T cells compared with ex vivo CD8 naive T cells. (A) Scatter plot of signal intensities obtained from hybridization of one of four gene expression microarray experiments is shown, in which sorted CD8+ TCRβrep cells from day 35 HSC/OP9-DL1 cocultures and thymuses from B6 mice (4–5 wk old) were labeled with CY3 (green) and CY5 (red), respectively, and used to hybridize onto an Agilent GPL4134 microarray. Selected fold change (gene ratios; color coded as in the scatter plot) represents the mean value of four independent hybridizations for the indicated genes to Agilent whole mouse genome oligo microarrays. (B) RNA transcript levels for the indicated genes, measured by QRT-PCR from sorted CD8+ TCRβrep cells derived from day 35 HSC/OP9-DL1 cocultures or ex vivo thymocytes, are shown. Results of replicate samples were normalized to β-actin expression. These data are representative of at least five independent experiments.

**Generation of self-tolerant CD8 T cells in vitro**

To determine whether BM-HSC/OP9-DL1 coculture-derived CD8 T cells can discriminate between self and nonself Ags, we performed MLR cultures using in vitro generated CD8 T cells as the responders (Fig. 4A). Of note, C57BL/6 (B6; H2b)-derived HSCs were used to generate CD8 T cells in OP9-DL1 cocultures, and these cells displayed increased proliferation in response to allogeneic CD1 (H2d)-irradiated splenocytes used as stimulators. In contrast, no increased proliferation in response to syngeneic B6 stimulator cells was detected (Fig. 4A). The generation of alloreactive CD8 T cells that also lack self-reactiveness suggests that in vitro generated CD8 T cells underwent a series of successful positive and negative selection events, two processes that are critical in the selection of a functional and self-tolerant T cell repertoire.

The absence of overt self-reactivity in the MLR assays does not fully address whether all potentially self-reactive T cell clones are indeed eliminated during CD8 T cell maturation in vitro. For instance, it is not clear whether a potentially limited abundance of self-peptides presented by MHCI on OP9-DL1 cells results in the elimination of all self-reactive T cells. To test this premise, we transferred 1–2 × 10⁶ CFSE-labeled in vitro generated or ex vivo CD8 T cells i.v. into syngeneic B6 mice. Three days later, donor CFSE-labeled T cell proliferation was evaluated by measuring CFSE dilution in vitro derived CD8 T cells in comparison with noninjected and CFSE-labeled cells. As shown in Fig. 4B (and Supplemental Fig. 2A), both in vitro and ex vivo CD8 T cells showed a very small number of cells that had diminished CFSE levels, as assessed by flow cytometry from lymph nodes (LNs), suggesting that CD8 T cells, generated in vitro from B6 HSC/OP9-DL1 cultures, failed to mount an overt antiserum response in vivo. However, flow cytometry analysis of CFSE-labeled in vitro generated CD8 T cells transferred into H2-mismatched host mice showed a substantial loss of CFSE (Supplemental Fig. 2B), confirming their ability to mount an effective alloresponse (Fig. 4A) and ability to become activated after in vivo transfer. Based on these findings, and consistent with the MLR assay analysis, the absence of CD8 T cell proliferation in vivo suggests that naive resting in vitro derived CD8 T cells can undergo tolerance induction.

To address whether in vitro generated CD8 T cells are able to undergo homeostatic T cell proliferation (51, 52), 1–2 × 10⁶ CFSE-labeled in vitro generated CD8 T cells were adoptively transferred into RAG2-deficient mice. Seven days after transfer, LNs and spleen were isolated and analyzed by flow cytometry. Notably, a population of donor T cells with little to no CFSE content was detected by flow cytometry (Fig. 4C, Supplemental Fig. 2C). These data suggest that in vitro generated CFSE-labeled CD8 T cells underwent several rounds of cell division after transfer into immunodeficient hosts. Of note, the transferred CD8 T cells showed slightly upregulated CD44 levels, but failed to upregulate CD25 expression (Supplemental Fig. 2C), a phenotypic feature of cells undergoing IL-7–driven homeostatic proliferation under lymphopenic conditions.

