CD8α+ Dendritic Cell Trans Presentation of IL-15 to Naive CD8+ T Cells Produces Antigen-Inexperienced T Cells in the Periphery with Memory Phenotype and Function

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Various populations of memory phenotype CD8\(^+\) T cells have been described over the last 15–20 y, all of which possess elevated effector functions relative to naive phenotype cells. Using a technique for isolating Ag-specific cells from unprimed hosts, we recently identified a new subset of cells, specific for nominal Ag, but phenotypically and functionally similar to memory cells arising as a result of homeostatic proliferation. We show in this study that these virtual memory (VM) cells are independent of previously identified innate memory cells, arising as a result of their response to IL-15 \textit{trans} presentation by lymphoid tissue-resident CD8\(^+\) dendritic cells in the periphery. The absence of IL-15, CD8\(^+\) T cell expression of either CD122 or eomesodermin or of CD8a\(^+\) dendritic cells all lead to the loss of VM cells in the host. Our results show that CD8\(^+\) T cell homeostatic expansion is an active process within the nonlymphopenic environment, is mediated by IL-15, and produces Ag-inexperienced memory cells that retain the capacity to respond to nominal Ag with memory-like function. Preferential engagement of these VM T cells into a vaccine response could dramatically enhance the rate by which immune protection develops. \textit{The Journal of Immunology}, 2013, 190: 000–000.
mice lacking IL-4 (31). This suggested that at least a portion of VM cells might be similar to innate memory cells. However, Akue et al. (31) also showed that most VM cells acquired their properties in the periphery and not the thymus, with the suggestion that the response of recent thymic emigrants to the lymphopenic neonatal environment may be responsible for promoting HP and the production of VM cells in the normal B6 host.

That being said, the precise mechanism by which VM cells experience HP, as well as the capacity of VM cells to participate in normal immune responses in the periphery remains largely unexplored. Cells having undergone HP possess both phenotypic and functional characteristics of true Ag-experienced memory cells (4, 5). Given that VM cells essentially represent a population of pre-existing memory phenotype cells specific for nominal Ag in the Ag-inexperienced host, their development and function must be considered in order for us to understand completely the nature of the primary immune response.

In this study, we confirm that the production of VM cells is independent of innate memory cell development in the thymus. Further, we show that VM cells develop as a result of CD122 stimulation on the T cells mediated by IL-15 encounter in the periphery. This occurs largely as a result of IL-15 trans presentation by CD8α+ dendritic cells (DCs) despite substantial production of IL-15 by tissue-derived DCs expressing or lacking CD103. These data indicate that HP is an active process during normal immune development (not just lymphopenia) and is mediated by IL-15 in the unmanipulated host. VM cells therefore represent a cell type within the unprimed repertoire that is one step closer to the formation of immunologic memory without the host ever having seen Ag.

Materials and Methods

Mice and reagents

Six- to 12-wk-old female B6 mice were purchased from the National Cancer Institute (Bethesda, MD). IL-4−/− and IL-15Ra−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Rag2−/−, CD122−/−, B220−/−, and CD49d−/− (kind gifts from Harald Von Boehm; Harvard Cancer Institute, Boston, MA) mice were bred in the National Jewish Biological Resource Center. Spleen cells from 1) the transgenic (Eomes) conditional knockout mice (Rag2−/−, Cd49d−/−, Cd122−/−, Tcrα−/−, Tcrβ−/−, B220−/−, IL-15Ra−/−, Thet−/−, IL-15Ra−/−, CD49d−/−; a kind gift from Charles Suri at the Scripps Institute, La Jolla, CA), B6/129 Batf3−/−, and B6 Batf5−/− (kind gifts from Ken Murphy at Washington University, St. Louis, MO) mice were bred in the National Jewish Biological Resource Center. Spleen cells from 1) the transgenic (Eomes) conditional knockout mice (Rag2−/−, Cd49d−/−, Cd122−/−, Tcrα−/−, Tcrβ−/−, B220−/−, IL-15Ra−/−, Thet−/−, IL-15Ra−/−, CD49d−/−; a kind gift from Charles Suri at the Scripps Institute, La Jolla, CA), B6/129 Batf3−/−, and B6 Batf5−/− (kind gifts from Ken Murphy at Washington University, St. Louis, MO) mice were bred in the National Jewish Biological Resource Center. Spleen cells from 1) the transgenic (Eomes) conditional knockout mice (Rag2−/−, Cd49d−/−, Cd122−/−, Tcrα−/−, Tcrβ−/−, B220−/−, IL-15Ra−/−, Thet−/−, IL-15Ra−/−, CD49d−/−; a kind gift from Charles Suri at the Scripps Institute, La Jolla, CA), B6/129 Batf3−/−, and B6 Batf5−/− (kind gifts from Ken Murphy at Washington University, St. Louis, MO) mice were bred in the National Jewish Biological Resource Center. Spleen cells from 1) the transgenic (Eomes) conditional knockout mice (Rag2−/−, Cd49d−/−, Cd122−/−, Tcrα−/−, Tcrβ−/−, B220−/−, IL-15Ra−/−, Thet−/−, IL-15Ra−/−, CD49d−/−; a kind gift from Charles Suri at the Scripps Institute, La Jolla, CA), B6/129 Batf3−/−, and B6 Batf5−/− (kind gifts from Ken Murphy at Washington University, St. Louis, MO) mice were bred in the National Jewish Biological Resource Center. Spleen cells from 1) the transgenic (Eomes) conditional knockout mice (Rag2−/−, Cd49d−/−, Cd122−/−, Tcrα−/−, Tcrβ−/−, B220−/−, IL-15Ra−/−, Thet−/−, IL-15Ra−/−, CD49d−/−; a kind gift from Charles Suri at the Scripps Institute, La Jolla, CA), B6/129 Batf3−/−, and B6 Batf5−/− (kind gifts from Ken Murphy at Washington University, St. Louis, MO) mice were bred in the National Jewish Biological Resource Center. Spleen cells from 1) the transgenic (Eomes) conditional knockout mice (Rag2−/−, Cd49d−/−, Cd122−/−, Tcrα−/−, Tcrβ−/−, B220−/−, IL-15Ra−/−, Thet−/−, IL-15Ra−/−, CD49d−/−; a kind gift from Charles Suri at the Scripps Institute, La Jolla, CA), B6/129 Batf3−/−, and B6 Batf5−/− (kind gifts from Ken Murphy at Washington University, St. Louis, MO) mice were bred in the National Jewish Biological Resource Center.

