Supplementary Materials for

Enzymatic synthesis of core 2 O-glycans governs the tissue-trafficking potential of memory CD8+ T cells


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Other Supplementary Material for this manuscript includes the following:
(available at immunology.sciencemag.org/cgi/content/full/2/16/eaan6049/DC1)

Table S2 (Microsoft Excel format). Raw data sets and statistical analyses.
SUPPLEMENTAL MATERIALS AND METHODS

Flow Cytometry and Antibodies

The following antibodies and corresponding isotype controls were used in this study: CD8α (53-6.7, Tonbo), glycosylated CD43 (1B11, BioLegend), CD43 (eBioR2/60, eBioscience), CD44 (1M7, BioLegend), PSGL-1 (2PH1, BD Biosciences), CD45.2 (104, Tonbo), CD62L (MEL-14, BioLegend), CD122 (clone, BioLegend), CD132 (clone TUGm2, BioLegend), Thy1.1 (OX-7, BioLegend), Thy1.2 (clone 53-2.1, BioLegend), KLRG1 (2F1, Tonbo), CD3ε (17A2, BioLegend), CCR7 (clone 4B12, BioLegend). H2-Db-GP33-41 and H2-Db-GP276-284 tetramers were generated at the University of Iowa. MCMV-specific tetramers were provided by the NIH tetramer core facility. Staining for surface antigens was performed in PBS/1% FBS for 15 minutes at 4°C. For tetramer binding, cells were incubated for 45 minutes at room temp. Data was acquired using either a BD Fortessa or BD LSR II Flow Cytometer. Flow cytometry data was analyzed using FlowJo software, version 9.9 or 10.

Viral Quantification from Skin

Quantification of viral load in the infected skin was determined using standard plaque assays on BSC-40 cells. Briefly, infected ears were removed and homogenized in 1 ml of RPMI supplemented with 1% FBS. Skin homogenates were then subjected to three rounds of freeze-thaw before serial dilutions were inoculated on BSC-40 cells in a 12-well plate that were then covered with 1% Seakem agarose in Modified Eagle Medium (Lonza). Plaques were visualized three days later following overnight incubation with Neutral Red dye.
Memory CD8+ T cell Purification and In Vitro Stimulation

Spleens of LCMV-immune mice containing memory Thy1.1 P14 CD8+ T cells were harvested and single cell suspensions were generated. Total splenocytes were incubated with PBS/1%FCS and anti-Thy1.1 (clone OX-7) or anti-CD8α (clone 53-6.7) antibody at 4°C for 15 minutes. Cells were washed and then incubated with anti-PE or anti-APC magnetic beads (Miltenyi) for 10 minutes, washed, and purified using the Miltenyi AutoMACS Pro cell separator. In some cases, viable cells were enriched with 35% Percoll in HBSS. For in vitro stimulation, 0.5-1.0 x 10^6 purified Thy1.1 memory P14 T cells or total CD8+ T cells were cultured in RPMI supplemented with 10% FBS and L-glutamine with recombinant murine IL-15 (Peprotech) as indicated. The O-glycan inhibitor Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (Sigma) was dissolved in DMSO and used at a 5 mM concentration during in vitro stimulation. For neuraminidase treatment, cells were fixed with Fixation Buffer (BD Bioscience) for 15 minutes at 4°C and then incubated with 5 mU of neuraminidase from vibrio cholera (Roche) in 100 μL of HBSS at 37°C for 3 hours.

Immunoblotting

Memory P14 CD8+ T cells were purified and cultured with recombinant murine IL-15 (Peprotech) as indicated. Following in vitro stimulation, cells were washed once with PBS and lysed (50 mM HEPES [pH 7.9], 1% NP-40, 125 mM NaCl, 2.5 mM CaCl2, plus protease inhibitors). Protein was quantified and samples were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Membranes were blocked with 4% BSA in TBS and immunoblotting was performed with antibodies specific for STAT5 (rabbit polyclonal), pSTAT5 Y694 (rabbit polyclonal), β-actin (all from Cell Signaling) or PSGL-1 (clone 4RA10,
eBioscience). Anti-rat and -rabbit IgG HRP were from Santa Cruz Biotechnology.

