Positive selecting cell type determines the phenotype of MHC class Ib-restricted CD8+ T cells

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Several studies have demonstrated an apparent link between positive selection on hematopoietic cells (HCs) and an “innate” T-cell phenotype. Whereas conventional CD8+ T cells are primarily selected on thymic epithelial cells (TECs) and certain innate T cells are exclusively selected on HCs, MHC class Ib-restricted CD8+ T cells appear to be selected on both TECs and HCs. However, whether TEC- and HC-selected T cells represent distinct lineages or whether the same T-cell precursors have the capacity to be selected on either cell type is unknown. Using an M3-restricted T-cell receptor transgenic mouse model, we demonstrate that not only are MHC class Ib-restricted CD8+ T cells capable of being selected on either cell type but that selecting cell type directly affects the phenotype of the resulting CD8+ T cells. M3-restricted CD8+ T cells selected on HCs acquire a more activated phenotype and possess more potent effector functions than those selected on TECs. Additionally, these two developmental pathways are active in the generation of the natural pool of M3-restricted CD8+ T cells. Our results suggest that these two distinct populations may allow MHC class Ib-restricted CD8+ T cells to occupy different immunological niches playing unique roles in immune responses to infection.

CD8+ T cells | H2-M3 | Innate lymphocytes | T-cell development

MHC class Ib genes, comprising the majority of the class I family, encode molecules that are expressed at lower levels than class Ia and have limited polymorphism. This large gene family includes but is not limited to MHC-linked H2-M3 (M3), Qa-1, and Qa-2 in mice; HLA-E, HLA-F, and HLA-G in humans; and MHC unlinked CD1 and MR1 molecules (1). CD1 molecules present lipid antigens to T cells, and CD1d, the only CD1 molecule found in mice, is required for the development a unique subset of T cells known as invariant natural killer T (iNKT) cells (2, 3). Unlike CD1 molecules, most MHC Ib molecules present peptide antigens to CD8+ T cells, and have been demonstrated to be involved in the immune response to a number of pathogens, including Mycobacterium tuberculosis (4–6), CMV (7), Salmonella enterica (8), Listeria monocytogenes (LM) (9–11), and Chlamydia pneumoniae (12).

M3 is one of the best-characterized MHC Ib molecules and preferentially binds N-formylated peptides (13). M3-restricted CD8+ T cells play a nonredundant role in antilisterial immunity, because M3-deficient (M3−/−) mice show increased susceptibility to listerial infection (14). Using T-cell receptor (TCR) transgenic (Tg) mice, we have shown that M3 is necessary and sufficient for the selection of M3-restricted T cells with no contribution from MHC Ia or other MHC Ib molecules (14, 15). Additionally, using a mouse model deficient in both MHC Ia and M3 molecules, we have shown that non-M3 MHC Ib-restricted CD8+ T cells are very similar to M3-restricted CD8+ T cells in terms of surface phenotype and expansion kinetics following LM infection (16). Based on this result, M3-restricted CD8+ T cells represent a reasonable model for the study of MHC Ib-restricted CD8+ T cells in general.

Most MHC Ib-restricted CD8+ T cells can be distinguished from MHC Ia-restricted T cells on the basis of a more “innate-like” phenotype. These cells express high levels of CD44 even in naive mice (17), are capable of rapidly producing cytokines after in vitro stimulation, and mount an immune response more quickly than MHC Ia-restricted CD8+ T cells following infection with LM (17, 18). Although the mechanisms responsible for the development of this innate-like phenotype are not fully understood, results from several studies suggest that the types of cells mediating selection of these T cells may contribute to their unique functional characteristics (1, 19).

Unlike MHC Ia-restricted T cells, which are positively selected by thymic epithelial cells (TECs) (20), iNKT cells are exclusively selected by CD1d-expressing thymocytes (21). Interestingly, a study using MHC Ia-deficient mice has demonstrated that MHC Ib-restricted CD8+ T cells, including M3-restricted CD8+ T cells, can be positively selected on hematopoietic cells (HCs) (19). However, it remains to be determined whether TEC- and HC-selected T cells represent two distinct T-cell lineages or whether certain T-cell precursors are capable of being selected by either pathway, expressing different phenotypes depending on how they are selected.

Using Tg mice expressing a TCR specific for the listerial peptide LemA presented by M3 (D7 Tg) (15), we show here that M3-restricted CD8+ T cells can be selected on both TECs and HCs. We demonstrate that D7 CD8+ T cells selected on HCs have more activated surface phenotypes and express increased amounts of Eomesodermin (Eomes), the T-box transcription factor shown to be up-regulated in a number of innate T-cell populations, including those found in mice deficient for IL-2-inducible T-cell kinase (22–24). Our results clearly demonstrate that M3-restricted CD8+ T cells can be generated from the same T-cell precursors selected on two distinct cell types and provide direct evidence that selecting cell type plays an important role in determining the phenotype and functional characteristics of these T cells.