Recipient mice were lethally irradiated with 900 rad following by grafting via the tail vein with 4 × 10⁵ total T-cell–depleted donor bone marrow cells suspended in 200 μl PBS. Bone marrow was depleted of T cells using magnetic removal of CD3+ cells (Miltenyi Biotec). Bone marrow chimeras were grafted at a ratio of 1:1, representing 2 × 10⁵ cells from CD45.1 wild-type (WT) and CD122−/− bone marrow. Chimeric mice were mated a minimum of 12 wk before being analyzed or immunized for experiments. Chimeric mice were fed trimethoprim-sulfamethoxazole–containing chow (Harlan Teklad 6596; Harlan) for 6 wk following reconstitution to reduce the risk of bacterial infection, but were switched to standard chow (Harlan Teklad 2919; Harlan) well before immunization.

Generation and DC analysis of IL-15TE reporter mice

We generated an IL-15 transgenic reporter mouse by modifying a bacterial artificial chromosome (BAC) and injecting it into pronuclei of C57BL/6J mice. A 200-kb-long BAC clone RP23-331F16, which included the entire IL-15 genomic locus, was obtained from Children’s Hospital Oakland Research Institute (Oakland, CA). Exon 8 of IL-15 was modified using a galk-based positive/negative selection system with reagents obtained from the National Cancer Institute Frederick campus (41). The modifications included addition of sequences coding for 2A peptide from Thosea asigna virus (TaV) RAEGRGSLTTCGDDVEENPGP, and EGFP to the end of IL-15. Our in vitro expression studies in COS-7 cells revealed that the WT sequence of the TaV 2A peptide made the IL-15 biologically inactive (data not shown). We overcame this problem by mutating C to S within the 2A peptide, a change that most likely prevented disulfide-bond formation of the IL-15 protein tagged with the 2A peptide (2A peptide remains attached to IL-15 after translation). The final construct contained a single open reading frame coding for IL-15, 2A from TaV (CisO), and EGFP peptides, a fusion designated as IL-15TE. Sequence of the final BAC construct was verified by restriction digests and direct sequencing of the modified exon 8 of IL-15. Next, IL-15 genomic sequences were separated from the vector sequences by restriction digestion, purified on a Sephadex G-25 column (Bio-Rad), and injected into pronuclei of C57BL/6J mice. Founder mice were screened by Southern Blotting, and IL-15TE founder #20 containing two copies of the transgene was selected as the final reporter mouse. For the rationale behind creation of this transgenic reporter and the graphic representation of this approach, see Supplemental Fig. 1 (42–45). DCs were isolated from CD11c+ EHS tumor cell media (Invitrogen) containing DNase (Worthington, Lakewood, NJ) and Collagenase D (Roche Diagnostics, Indianapolis, IN) as described (32). Fluorochrome-conjugated Abs against CD11c, CD11b, CD8, CD103, B220, CD40, class II, and CD326...
were all purchased from eBioscience, and DC stains and analysis were performed as previously described (46).

