IMMUNOLOGICAL MEMORY

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

Jossef F. Osborn,[1]* Jana L. Mooster,[1]* Samuel J. Hobbs,[1] Michael W. Munks,[1] Conrad Barry,[1] John T. Harty,[2] Ann B. Hill,[1] Jeffrey C. Nolz[1,3,4]†

Trafficking of memory CD8$^+$ T cells out of the circulation is essential to provide protective immunity against intracellular pathogens in nonlymphoid tissues. However, the molecular mechanisms that dictate the trafficking potential of diverse memory CD8$^+$ T cell populations are not completely defined. We show that after infection or inflammatory challenge, central memory (T_{CM}) CD8$^+$ T cells rapidly traffic into nonlymphoid tissues, whereas most effector memory cells remain in the circulation. Furthermore, we demonstrate that cellular migration of memory CD8$^+$ T cells into nonlymphoid tissues is driven by interleukin-15 (IL-15)–stimulated enzymatic synthesis of core 2 O-glycans, which generates functional ligands for E- and P-selectins. Given that IL-15–stimulated expression of glycosyltransferase enzymes is largely a feature of T_{CM} CD8$^+$ T cells, this allows T_{CM} to selectively migrate out of the circulation and into nonlymphoid tissues. Collectively, our data indicate that entry of memory CD8$^+$ T cells into inflamed, nonlymphoid tissues is primarily restricted to T_{CM} cells that have the capacity to synthesize core 2 O-glycans.

INTRODUCTION

Trafficking of leukocytes out of the circulation and into lymphoid and nonlymphoid tissues requires the collective action of a variety of receptor-ligand interactions (*1*). After maturation in the thymus, naïve CD8$^+$ T cells enter the periphery but are confined to the circulation and lymphoid organs, because their gene expression profile limits their trafficking to these compartments. In contrast, antigen-experienced, long-lived memory CD8$^+$ T cells can directly infiltrate nonlymphoid tissues during episodes of local inflammation (*2*–*4*). Furthermore, the capacity for memory CD8$^+$ T cells to traffic directly into inflamed tissues occurs independent of antigen restimulation and before the reexpansion of memory CD8$^+$ T cells in lymphoid organs during an infection (*5*). This feature of memory CD8$^+$ T cells contributes substantially to antigen-specific protective immunity in nonlymphoid tissues (*6*, *7*). Although understanding the factors that govern the tissue-trafficking potential of memory CD8$^+$ T cells is highly relevant for vaccine design and host defense, the molecular and biochemical mechanisms that contribute to trafficking of memory CD8$^+$ T cells are largely undefined.

Memory T cells are often classified on the basis of the expression of receptors required for lymph node homing (*8*, *9*). Naïve and central memory (T_{CM}) CD8$^+$ T cells express the chemokine receptor CCR7 and L-selectin (CD62L), which are required for T cells to extravasate across high endothelial venules and into lymph nodes. Effector memory (T_{EM}) CD8$^+$ T cells do not express these receptors, which exclude them from entering lymph nodes directly from the circulation. Because T_{EM} cannot enter lymph nodes, it has been predicted that these cells actively patrol and rapidly infiltrate nonlymphoid tissues after infection or tissues injury. T_{EM} CD8$^+$ T cells express a variety of inflammatory chemokine receptors such as CCR5 and CX3CR1 (*8*, *10*, *11*), which could cause them to infiltrate nonlymphoid tissues during episodes of inflammation. T_{EM} CD8$^+$ T cells are also rich in granzymes, are highly cytolytic, and provide robust protective immunity against some infections (*12*–*14*). Nevertheless, studies directly comparing the trafficking potential of T_{CM} and T_{EM} CD8$^+$ T cell subsets or identifying the mechanisms that dictate their trafficking into nonlymphoid tissues during inflammatory challenges have not been rigorously performed.

After infection, tissue damage, or other inflammatory event within nonlymphoid tissues, the associated vascular endothelium becomes activated and expresses adhesion molecules and chemokines that function to recruit circulating leukocytes. E- and P-selectins are C-type lectin, oligosaccharide-binding proteins that function to capture circulating leukocytes as the first step of the extravasation process (*15*). The capacity for CD8$^+$ T cells to generate functional ligands for E- and P-selectins relies on posttranslational O-linked glycosylation of cell surface proteins such as P-selectin glycoprotein ligand 1 (PSGL-1), E-selectin ligand 1 (ESL-1), CD44, and CD43 (*16*). Specifically, core 2 O-glycans decorated with sialyl Lewis x (sLe^x) provide the ligand-binding site for the C-type lectin domains of E- and P-selectins (*17*). Enzymatic synthesis of core 2 O-glycans in CD8$^+$ T cells requires core 2 β1,6 *N*-acetylglucosaminyltransferase-I [C2GlcNAcT-I (*Gcnt1*)] to synthesize the complex core 2 O-glycans that function as E- and P-selectin ligands (*18*). Thus, expression of *Gcnt1* and the mechanisms that regulate core 2 O-glycan synthesis in memory CD8$^+$ T cells may ultimately control their tissue-trafficking potential.