Results

Phenotype and Effector Function of CD8+ T Cells in D7 TCR Tg Mice. Previous studies have demonstrated that the majority of MHC Ib-restricted CD8+ T cells exhibit an activated phenotype in naive mice (17). We therefore examined the phenotype and effector functions of CD8+ T cells from D7 TCR Tg mice (D7 Tg: TCR Vα10 and Vβ5, specific for M3/LemA) (15). Similar to other MHC Ib-restricted CD8+ T cells, a significant proportion of CD8+ T cells from both the thymus and spleen of naive D7 Tg

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mice exhibited an activated phenotype (CD44hi, CD62Lhi, β7 integrinhi, and Ly6Chi). In contrast, only a very small proportion of CD8+ T cells from age-matched WT B6 mice had an activated phenotype (Fig.1A). Consistent with their surface phenotype, D7 CD8+ T cells demonstrated a more potent IFN-γ response following in vitro stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin with CD8+ T cells from B6 mice (Fig. 1B). Several recent studies have identified the transcription factor promyelocytic leukemia zinc finger (PLZF) as a key regulator in the development of a number of innate T-cell populations (25–27). However, we found that PLZF expression levels in D7 CD8+ T cells were much lower than in INKT cells and more similar to the levels in conventional T cells (Fig. 1C).

**D7 TCR CD8+ T Cells Can Be Selected by Either HCs or TECs.** Unlike conventional CD8+ T cells, which are selected on MHC Ia-expressing TECs (20), it is well established that some MHC Ib-restricted CD8+ T cells can be selected on HCs (19). To determine whether M3-restricted CD8+ T cells are selected exclusively on HCs or can be selected on both HCs and TECs, we generated chimeric mice by adoptive transfer of bone marrow (BM) cells from D7 Tg M3−/− Rag−/− (D7M3−/−) and D7 Tg M3−/− Rag−/− (D7M3−/−) donors into irradiated M3−/− Rag−/− mice. Thymocytes and splenocytes from indicated chimeric mice were stained with antibodies specific for CD8β, CD4, and Vβ5. (A) Upper Numbers represent percentages of total thymocyte population within each quadrant. (Lower) Percentages of CD8+Vβ5+ cells within the total thymocyte population. Because both donor and recipient mice are on the Rag−/− background, all Vβ5+ cells in chimeric mice are D7 T cells. (B) Percentage of CD8 single positive cells within the total thymocyte population from various BM chimeras. (C) Mean percentage of CD8+Vβ5+ cells in the total thymocyte populations from the lymph node, spleen, and small intestine ± SEM. *p < 0.05; **p < 0.01. Data shown are representative of four independent experiments with two to three mice per group. IEL, intestinal epithelial lymphocytes.

**D7 CD8+ T Cells Selected on HCs Are More Activated Than Those on TECs.** To assess differences in effector function between these two subsets of D7 CD8+ T cells, splenocytes from chimeric mice were stimulated in vitro with LeuA and the levels of intracellular IFN-γ and extent of degranulation (as measured by CD107a expression) were determined by flow cytometry. Although both D7HCs and D7TECs responded to LeuA stimulation, consistent

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with their surface phenotypes, D7HCs mounted a significantly more robust response (Fig. 3C). D7HCs also demonstrated higher proliferative capacity (measured by carboxyfluorescein succinimidyl ester dilution) following stimulation with LemA (Fig. S1).

To compare the functional properties of D7TECs and D7HCs, we adoptively transferred each subset of T cells into naive B6 congenic mice and examined their effector functions following infection with LM. Seven days post-infection, splenocytes and hepatic leukocytes were harvested from infected mice and stimulated in vitro with LemA, and their IFN-γ responses were determined. Similar to our earlier results, although both groups of T cells responded in an antigen-specific manner, D7HCs mounted a more potent response (Fig. 4A).

During LM infection, M3-restricted T cells have been shown to expand faster than conventional CD8+ T cells but are incapable of mounting a robust secondary response on rechallenge (29). Because conventional CD8+ T cells are almost exclusively selected on TECs, we were interested in comparing the kinetics of D7TECs and D7HCs expansion after infection. Somewhat surprisingly, although the magnitude of the D7TEC response was lower than the D7HC response, the two T-cell populations exhibited identical kinetics in response to LM infection and neither group was capable of significant expansion following secondary infection (Fig. 4B). Taken together, these results demonstrate that although D7 CD8+ T cells develop distinct surface and effector phenotypes depending on what cells mediate their selection, certain characteristics that distinguish MHC Ib-restricted T cells from conventional T cells are unaffected by selection pathway.