Results
VM cells are distinct from Ag-experienced memory cells and present in the CD8, not CD4, T cell subset

Using magnetic columns to enrich for tetramer-stained T cells, we and others (15, 47, 48) characterized the naive T cell repertoire specific for different Ags within the unprimed host. This method revealed a unique population of memory phenotype T cells (VM cells) bearing the phenotype of cells having undergone HP (15). VM cells expressed intermediate levels of LFA-1 and high levels of CD44, Ly6C, and CD122 (15) (Fig. 1A, 1B). However, in contrast to memory cells resulting from antigenic stimulation, VM cells expressed low levels of the α4-integrin CD49d (Fig. 1A, 1B) (15). One quality of VM cells we previously noted was their high expression of CD122 (15). Indeed, closer examination revealed that VM cells expressed even higher levels of CD122 than Ag-experienced memory cells of the same specificity (Fig. 1C, 1D). CD8+ T cells, both human and mouse, are well documented to have overall higher levels of CD122 expression than CD4+ T cells, and so it was not surprising to find that the VM subset was present only within the unprimed CD8+ and not the CD4+ T cell repertoire (Fig. 1E). Although there are CD122-expressing CD4+ T cells present in the unprimed host, they are essentially all CD49d+. These data suggest that the majority of CD122hiCD4+ T cells are the result of

FIGURE 1. VM cells are a distinct subset of CD8+ T cells with memory-like phenotype and function. (A) B8R-specific T cells were isolated from naive B6 and B6 mice challenged with VV 30 d previously using magnetic enrichment of tetramer-stained cells as described previously (15) and in the Materials and Methods. (B) CD44lo and CD44hi cells, from both naive and VV-challenged hosts as shown in (A), were analyzed by FACS for CD122 and CD49d expression. (C and D) Overlay and graph of CD122 expression of the cells from the regions described in (A) and (B). Data are shown as mean fluorescence intensity (MFI) of CD122 staining of each population. (E) FACS analysis of spleen CD4 and CD8+ spleen cells for the distribution of CD122 and CD49d staining. (F) Overlay of CD122 expression from the regions shown in (E). (G and H) Naive and VM T cell transfer and immune protection study. CD44lo and CD44hiCD49dlo cells were sorted by FACS and transferred into separate naive hosts, which were then challenged with LMova. Five days later, the bacterial load in the spleens was determined as previously described. All data are representative of two to five experiments performed. Data points are from three to four mice per group. Error bars represent SD. All experiments shown were performed two to five times. *p < 0.05, ***p < 0.0001, unpaired t test.
Ag stimulation and not the result of the cause(s) underlying CD8+ VM cell development. Consistent with this, the levels of CD122 expression on CD44hiCD8+ T cells were similar to that observed for CD49dhiCD8+ T cells but lower than that observed in the CD49dhi VM cell subset (Fig. 1F).

HP memory cells derived from a lymphopenic environment have an identical phenotype to that of VM cells and were previously shown to mediate enhanced immunologic protection against infectious challenge (40). We used the same experimental system to determine whether VM cells, in the absence of any additional stimulation, could also mediate enhanced immunologic protection (Fig. 1G). Naïve phenotype (CD44lo, CD49dlo) and VM phenotype (CD44hi, CD49dhi) CD45.1+ OT1 cells were sorted and transferred into separate naive CD45.2 B6 recipients. These recipients were then challenged with L. monocytogenes, and 4 d later, the bacterial load in the spleen was determined. Mice transferred with VM cells were better protected against LM challenge than either naive OT1-transferred hosts or nontransferred controls (Fig. 1H). We conclude from these data (Fig. 1) that VM cells represent a pool of T cells within the unprimed host that are: 1) unique to CD8+ T cells; 2) distinct from Ag-experienced memory cells; and 3) phenotypically and functionally similar to HP memory cells.

VM cell development in B6 mice is largely independent of IL-4, NKT cells, PLZF, and Tbet

Memory phenotype CD8+ T cells arise in the thymus of a number of genetically modified mouse strains. Cells originally dubbed innate memory cells were found in elevated numbers in itk−/− (19, 20), id3−/− (24, 26–29), and If/2−/− mice (17, 30). These innate memory cells were recently shown to be dependent on IL-4 production by PLZF+ NKT cells within the thymus (17). Given the phenotypic similarity of our VM cells to innate memory cells, we first wished to examine whether VM cells might also arise via a similar NKT/IL-4/PLZF-dependent mechanism.