**In vitro generated CD8 T cells display a broad TCR-Vβ CDR3 diversity**

In an attempt to characterize the TCR repertoire that is present in CD8 T cells generated in vitro, we performed a CDR3 length spectratyping analysis of in vitro and ex vivo CD8 T cells. The analysis of 22 of 24 Vβ-chains (Vβ1-Vβ20) showed that in vitro generated CD8 T cells displayed a broad and highly diverse TCR-Vβ usage, with CDR3 lengths showing a normal Gaussian distribution comparable to that of ex vivo CD8 T cells (Fig. 5). Notably, we found that nearly all of the Vβ-chains showed polyclonal usage with similar CDR3 lengths to that of ex vivo CD8 T cells. These findings were supported by surface expression of TCRα and TCRβ tested at protein level (data not shown). Taken together, these results provide an important insight by demonstrating that a broad and diverse TCR repertoire is produced during in vitro T cell differentiation.

**Generation of an Ag-specific response by CD8 T cells generated in vitro**

Based on the above findings showing that a broad TCR repertoire is generated in vitro, and that these CD8 T cells are functionally mature with a powerful effector machinery capable of recognizing and destroying cells expressing viral or tumor-associated Ags, we sought to assess the premise that an Ag-specific response can be developed entirely in vitro. To this end, we examined whether a specific response can be generated against a tumor-associated Ag and viral Ag, the hTRP-2 (also known as dopachrome tautomerase) and LCMV-associated Ag gp33, respectively.

For this approach, a bulk population of in vitro generated CD8 T cells was stimulated (or primed) with BM-derived DCs that were induced to express full hTRP-2 protein or LCMV-specific gp33 epitope (KAVYNFEAT) following infection with a recombinant adenovirus-based Ag delivery (Fig. 6). The Ag-specific response was measured by MHCI-specific tetramers (tet) containing immunodominant CD8 epitopes for both mouse and hTRP-2, SVYDFFVWL (residues 180–188) and LCMV-specific gp33–41. At the peak of the response (day 14), the frequency of gp33-tet+ cells in vitro derived CD8 T cells reached an average of ~10%, which was higher, but not statistically different, than that observed from ex vivo CD8 T cells (Fig. 6A). Similarly, frequency of TRP-2-tet+ cells was higher, but not statistically different, for in vitro derived CD8 T cells than that observed from ex vivo CD8 T cells (Fig. 6B).
Flow cytometric analysis using control tetramer peptides (gp276–286, or ova257–264) was used to show the specificity of the responding T cells, which was low and not statistically different between each Ag-specific population. Taken together, these findings clearly demonstrate that the use of a Notch ligand-expressing stromal cell line supports the generation of CD8 T cells that are functionally competent, self-tolerant, bearing a diverse TCR repertoire, and capable of mounting an Ag-specific response.

**FIGURE 3.** In vitro derived CD8+ TCRβhigh cells display similar functional properties to ex vivo CD8+ TCRβhigh cells. (A) Bar graphs show [3H]Tdr incorporation measured from anti-CD3/CD28 mAb-stimulated (ST) and unstimulated (UST) CD8+ TCRβhigh T cells, obtained in vitro (day 35 BM-HSC/OP9-DL1 cocultures) or ex vivo (6-wk B6 thymocytes). Data represent the average values, and error bars represent SEM from triplicate cultures. (B) Flow cytometry analysis indicating the number of cell cycle divisions detected by labeling sorted CD8+ TCRβhigh T cells with 0.5 μM CFSE. Number of cell divisions (at top) was calculated by FlowJo software using the same constraints for all samples. Proliferation indexes (numbers at bottom left or right of each histograms) were calculated using the same parameters. Representative plots of stimulated and unstimulated cells are shown. (C) Bar graphs showing the changes in MFI values (from triplicate samples) of the indicated cell surface markers from stimulated (ST: white and black bars) or unstimulated (UST: light and dark gray bars) CD8+ TCRβhigh cells, obtained as in (A), measured at 24, 48, and 72 h. Data are representative of at least three independent experiments. (D) Bar graph showing IFN-γ production detected from culture supernatants of stimulated (ST) or unstimulated (UST) CD8+ TCRβhigh T cells, in vitro (white bars) and ex vivo (black bars, thymocytes). (E) Flow cytometric analysis of Gzm-B and perforin expression of stimulated (black lines) or unstimulated (shaded) CD8+ TCRβhigh T cells for 72 h, as indicated. Data are representative of at least five separate experiments.
Absence of overt self-reactivity by in vitro derived CD8$^+$ T cells. (A) Bar graph shows $[^{3}H]$Tdr incorporation measured from a 3-d MLR assay using sorted CD8$^+$ TCR$^{[\beta]_{\text{high}}}$ T-cells isolated from HSC/OP9-DL1 day 35 cocultures (in vitro) or from B6 thymocytes (ex vivo) mixed with either syngeneic (Syn) or allogeneic (Allo) irradiated splenocytes as stimulators. Data represent the average values, and error bars represent SEM from triplicate cultures. (B and C) Flow cytometric analysis of CFSE levels from labeled CD8$^+$ TCR$^{[\beta]_{\text{high}}}$ T-cells, obtained in vitro (B6 BM-HSC/OP9-DL1 coculture, day 35) or ex vivo (B6 mice splenocytes), i.e. injected into nonirradiated B6 mice (B) or RAG$^{2-/-}$ mice (C). Analysis of CFSE expression by CD8$^+$ cells from LNs of injected (middle and bottom panels) or noninjected (top panel) mice after 3 d (B) or 7 d (C). The data are representative of two to three mice analyzed individually. Experiment was repeated at least three times.