**Fig. 3.** D7 CD8+ T cells selected on HCs are more activated and possess more potent effector function than those selected on TECs. Expression of activation markers on lymphocytes isolated from chimeric mice. The percentages of CD8+Vβ5+ cells (A: lymph node) and HSA+CD8+ cells (B: thymus) displaying each activation marker are shown. Data shown are representative of four independent experiments with two mice per group. (C) Splenocytes from D7TECs and D7HCs were stimulated with LemA. Intracellular IFN-γ production and CD107a expression were analyzed. Data shown are representative of two independent experiments with three mice per group.

**Fig. 4.** D7 T cells selected on TECs and HCs respond with similar kinetics but different magnitudes during LM infection. Sorted D7TECs and D7HCs (CD45.2+) were adoptively transferred into CD45.1 B6 congenic mice and recipient mice infected with LM. (A) Seven days postinfection, splenocytes and hepatic leukocytes from recipient mice were stimulated with LemA. The percentages of cells producing IFN-γ within the CD45.2+CD8+ LemA-specific D7TEC or D7HC population are shown. (B) Percentages of Vβ5+CD45.2+ T cells detected in the blood of recipient mice at different time points following primary and secondary LM infection. *P < 0.05. Data shown are representative of two independent experiments with three to four mice per group.

**D7 CD8+ T Cells Selected on HCs Up-Regulate a Specific Transcription Factor Involved in Innate T-Cell Development.** The T-box transcription factor Eomes is significantly up-regulated in innate CD8+ T cells compared with conventional CD8+ T cells (23). It is therefore possible that Eomes plays a role in determining the phenotype of M3-restricted CD8+ T cells. In addition to comparing Eomes expression between D7TECs and D7HCs, we were interested in comparing the expression levels of CD122 (the receptor specifying IL-15 responsiveness), which, similar to CD44, is expressed at a high level on innate T cells under naive conditions (30).

We harvested liver leukocytes and thymocytes from chimeric mice; depleted immature thymocytes; and, in addition to assessing CD122 expression, determined the relative amounts of intracellular Eomes via intracellular staining (Fig. 5 A and B). We also isolated total RNA from both sets of D7 CD8+ T cells and analyzed Eomes expression by real-time RT-PCR (Fig. 5C). Although a significant proportion of D7HC thymocytes are CD122+Eomes+, this population is almost absent in the thymus of D7TECs. Our finding that D7HCs express higher levels of Eomes suggests a potential role for the transcription factor in determining the innate-like phenotype of M3-restricted CD8+ T cells selected on HCs.

**Natural Pool of H2-M3–Restricted CD8+ T Cells Consists of T Cells Selected by Both HC- and TEC-Mediated Pathways.** Unlike CD1d-restricted iNKT cells, which can be easily detected in naive mice using CD1d/α-galactosylceramide (αGalCer) tetramer, low frequency and a diverse TCR repertoire complicate tetramer-based detection of M3-restricted CD8+ T cells in naive mice. Although the D7 Tg mouse is useful, it was important to determine the phenotype of M3-restricted CD8+ T cells in naive B6 mice and to identify what cells support selection of these T cells under more accurate physiological conditions.

To characterize the naive M3-restricted LemA-specific T-cell population, we used a tetramer-based enrichment approach that
has been developed recently to detect low-frequency antigen-specific T cells in naive mice (31). Whereas conventional CD8+ T cells were predominantly CD44hi, Ly6Cch, and CD62Lch, a significant proportion of M3-restricted LemA-specific CD8+ T cells in naive B6 mice appeared to possess an innate-like phenotype. These M3/LemA− CD8+ T cells (CD44hi, Ly6Cch, and CD62Lch) exhibited a surface marker expression profile that closely resembled the pattern observed in D7 Tg mice (Fig. 6A).

To examine which cell types mediate selection of M3-restricted CD8+ T cells under polyclonal conditions, we generated BM chimeric mice by transferring either B6 or M3−/− BM into irradiated B6 or M3−/− recipients. Because significant numbers of antigen-specific M3-restricted CD8+ T cells are only detectable after antigen-induced cellular expansion, BM chimeric mice were immunized with LemA-pulsed BM-derived dendritic cells (BMDCs) 10 wk following adoptive transfer. Seven days post-immunization, a significant number of M3/LemA tetramer+CD8+ T cells were detected in the spleen and liver of both B6→M3−/− (HC-selected) and M3−/−→B6 (TEC-selected) chimeric mice but not in the negative control group (M3−/−→M3−/−).