We therefore examined the secondary lymphoid tissues of IL-4−/−, PLZF−/− (deficient in PLZF expression in lymphocytes), and NKT-deficient mice (CD1d−/−, Jca1−/−). Though all of these strains have reduced innate memory cells (16), a substantial pool of VM cells was still present (Fig. 2A, 2B). Closer examination revealed that the IL-4−/−, CD1d−/−, Jca1−/−, and PLZF−/− strains did have modest, but statistically significant, reductions in VM phenotype cells as compared with the WT controls (Fig. 2C), consistent with recently published data (31). In addition, Tbet−/− mice displayed no loss of VM cells in the naïve T cell repertoire (Fig. 2C), indicating that this transcription factor is also not required for the production of VM cells (49). Tetramer staining and magnetic bead pulldown for each strain as described in Fig. 1 revealed similar results for the B8R-specific T cell pool as compared with the bulk T cell pool (data not shown).

id3−/− mice have elevated numbers of innate memory cells in the periphery (28, 29). Our examination of the CD8+ T cells in the secondary lymphoid tissue of id3−/− hosts was consistent with these reports, showing an elevated percentage of CD44hi, CD122hi CD8+ T cells in the periphery (Fig. 2D). However, these cells in the id3−/− expressed higher levels of CD49d (Fig. 2D), in contrast to VM cells. These data suggested that one of the potential means

**FIGURE 2.** VM cells are a population independent from innate memory cells. CD8+ spleen cells from the indicated strains of mice were isolated and stained for CD44, CD122, and CD49d expression. (A and B) data shown were gated on all live, B220−, CD8+ events. Numbers indicate percent of CD8+ T cells in each quadrant. (C) Graph of data gated as in (A), each data point representing an individual, age-matched mouse. Data are shown as percent of VM cells out of total CD8+ T cells from each strain. (D) Dot plots and histograms of CD8+ T cells from WT and id3−/− mice. Histograms are of all CD44hi cells shown in the dot plots. Data shown are representative of four independent id3−/− spleens. (E) Spleen and thymus from B6 hosts were isolated, and CD49d expression was determined on all B220−CD4−CD8+CD44hi CD122hi cells in each tissue. Gating strategy is shown in the dot plots and histogram and mean fluorescence intensity (MFI) of CD49d staining is shown in the graph, each data point representing an individual. All data are representative of two to five experiments performed. Error bars represent SD. *p < 0.05, **p < 0.001, unpaired t test.
by which innate memory and VM cells might be distinguished could be their level of CD49d expression. We therefore examined the phenotype of innate memory cells in the thymus of normal B6 mice and compared their CD49d expression to that of VM cells found in the periphery. Though B6 mice have far fewer innate memory cells in the thymus as compared with BALB/c mice (16, 17), they are detectable as a small population of CD8+CD44hiCD122hi cells (Fig. 2E). Similar to the innate memory cells found in the id3−/− host, innate memory cells in the thymus express a statistically significant higher level of CD49d as compared with VM cells or naive T cells in the periphery (Fig. 2E). Collectively, our results are consistent with those recently reported (31) and support the conclusion that VM cells represent a subset of memory phenotype CD8+ T cells that are distinct from the innate memory cells described previously. Further, our data indicate that CD49d expression is a distinguishing marker between VM cells and innate memory cells.

VM cell development is CD122 and IL-15 dependent

We next wished to determine what mediators might be responsible for the appearance of VM cells in the unprimed host. We focused our attention on IL-15 and the IL-2Rγ-chain (CD122), for the appearance of VM cells in the unprimed host. We focused

Secondly, whereas resting naive phenotype CD8+ T cells express a higher level of CD122, than even Ag-driven memory cells do (Fig. 1). Thirdly, a previous report described a unique form of HP, VM cell development is CD122 dependent. (**FIGURE 3.** VM cell development is CD122 dependent. (A) WT × CD122−/− bone marrow chimeras were made as previously described and as in the Materials and Methods. Twelve weeks after reconstitution, spleen CD8+ T cells were analyzed for CD44 and CD49d expression. (B) Percent of VM phenotype cells out of total CD8+ T cells from each background, WT or CD122−/−, in the bone marrow chimera mice shown in (A). Each data point represents a separate chimeric host. Data are representative of three independent mixed chimera experiments, each with three to five chimeras. (C) B8R-specific T cells were isolated from the chimeric mice using magnetic enrichment of tetramer-stained cells as described previously (15) and in the Materials and Methods. The column-bound CD8+ cells were gated into CD45.1 (WT) and CD45.2 (CD122−/−) backgrounds and analyzed for CD44 expression and tetramer staining. Two representative mice are shown (top and bottom plots, respectively). (D) Peripheral blood from WT × CD122−/− chimeras before and after immunization with B8R peptide in conjunction with polyinosinic-polyctydilic acid and anti-CD40 as previously described (36). Data shown are gated on all B220−CD8+CD45.1+ (WT) and B220−CD8+CD45.2+ (CD122−/−) events. Data shown are representative of four immunized chimeric mice. **p < 0.001. CD122−/− mice are marked by extreme lymphoproliferative syndrome similar to that observed in CD25−/− and scurfy/Foxp3−/− hosts (51). To avoid these issues associated with the intact CD122−/− mice, we made mixed bone marrow chimeras by transferring a mixture of bone marrow from CD122−/− (CD45.2) and WT (CD45.1) mice into irradiated Rag2−/− recipients. We assessed the unprimed bulk and Ag-specific CD8+ T cell repertoire in the reconstituted mice for the presence or absence of VM phenotype cells derived from the WT and CD122−/− backgrounds. Not surprisingly, whereas VM cells derived from the WT bone marrow were abundant, essentially no VM cells derived from the CD122−/− bone marrow were observed (Fig. 3A, 3B). Furthermore, tetramer staining and magnetic column enrichment of all B8R-specific T cells [dominant Ag derived from VV (52)] from the chimeric hosts revealed a similar deficit in the B8R-specific VM cells derived from the CD122−/− background (Fig. 3C). Interestingly, CD49d+CD44hi cells were readily observed in the bulk CD8+ T cells derived from the CD122−/− background (Fig. 3A), suggesting no defects in the capacity of CD122−/− cells toward Ag-experienced memory formation, presumably against endogenous gut- or food-related Ags. Indeed, immunization of WT:CD122−/− chimeric mice resulted in the expansion of Ag-specific CD122−/− cells that was five to six times that of the WT Ag-specific T cells (Fig. 3D). This suggests that CD122−/− cells may have an increased sensitivity to TCR-mediated expansion, a feature of CD122−/− cells previously overshadowed by the larger regulatory T cell defect in these mice. Regardless, these data confirm that VM cell formation in the periphery requires CD8+ T cell CD122 expression.