**Discussion**

Whereas several previous studies have concluded that functionally mature CD8$^+$ SP T-cells can be generated from mouse FLs and embryonic stem cells, as well as human cord blood-derived CD34$^+$ HPCs, in this study, we demonstrate that OP9-DL1 cells support efficient T cell differentiation from adult BM-derived HSCs, giving rise to functionally mature CD8 SP cells.

Recent studies have demonstrated phenotypic classification of CD8 T cells based on the expression of activation markers into innate CD8$^+$CD44$^+$CD122$^+$ or CD8$^+$NK1.1$^+$CD44$^+$CD122$^+$CD244$^+$NK-T-like CD8$^+$ T cells versus conventional CD8$^+$CD44$^-$CD122$^-$NK1.1$^-$ cells; CD8$^+$CD28$^+$ suppressor versus CD8$^+$CD28$^-$ effector cells (42, 44, 53, 54). An inclusive phenotypic comparison in this study demonstrates that in vitro derived CD8$^+$ T cells exhibit all aspects of ex vivo conventional CD8$^+$ TCR$^{[\beta]_{\text{high}}}$ cells recognized by high expression of CD5, CD27, CD28, Qa2, and MHC-I, and lack of expression of innate phenotypic markers such as CD44, CD122, and CD244. This analysis indicates that the in vitro derived CD8$^+$ cells appear to conform to a conventional CD8 phenotypic profile.

Conversely, several distinctive phenotypic features of in vitro derived in comparison with ex vivo CD8$^+$ T cells are lower levels of CD5 expression, and the lack of downregulation of CD24 (heat-stable Ag), which has been associated with the mature status of T cells. CD5 levels have been shown to correlate with the intensity of TCR signals mediating positive selection (55), which suggests that cells differentiating in vitro may experience TCR signals of lower intensity, but sufficiently strong to allow for the generation of CD8$^+$ T cells.

In terms of CD24 expression, one study has shown that recent thymic emigrants retain high expression of CD24, and that downregulation takes place in the periphery (56). Despite the evidence that functional properties might be diminished in CD8$^+$ CD24$^+$ cells (e.g., reduction in proliferation and cytokine production in response to stimulation) (56), our data support the notion of functional autonomy despite the expression of CD24. Additionally, the functional significance of this trait resides in the finding that CD24 expression is required for optimal T cell proliferation in lymphopenic host (57). Accordingly, we find that in vitro CD8$^+$CD24$^+$ when compared with ex vivo CD8$^+$CD24$^-$ derived T-cells undergo enhanced proliferative responses in RAG2$^{2-/-}$ host mice. Although the role of CD24 on T cells is largely unknown, our current understanding highlights the finding that functional CD8$^+$ T cell responses are not dependent on downregulation of CD24 maturation marker per se. Of note, this evidence is also supported by the ability of CD8$^+$ T cells differentiated from FL progenitors in OP9-DL1 cocultures to maintain functional competence (e.g., undergo proliferation, upregulation of activation markers, and generation of Gzm-B upon activation; data not shown).