In agreement with our D7 Tg mouse model, HC-selected cells appeared to mount a more robust response than TEC-selected cells, most noticeably in the liver of immunized animals (Fig. 6B).

To examine the functional properties of these two types of M3-restricted CD8+ T cells further, splenocytes isolated from each group of chimeric mice were stimulated with LemA-pulsed antigen presenting cells (APCs), and the number of IFN-γ-producing cells was measured by enzyme-linked immunospot assay. Similar to results from the D7 Tg mouse, both HC- and TEC-selected M3-restricted CD8+ T cells were capable of producing IFN-γ in an antigen-specific manner (Fig. 6C). These results demonstrate that the natural pool of M3-restricted CD8+ T cells consists of two phenotypically distinct populations resulting from selection by either TEC- or HC-mediated pathways.

**Discussion**

The results presented here provide direct experimental evidence that selecting cell type plays an important role in determining the phenotype of M3-restricted CD8+ T cells. Using M3/LemA-specific TCR Tg mice, we demonstrate that M3-restricted CD8+ T cells with identical TCRs can be selected on either TECs (D7TECs) or HCs (D7HCS), giving rise to two phenotypically and functionally divergent populations. Interestingly, recent studies have suggested a model in which IL-4-producing PLZF+ T cells are responsible for regulating the innate phenotype of MHC Ia-restricted CD8+ T cells found in KL2F- and Id3-deficient animals (32, 33). We were, however, unable to detect PLZF-expressing T cells in the thymus of D7 Tg mice, suggesting that differences in selecting cell type are sufficient to regulate the development of an innate-like phenotype in M3-restricted CD8+ T cells. To corroborate our findings on the polyclonal level, we characterized the M3-restricted LemA-specific CD8+ T-cell...
population in naïve B6 mice, demonstrating that this population appeared to consist of two phenotypically distinct subsets. Further studies using BM chimeras showed conclusively that selection of M3-restricted CD8 T cells under polyclonal conditions occurs efficiently on either TECs or HC.

Although the unique phenotypic characteristics of M3-restricted CD8 T cells are dependent on their selecting cell types, the question remains as to why MHC Ib-restricted CD8+ T cells can be selected on both TECs and HC, whereas conventional T cells and iNKT cells are only efficiently selected on TECs and HC, respectively (20, 21, 34). Although conventional T-cell selection is thought to be dependent on certain specialized cell-surface molecules and cytokines provided by TECs (35), there is some evidence to suggest that cells with high affinities for self-MHC molecules may be less dependent on these TEC-provided signals and, as such, are capable of being selected on HC (19, 36). The fact that iNKT cells are solely selected on HC and are thought to have higher affinities for selecting ligands than conventional T cells (2, 37, 38) appears to support the notion that TCR affinity plays an important role in determining which cells are selected on HC.

To test if this hypothesis could explain M3-restricted T-cell selection, we compared the TCR affinities of iNKT cells with D7 Tg T cells. Using tetramer binding and decay experiments, we determined that D7 CD8 T cells bind M3–LemA complexes with lower affinity than iNKT cells; however, based on comparisons with previously published reports (37, 38), D7 CD8+ T cells still have significantly higher affinity for peptide–MHC complexes than conventional CD8+ T cells (Fig. S2). This intermediate TCR affinity (most likely reflective of the limited repertoire of N-formylated “self” peptides necessary for positive selection) might explain why M3-restricted CD8+ T cells are efficiently selected on both TECs and HC.

Our results suggest a basic model whereby MHC Ib-restricted CD8+ T cells initiate a specific developmental program based on specific signals received from the selecting cells. In the case of cells selected on HC, this signaling cascade results in the up-regulation of transcription factors, including but not necessarily limited to Eomes, resulting in the development of a more innate pre-activated phenotype. Another transcription factor associated with CD8+ T-cell effector function, T-bet, was similarly up-regulated in D7+CD8+ T cells. The vast majority of these T cells express an activated phenotype, the D7 Tg model appears to be “programmed” depending on the cell types that mediate their positive selection. Our data not only confirm the role played by HC in determining the phenotype of MHC Ib-restricted CD8+ T cells but further suggest that both selecting pathways may have physiologically significant roles. Specifically, M3-restricted CD8+ T cells selected on TECs have higher levels of β7 integrin expression and, as a result, traffic more efficiently to the gut. It is therefore possible that these two different selecting pathways allow M3-restricted CD8+ T cells, and possibly other MHC Ib-restricted CD8+ T cells, to occupy a number of different immunological niches playing unique roles in mediating immune function.