We next examined the potential role of IL-15 in VM cell development. IL-15−/− and IL-15Rα−/− mice have a deficit in their capacity to maintain memory CD8+ T cells over time (53–56).
However, the T cell repertoire in these mice had never been analyzed for the presence of cells bearing a VM phenotype. We therefore examined IL-15Rα−/− mice for the presence or absence of VM phenotype cells, both in the bulk CD8+ T cell pool as well as within Ag-specific T cells enriched from the unprimed repertoire. As previously described, the bulk CD8+ T cell pool had a reduction in the percentage of CD44hi cells (Fig. 4A). However, closer examination revealed that CD44hiCD122hiVLA4lo VM cells were present in frequencies similar to those in WT hosts and that the major deficit in CD44hi cells was due to a loss of the CD44hiCD122hiVLA4lo VM cells (Fig. 4A, 4B). We observed this loss of VM phenotype cells both in the bulk CD8+ T cell pool (Fig. 4B) as well as in the collection of Ag-specific T cells, in this case either for B8R (Fig. 4C, 4D) or HSVgB (not shown). Two conclusions can be made from these data. First, the development of VM cells within the normal unprimed host is largely IL-15 dependent. Second, though VM cells can be found at some of the earliest time points in neonates (31), the lack of VM cells in the IL-15Rα−/− host indicates that homeostatic stimuli typically associated with lymphopenia (i.e., IL-7) do not contribute substantially to VM cell production.

**VM cells expand in vivo to IL-15Rα/IL-15 complexes**

IL-15 stimulates cells by binding to IL-15Rα complexes. We injected WT hosts with IL-15/IL-15Rα complexes (34) and examined the resulting impact on bulk and Ag-specific CD8+ T cells in the unprimed host. IL-15/IL-15Rα injection into B6 mice resulted in a dramatic expansion of CD8+ T cells with the largest expansion manifest by CD44hiCD122hiVLA4lo VM cells (Fig. 5A). This effect was observed when examining either bulk CD8+ T cells (Fig. 5A) or the Ag-specific T cells isolated by magnetic column enrichment (Fig. 5B). Importantly, the Ag-specific cells expanded by IL-15/IL-15Rα treatment maintained low expression of CD49d (Fig. 5C), consistent with HP devoid of overt TCR stimulation (15). Examining the total numbers of CD8+ T cells within each subset before and after IL-15 injection revealed a substantial fold increase in both memory and VM cells without any corresponding decrease in naive phenotype cells (Fig. 5D). These data suggest that the increase in VM cells occurred largely as a result of the expansion of the pre-existing VM cell pool. VM cell expansion occurred over a broad range of IL-15/IL-15Rα treatment and almost exclusively affected the CD8+ T cells and not the CD4+ T cells (Fig. 5E). The small increase in the percentage of CD44hiCD4+ T cells that occurred at higher doses of IL-15/IL-15Rα complexes was due to an increase in frequency of CD44hiCD49dlo cells (not shown), consistent with the CD122 expression profile of these cells. In addition, even the strains of mice with a modest reduction in VM cells (Fig. 2C) responded to IL-15/IL-15Rα complex injection with a dramatic expansion of VM-phenotype cells (Fig. 5F). These data show that IL-15–mediated stimulation of the CD8+ T cells in the unprimed host dramatically increases the frequency of VM cells. Further, the proliferation these cells experience does not influence their expression of CD49d, consistent with an absence of Ag/TCR stimulation in this process.