Additionally, when compared directly, we find a similar pattern of expression of the memory differentiation markers by in vitro derived CD8$^+$ T cells to what is observed by ex vivo CD8$^+$ T cells. However, one of the differences was the altered expression of...
CD279 (PD1) by in vitro derived CD8 T cells (Supplemental Fig. 3A, 3B). Although expression of other inhibitory receptors such as CD244 (2B4), CD160, CD152 (CTLA4), and Lag3 was not tested, induction of inhibitory receptor PD1 correlates with T cell exhaustion (58). Whereas the mechanism that controls PD1 expression is largely unknown, it remains to be investigated whether Notch signaling plays a role in the induction of this inhibitory receptor. With this in mind, we noted that, following TCR stimulation, the addition of Notch signaling promoted expression of PD1 (Supplemental Fig. 3B). Despite this finding, our results also support previous reports that demonstrate Notch ligand-induced activation, whereby higher expression of activating receptors (e.g., CD28) and cytolytic molecules (e.g., granzyme A, Gzm-B, and perforin) may balance or counterbalance expression of the inhibitory receptors resulting in unaltered or even enhanced CD8 T cell-mediated overall response (59). Thus, the ability to fully evaluate the differentiation status of in vitro derived CD8 T cells might have an important implication in the prospective use of in vitro derived human CD8 T cells for immune reconstitution.

In this study, we also analyzed a small fraction of genes obtained from the gene expression microarray performed between in vitro derived and ex vivo CD8 T cells. We noted that 52 genes were upregulated and 87 genes were downregulated by in vitro derived CD8 T cells. In-depth analysis and conformation of differentially expressed genes by in vitro derived CD8 T cells will be characterized in detail elsewhere. Nevertheless, this initial analysis points to a potential role of Notch signaling in regulating the expression of this effector-function gene. Other differences obtained from the gene microarray analysis may be due to the presence of contaminating OP9 cells, or due to ongoing Notch or cytokine signaling, from the exogenously added IL-7.

Despite a number of studies that have demonstrated induction of CD8 T cell differentiation from different mouse or human progenitors using OP9-DL1 cells, the process of positive and negative selection of in vitro derived CD8 T cells is mainly discussed rather than experimentally demonstrated. Moreover, insufficient numbers of CD8 T cells were used for in vitro analyses only (22, 29, 60). In this study, we reveal that in vitro derived CD8 T cells when transferred into syngeneic host mice remain resting, which suggests that OP9-DL1 cells express an array of self-Ags at a sufficient level to eliminate potentially autoreactive clones.

Moreover, previous studies have demonstrated that, in the periphery, mature naïve T cells, for their survival and homeostatic proliferation, require low-affinity interactions with self-pMHC molecules involved in positive selection. This observation was confirmed by one study, which elegantly identified that the homeostatic proliferation of adaptively transferred CD8 T cells is abrogated in MHC-I-deficient irradiated host (52). This would suggest that in an immunodeficient host, proliferation of in vitro derived CD8 T cells is attained through the TCR interaction(s) with self-pMHC and the trophic effects of available cytokines (e.g., IL-7, IL-15). In light of this finding, we hypothesize that homeostatic proliferation of in vitro derived CD8 T cells was mediated through contact(s) of TCR expressed by in vitro derived CD8 T cells with pMHC expressed either by APCs such as DCs in the periphery. Surprisingly, homeostatic proliferation of in vitro derived CD8 T cells seems to be augmented in immunodeficient host. This may be due to either increased concentration of available IL-7 in vivo, high expression of CD24 (previously discussed), or overt activation of TCR with pMHC expressed in the periphery, although the latter possibility is not supported by the lack of CD25. Nevertheless, this finding highlights the importance of the TCR/self-pMHC recognition, which allows for proliferation of in vitro derived CD8 T cells to occur in the periphery.

In addition to the ability of in vitro derived CD8 T cells to display self-tolerance and homeostatic proliferation in vivo, we demonstrate that in vitro derived CD8 T cells also express a diverse TCR repertoire, an attribute ascribed to conventional CD8 T cells, which can recognize peptides in the context of classical MHC-I molecules.
(61). Of note, although innate CD8 T cells play an important role in the immune responses against several bacteria, viruses, and parasites, their TCR repertoire diversity is limited (62). In contrast, we demonstrate a broad and functional heterogeneity among CD8 T cells. Importantly, we also show that these in vitro derived CD8 T cells have the ability to differentiate into Ag-specific, gp33, and TRP-2, CD8 T cells.

In conclusion, important initial steps were made in identifying phenotypic, molecular, and functional nature of in vitro derived CD8 T cells generated from BM-HSC/OP9-DL1 cocultures. Further investigation regarding primary and/or secondary Ag-specific responses by in vitro derived CD8 T cells in vivo may enhance our understanding of additional inputs necessary for developing novel approaches for manipulating immune responses against foreign Ags.

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Disclosures

The authors have no financial conflicts of interest.

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