Materials and Methods

Mice. C57Bl/6 (B6), CD45.1 congenic B6, and Rag–2–deficient mice (Rag−/−) were purchased from Jackson Laboratories. D7 Tg (15) and M3−/− (14) mice were generated in our laboratory and were backcrossed at least 10 times to the B6 background. D7 Tg mice were crossed onto the Rag−/− background and further crossed with M3−/− mice for these studies. All animal work was approved by the Institutional Animal Care and Use Committee.

Antibodies and Tetramers. FITC-conjugated anti-CD8β, CD44, Ly6C, CD24, VJ51, CD45.1, CD45.2, and CD107; phycoerythrin (PE)-conjugated anti-CD8α, B220, CD122, and Eomes; peridinin chlorophyll protein complex (PerCP)-conjugated anti-CD4, B220, and Ly6c; allophycocyanin-conjugated IFN-γ, and CD11c; PerCP Cy5.5-conjugated anti–T–CR–J; p biển conjugated anti-B220, CD11b, and CD11c; and biotin-conjugated anti–CD62L, CD45.1, and CD45.2 were purchased from BD Biosciences or eBioscience. Anti-PLZF was purchased from Santa Cruz. CD1d–GalCer tetramers were generated in our laboratory, and H-2M3Lema tetramers were provided by the National Institutes of Health tetramer core facility.

Flow Cytometry. Single-cell suspensions were prepared by standard procedures and stained with the appropriate combinations of mAbs. PLZF expression was analyzed via intracellular staining using the FoxP3 staining buffer set (eBioscience) with 4 μg/mL anti-PLZF mAb. For Eomes staining, thymocytes were first depleted of immature cells using anti-CD24 mAb and a magnetic affinity cell sorting separation column. Cells were fixed, permeabilized, and then stained with anti-Eomes mAb. Flow cytometry was performed with a FACSJuno BD(0) (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc.).

BM Chimeras. Donor BM cells were depleted of mature T cells using anti-Thy1.2 mAb (AT83.A-6) and rabbit complement. A total of 1 × 10^6 T-cell-depleted BM cells were injected i.v. per irradiated recipient. Chimeras were analyzed by flow cytometry or used for immunization 8–10 wk later.

BMDC Generation and DC Immunization. BMDCs were prepared as described previously (16). DCs were pulsed with 1 μM LeuM peptide (FMIGWII) for 6 h and injected i.v. into BM chimeric mice (1 × 10^6 peptide-coated DCs per mouse).
Bacteria and LM Infection. The recombinant LM strain rLM-ovalbumin (OVA) was grown in brain-heart infusion broth supplemented with 5 μg/mL erythromycin. For primary infections, mice were infected i.v. with 5 × 10^7 cfu rLM-OVA. When necessary, secondary infection was performed 1 mo after primary infection with 1 × 10^6 cfu rLM-OVA.

Intracellular Cytokine Staining and Degranulation Assay. Splenocytes were stimulated with PMA (20 ng/mL) and ionomycin (1 μM) or 5 μM LeuA peptide for 3–5 h in the presence of 10 μg/mL monensin and, in relevant experiments, anti-CD107a mAb. Cells were washed and stained for cell surface markers. After fixation with 4% paraformaldehyde and permeabilization with 0.1% saponin, cells were stained with anti-IFN-γ mAb.

Enzyme-Linked Immunosorbent Assay. Multiscreen-IP filter plates (Millipore) were coated with anti-IFN-γ mAb, washed, and blocked with RPMI-10. Splenocytes from various BM chimeras were cultured with LeuA-pulsed or unpulsed BMDCs. Plates were incubated for 18 h, and IFN-γ-producing cells were quantified with an ImmunoSpot reader (Cellular Technology).

RNA Extraction and Quantitative Real-Time PCR. D7 T cells (CD8+VJ5+ cells) from BM chimeras were sorted via MoFlo (Beckman Coulter Inc.). Total RNA was reverse-transcribed using SuperScript II RT (Invitrogen). Real-time PCR was performed with forward and reverse primers and normalized to GAPDH (forward primer: 5′-TGAATGACCTCAGACTCAGA-3′) and reverse primer: 5′-TTCAACCATGGAAGAGGCC-3′.

Statistical Analysis. Statistical analyses were performed using PRISM software (GraphPad). Statistical significance of differences was calculated using Student t tests. P values <0.05 were considered significant (*P < 0.05; **P < 0.01; ***P < 0.001).

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