**VM cell development is compromised in Eomes−/− hosts**

The maintenance of long-lived Ag-experienced memory cells requires IL-15–mediated stimulation through CD122/CD132 (55, 63), which leads to induction of the transcription factor Eomes.
Interestingly, CD122 expression itself is largely dependent upon Eomes (64), which VM cells overexpress compared with naive cells (Fig. 6A). Analysis of the different subsets of CD8\(^+\) T cells in the unprimed host revealed that, whereas CD49dh\(^+\) and CD49dl\(^+\) CD8\(^+\) T cells expressed roughly equivalent levels of Tbet, VM cells expressed elevated Eomes even compared with Ag-experienced memory cells (Fig. 6A). We speculated that this Eomes expression may be important in the development of VM cells such that Eomes deficiency would result in a loss of VM cells from the unprimed host. Indeed, CD8\(^+\) T cells in a T cell–specific conditional Eomes\(^{-/-}\) host (CD4-Cre \(\times\) Eomes\(^{lox/lox}\)) displayed a substantial loss of VM cells from the periphery (Fig. 6B, 6C) in both percentage and total numbers (Fig. 6C). Further, the few VM-phenotype cells present in these mice had lower expression of CD122 as compared with VM cells from WT mice, as might be expected. Although a decrease in CD49dh\(^+\)/CD8\(^+\) T cells in the Eomes conditional knockout, as well as the Eomes heterozygous mice, was already well established (64–66), we show in this study that the major deficit in these mice (similar to the IL-15\(^{-/-}\) hosts) is the loss of VM cells.

**Steady-state IL-15 reporter expression in vivo by CD8a\(^+\) and tissue-derived DCs**

We next wished to determine the source of IL-15 expression/presentation that was responsible for generating VM cells from the pool of naive phenotype cells. IL-15 expression has been described in a variety of DCs and macrophage/monocyte subsets (57, 67, 68). Recent data using a transgenic IL-15 reporter system showed substantial expression/presentation of IL-15 in the CD8a\(^+\) DC subset (69). We also recently generated an IL-15 reporter system using a cotranslational reporter mouse for IL-15 by introducing, as a transgene, a modified BAC containing the IL-15 genomic locus in which IL-15 is linked via a 2A peptide to GFP (see Supplemental Fig. 1 and Materials and Methods). We isolated cells from the peripheral LN and spleens of these mice and analyzed the various DC populations for expression of the IL-15 reporter. Similar to the recently published data (69), minimal expression of GFP was noted in the resident CD11b\(^+\) DC subset in LN or spleen (Fig. 7A, 7C). In contrast, the resident CD8a\(^+\) DCs as well as the tissue-derived DC subsets displayed high levels of GFP expression (Fig. 7B, 7B). In particular, Langerhan cells as well as the skin- and tissue-derived CD103\(^+\) DCs showed some of the highest GFP expression (Fig. 7B). Analysis of splenic DC subsets mirrored that seen in the LN, with the dominant GFP\(^+\) DC subset being the CD8a\(^+\) DCs (Fig. 7C). Thus, skin/tissue-derived DCs as well as lymphoid-derived CD8a\(^+\) DCs demonstrated substantial IL-15 expression/presentation in the resting host. These results are again fully consistent with published data using a different IL-15 reporter system (69).

**VM cell development is compromised in CD8a\(^+\) DC-deficient hosts**

Our observation of IL-15 expression in the CD8a\(^+\) and CD103\(^+\) DCs suggested that these subsets might play an important role in presenting IL-15 to support VM cell development. To explore the importance of these DC subsets, we used the BatF3\(^{-/-}\) mouse, which has a significant defect in the development of both CD8a\(^+\) and CD103\(^+\) DCs (70–72). Even though IL-15 is still present within these hosts, we reasoned that if these DC subsets were important in IL-15 presentation to the naive T cell pool, then we might expect to see a reduction in VM cells. Consistent with this expectation, we found a loss of VM cells (Fig. 7D) roughly equiv-
alent to that seen in the Eomes−/− hosts (Figs. 6C, 7E). This is again despite the fact that all other DC subsets and non-DC cell types capable of IL-15 expression (67, 68) are still present in these hosts. The BatF3−/− mice we initially used were on a B6/129 hybrid background, resulting from the intercrossing of F1-F2 B6/129 mice. We therefore repeated the experiment using BatF3−/− mice backcrossed to B6 for at least 10 generations (a kind gift from Dr. Ken Murphy, Washington University, St. Louis, MO). To our surprise, we found that the B6 BatF3−/− mice had a full complement of VM cells relative to WT B6 hosts (Fig. 8A, 8B). However, we also observed that the lymphoid-resident CD8α− DCs were also present in almost normal numbers in these mice (Fig. 8C). Of note, the CD103+ DCs were absent in BatF3−/− mice on either the B6 or B6/129 backgrounds (not shown). Although it is unclear how the loss of BatF3 has such different effects on the presence of CD8α− DCs in each background, the fact that the recovery of this DC subset corresponds with the full recovery of VM cell development indicates that VM cell development occurs largely as a result of the presentation of IL-15 by the CD8α− DC subset and does not require IL-15 production by the CD103+DCs.