**Quantitative PCR**

Memory P14 CD8+ T cells were purified as described and incubated in vitro for 3 days in 10% FBS-RPMI media alone or supplemented daily with 500 ng/ml murine IL-15 (Peptrotech). RNA was purified using the RNeasy mini kit (Qiagen) and cDNA was synthesized from 125 ng of RNA using the SuperScript III First Strand kit (Invitrogen) according to the manufacturers’ protocol. qPCR was performed using the Power SYBR green PCR Master Mix (ThermoFischer) and reactions were analyzed on a Step One Plus Real Time PCR system (Applied Biosciences). Changes in gene expression were quantified using the ΔΔCt method, normalized to expression of TATA binding protein (TBP). Primers to detect changes in gene expression were from Integrated DNA Technologies and described in Table 1.
Supplemental Figure 1: Trafficking of memory CD8+ T cells into VacV-infected skin. (A) To quantify trafficking of memory CD8+ T cells, naïve B6 mice were infected with LCMV by I.P. injection. At 60 days post-infection, LCMV-immune or naïve, age-matched B6 mice were infected with VacV and trafficking of CD8+ T cells into the infected skin was quantified on day 3 post-infection. (B) Representative examples of number of CD8+ T cells (CD8α+/CD3ε+) in the skin of naïve or LCMV-immune mice infected with VacV.
Supplemental Figure 2: CD8+ T cells require *Gcnt1* to synthesize core 2 O-glycans for binding to E- and P-selectins. (A) WT or *Gcnt1*<sup>−/−</sup> C57Bl/6 mice were infected with LCMV Armstrong. On day 7 post-infection, expansion of GP33- and GP276-specific CD8<sup>+</sup> T cells in the blood were identified by MHC-I tetramers. (B,C) Quantification of expansion of GP33- and GP276-specific CD8<sup>+</sup> T cells shown in (A). (D) GP33- and GP276-specific CD8<sup>+</sup> T cells from (A) were analyzed for expression of core 2 O-glycosylated CD43 (1B11) and binding to both P- and E-selectin. (E,F) Quantification of data shown in (D).
Supplemental Figure 3: Trafficking of memory CD8$^+$ T cells into VacV-infected skin requires interactions with E- and P-selectins. Naïve P14 CD8$^+$ T cells were transferred into naïve B6 mice and infected with LCMV. On day 90 post-infection, LCMV-immune mice were treated with either control IgG or blocking antibodies against P- and E-selectin (on days -1, 1, and 2 post-infection) and infected with VacV-GP33 or VacV-OVA. On day 3 post-VacV infection, trafficking of memory P14 CD8$^+$ T cells into the skin was quantified.
Supplemental Figure 4: IL-15 regulates E- and P-selectin binding during VacV infection.

(A) Same as Figure 2D, except expression of St3gal6 and Fut4 was determined by qPCR. (B) LCMV-immune mice received control IgG or IL-15 neutralizing antibody for 10 days and then infected with VacV-GP33 on the skin of the left ear. On day 3 post-infection, memory P14 CD8+ T cells from the blood were analyzed for binding to E-selectin and P-selectin. (C,D) Quantification of (B)
Supplemental Figure 5: T<sub>CM</sub> CD8<sup>+</sup> T cells synthesize core 2 O-glycans and maintain expression of CD62L. (A) Central memory (CD62L<sup>+</sup>,KLRG1<sup>-</sup>) P14 CD8<sup>+</sup> T cells from Figure 3F were analyzed for expression of CD62L, KLRG1, and core 2 O-glycosylated CD43 (1B11) following in vitro culture with 250 ng/ml IL-15. (B) Memory P14 CD8<sup>+</sup> T cells from the spleen 120 days after LCMV-Army Armstrong infection were cultured as described for (A) and analyzed for
expression of CCR7 and 1B11. (C) Same as (B) except memory P14 CD8\(^+\) T cells were from the inguinal lymph node and analyzed for expression of core 2 O-glycans (1B11) and binding to P- and E-selectin following culture with 250 ng/ml of IL-15
Supplemental Figure 6: Phenotype of terminally differentiated tertiary memory CD8+ T cells using repetitive LCMV-Armstrong infection. (A) Protocol for generating either primary or tertiary memory P14 CD8+ T cells with adoptive transfers and LCMV infection. (B) Percentage of T_CMO in the P14 CD8+ T cell population (based on expression of CD62L and KLRG1) following either primary or tertiary LCMV infection. (C) Example of CD62L and KLRG1 expression from (B). (D) Frequencies of primary and tertiary memory P14 CD8+ T cells in the circulation on day 80 after LCMV infection. (E) Expression of PSGL-1, CD44, and CD43 on cells from (D).
Supplemental Figure 7: Primary memory CD8+ T cells are recruited into the skin better than tertiary memory CD8+ T cells during contact hypersensitivity. (A) LCMV-immune mice with memory P14 CD8+ T cells were sensitized with DNFB on the shaved abdomen and subsequently challenged with DNFB on the ear skin 5 days later. Trafficking of memory P14 CD8+ T cells into the skin was quantified compared to unchallenged mice or mice that were challenged on the ear skin, but not sensitized. (B) Quantification of (A). (C) Mice containing equal frequencies of primary and tertiary memory P14 CD8+ T cells in the circulation were
sensitized and challenged with DNFB. Trafficking of memory P14 CD8+ T cells into the skin was analyzed on day 3 after challenge. (D) Quantification of (C) on day 2 and 3 post-challenge. (E) Expression of core 2 O-glycosylated CD43 on primary and tertiary memory CD8+ T cells in the circulation and skin on day 2 post-DNFB challenge. (F) Quantification of (E).
SUPPLEMENTAL TABLE 1. Primer pairs for gene expression analysis in Fig. 2 and fig. S4.

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