Discussion

Our data provide mechanistic insight into the influence of IL-15 in mediating a form of HP that is active during normal immune development, independent of lymphopenia, and responsible for the generation of a unique pool of CD8+ T cells capable of enhanced responses to Ag. This subset exists only within the CD8+ T cell pool, probably because CD8+ T cells express higher levels of CD122 than naive CD4+ T cells (Fig. 1) and are therefore capable of responding to IL-15 trans presentation in the absence of overt Ag stimulation. Our data also confirm recently published data (69) showing steady-state IL-15 expression by CD8+ DCs, and we extend those findings to identify a novel function for this IL-15 trans presentation, namely that of facilitating VM cell formation in the periphery. Collectively, our data suggest a model in which elevated Eomes expression in a subset of naive phenotype cells leads to greater expression of CD122 and therefore greater sensitivity to IL-15 presented by CD8α− DCs, resulting in subsequent proliferation of the T cell and conversion to VM phenotype cells.

It remains an outstanding question as to what mediates the cellular decision of any given naive phenotype CD8+ T cell to increase CD122 expression and respond to the IL-15 trans presentation by the CD8α− DCs. In addition to cytokine-mediated signals, there is evidence that increased so-called tonic TCR stimulation has an influence on the degree of HP experienced by T cells in a lymphopenic environment (5, 12–14). Indeed, more recent data indicate that the T cell response specifically to IL-15 is heavily influenced by TCR/MHC affinity (73). These data suggest that the VM repertoire may have subtle differences in TCR usage that confer an increase in TCR/self-peptide/MHC interaction sufficient to contribute to VM cell formation. Cursory analysis using the available Abs for TCR Vβ and Vα showed no difference between naive and VM cells of the same specificity (R.M. Kedl, unpublished observations). However, the resolution of this method is almost surely inadequate to resolve these differences, and deep sequencing is currently underway in pursuit of the full characterization of the naive and VM cell repertoires.

Independent of repertoire issues, we can at least conclusively say that the decision to convert from naive phenotype to VM phenotype largely requires the expression of Eomes. Though our and others’ (31) data firmly establish the derivation of VM cells in the periphery as unrelated to the generation of innate memory cells in the thymus, a requirement for Eomes expression is a shared feature for all CD8 memory cells, regardless of means by which they are created (1, 16). IL-15–mediated stimulation through CD122 leads to the induction of Eomes (55, 63), providing an effective link to all of the factors required for VM cell development. However, CD122 is itself dependent upon Eomes expression (64), calling into the
question as what comes first, increased Eomes expression or elevated CD122? To resolve this dilemma, it is tempting to speculate on a potential role for the Wnt pathway. Eomes expression in Ag-driven memory CD8+ T cells is at least partially maintained by the HMG-box transcription factor, TCF-1 (*tcf7*) (74–76). Two major isoforms of TCF-1, p45 and p33, are expressed in naive T cells with both positive (p45) and negative (p33) regulatory functions based on their association with either coactivators (*β*-catenin) or repressors (groucho), respectively (77–83). In cooperation with *β*-catenin, the p45 isoform contributes to Eomes expression, which induces the subsequent expression of CD122 and sensitivity to IL-15 stimulation (76). It is interesting to note that ~90% of all CD8+ T cells in the TCF-1−/− host are VM phenotype (data not shown). The largely memory phenotype of the T cells in this host is well documented (77, 84) but is generally attributed to the lymphopenic periphery caused by aberrant thymic selection. However, a variety of other peripheral lymphopenic strains have typically 40–50% VM phenotype CD8+ T cells in the periphery (R.M. Kedl, unpublished observations), suggesting a more direct connection between the loss of these transcription factors and VM cell formation.

Curiously, the TCF family member encoded by *tcf7l2* (also known as TCF-4) similarly uses *β*-catenin as a coactivator, targets a very similar DNA binding motif, and is already known to associate tightly with the Eomes promoter (85). Taken together, these
data support the hypothesis that the loss of TCF-1 results in the loss of both activating and repressor elements within the naive T cell, allowing the cells to use other β-catenin responsive TCF transcription factor(s), such as TCF-4, to produce elevated Eomes expression, facilitating the cellular response to IL-15 and subsequent differentiation of both activating and repressor elements within the naive T cell, 

Perhaps the most significant application of our data is the fact that we have identified a physiologically relevant, lymphopenia-independent form of T cell homeostatic expansion. Although the reality of HP in extreme lymphopenia is well established (5–11), the physiological relevance of HP outside of bone marrow transplantation is unclear, as is the representation of HP memory T cells within a normal, unmanipulated host. Our data address both of these gaps in the knowledge base first by identifying nominal Ag-specific T cells within the pool of memory phenotype cells in the unprimed host and second by clarifying the mechanism by which these cells come into existence. We can now say that HP, at least for a subset of CD8+ T cells, is an active process during normal immune development and not requiring a state of lymphopenia. Furthermore, this HP is largely mediated by IL-15 presentation by CD8α+ DCs in the unmanipulated host. The result of this HP is the generation of a pool of CD8+ T cells that can participate in de novo responses to nominal Ags but that have a functional capacity more similar to Ag-experienced memory cells.

Given that VM cells can mediate protection against infectious challenge (Fig. 1), it is possible that they could be manipulated to provide increased immune protection, even in an Ag-inexperienced host. As we previously demonstrated that VM cells, at least in vitro, initiate proliferation more rapidly than naive phenotype cells (15), the descendants of VM precursors may be over represented in the pool of cells responding to antigenic challenge, ultimately shaping (if not mediating) such familiar features of the memory response as immunodominance (87) and population affinity maturation (88–90). Additionally, VM cells may access peripheral sites of inflammation even before their phenotypically naive counterparts have had the opportunity to respond within the draining lymphoid tissue. This trafficking capacity might be expected to restrict the growth rate of the infectious organism in situ, subsequently affecting the overall Ag load and the inflammatory environment, which ultimately controls the response of the naive phenotype cells. Thus, the function of VM cells must be considered to completely understand the nature of the primary immune response.

Finally, these data confront our basic assumptions of what constitutes the naive and memory T cell pools. Although it is well agreed upon that the memory cell pool has a number of functionally distinct subsets (1), the pool of Ag-inexperienced (naive) T cells has always been considered relatively homogeneous. Most studies assume this functional and phenotypic homogeneity and focus instead on elucidating the mechanisms underlying the functional advantages within the various subsets of Ag-experienced memory cells. The data we present in this study are in stark contrast to this assumption and indicate that the primary CD8+ T cell response is actually the collective response of both naive and memory phenotype T cells coexisting within the same unprimed host. Further work must be done to more carefully clarify the functional characteristics of VM cells, their participation in primary and secondary responses, their role in population affinity maturation and immunodominance, and their ultimate role in promoting protective immunity.

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Disclosures

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References


Supplemental Figure 1. A) Rationale for construction of translational reporter for IL-15. Studies from Waldman group implicated several regulators of translation contained within the 5‘ untranslated region (UTR), in the signal peptide (SP), and in the coding region of IL-15. In order to generate a reporter that truly reflects all aspects of IL-15 regulation of expression, we included all these elements and also added native 3’UTR. To preserve translational control, we utilized the functionality of a viral 2A-peptide that allows for co-translational expression of two polypeptides from a single mRNA. We incorporated a modified sequence of a 2A peptide from Thosea asigna virus, called TaV(CtoS), between the end of IL-15 coding sequence and Enhanced Green Fluorescence Protein (EGFP) (see Materials and Methods). This reporter construct encodes a single open-reading-frame of IL-15/2A-TaV/EGFP(IL-15TE). 2A peptide causes translational pause that results in hydrolysis of the nascent IL-15 polypeptide, followed by translation of EGFP. This tight coupling of translation allows for monitoring of IL-15 production by detection of EGFP. In addition, IL-15 protein is translated into the secretory pathway since it has a signal peptide, while EGFP accumulates in the cytosol. This phenomenon has been reported by de Felipe and confirmed by us (data not shown). Retention of the reporter protein in the cytosol may increase sensitivity of detection. B) Generation of IL-15 translational reporter mouse IL-15TE. Given that production of IL-15 protein is controlled transcriptionally and translationally, we generated a transgenic reporter mouse that would reflect both levels of this regulation. To preserve transcriptional control, we selected a Bacterial Artificial Chromosome (BAC) RP23-331F16 that contained the intact genomic locus of IL-15 flanked by large regions of genomic DNA (43 kb upstream and 90 kb downstream, respectively), as the backbone for our transgenic construct. To preserve translational control, we utilized the functionality of a viral 2A-peptide (see above). Thus, our targeting construct contained in-frame sequences of IL-15, 2A, and EGFP, followed by a short stretch of IL-15 3’UTR. The resulting targeted BAC encoded a single open-reading-frame of IL15/2A/EGFP.