Previously, we have demonstrated that core 2 O-glycan synthesis is dynamically regulated in memory CD8$^+$ T cells in an interleukin-15 (IL-15)–dependent, antigen-independent manner (*7*). Because core 2 O-glycan synthesis is critical for memory CD8$^+$ T cells to traffic into inflamed nonlymphoid tissues, we investigated whether the capacity to synthesize core 2 O-glycans was differentially regulated within the memory CD8$^+$ T cell compartment. Unexpectedly, we found that in disagreement with the T_{CM}/T_{EM} trafficking paradigm, core 2 O-glycan synthesis was highly active in T_{CM} but was less active or even absent in T_{EM} subsets. Furthermore, we show that in response to repetitive rounds of antigen-stimulated differentiation, memory CD8$^+$ T cells become KLRG1$^+$ T_{EM} that lose the capacity to synthesize core 2 O-glycans and are therefore unable to effectively generate functional ligands for E- and P-selectins, and trafficking into inflamed, nonlymphoid tissues is

[1]Department of Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, OR 97239, USA. [2]Department of Microbiology, University of Iowa, Iowa City, IA 52242, USA. [3]Department of Cell, Developmental and Cancer Biology, Oregon Health and Science University, Portland, OR 97239, USA. [4]Department of Radiation Medicine, Oregon Health and Science University, Portland, OR 97239, USA.
*These authors contributed equally to this work.
†Corresponding author. Email: nolz@ohsu.edu

diminished. Overall, these results identify core 2 O-glycan synthesis as a critical regulator of memory CD8$^+$ T cell trafficking and demonstrate that T_{CM} CD8$^+$ T cells, and not T_{EM}, are the major subset that infiltrates nonlymphoid tissues in response to inflammation.

RESULTS

Core 2 O-glycan–expressing memory CD8$^+$ T cells traffic into inflamed skin in an antigen-independent manner

It has recently been demonstrated that naïve, pathogen-free mice may not accurately model typical inflammatory responses during infections because these animals have never acquired a mature, functional memory T cell compartment (*19*). Thus, to define the extent of memory CD8$^+$ T cell trafficking that occurs in nonlymphoid tissues during a viral infection, we compared the quantity of CD8$^+$ T cells in the skin of naïve, pathogen-free B6 mice infected with vaccinia virus (VacV) versus mice that had been previously infected 60 days earlier with lymphocytic choriomeningitis virus (LCMV) (fig. S1A). Because LCMV and VacV do not share any T cell epitopes, this strategy allowed us to specifically quantify the trafficking of LCMV-specific memory CD8$^+$ T cells into an inflamed tissue. On day 3 after infection, there were 5 to 10 times more CD8$^+$ T cells in the VacV-infected skin of mice that were LCMV-immune compared with naïve controls (Fig. 1A and fig. S1B). LCMV-specific memory CD8$^+$ T cells (H2-D^b-GP33 and H2-D^b-GP276) trafficked into the VacV-infected skin and were reactive to the monoclonal antibody 1B11 (Fig. 1, B and C), which binds to the core 2 O-linked glycosylated isoform of CD43 and identifies CD8$^+$ T cells that can bind to E- and P-selectins (*7*, *20*). This demonstrates that during a local inflammatory event, core 2 O-glycan–expressing memory CD8$^+$ T cells, regardless of specificity, traffic into the skin in an antigen-independent manner.

Previous studies have demonstrated that activated CD8$^+$ T cells require *Gcnt1* to initiate synthesis of core 2 O-glycans (*17*, *18*). After acute LCMV infection, antigen-specific *Gcnt1*$^{-/-}$ CD8$^+$ T cells expanded the same as wild-type (WT) controls but did not express the 1B11 epitope, and binding to both E- and P-selectins was significantly reduced (fig. S2, A to F). Because *Gcnt1*$^{-/-}$ CD8$^+$ T cells were deficient in generating functional ligands for E- and P-selectins, we next tested whether *Gcnt1* was required for trafficking of memory CD8$^+$ T cells into the skin. LCMV-immune WT and *Gcnt1*$^{-/-}$ mice were infected with VacV, and trafficking of GP33- and GP276-specific CD8$^+$ T cells into the skin was analyzed. Similar numbers of GP33- and GP276-specific memory CD8$^+$ T cells were present in the spleens of WT and *Gcnt1*$^{-/-}$ mice, but trafficking of *Gcnt1*$^{-/-}$ memory CD8$^+$ T cells into the skin was significantly reduced compared with WT controls (Fig. 1, D and E). Thus, the capacity to synthesize core 2 O-glycans is critical for memory CD8$^+$ T cells to traffic into the skin in response to local inflammation.

To determine whether the antigen-independent trafficking of memory CD8$^+$ T cells into VacV-infected skin was critical for these cells to provide protective immunity, naïve T cell receptor–transgenic (TCR-tg) P14 CD8$^+$ T cells (specific for the LCMV epitope H2-D^b-GP33) were transferred into naïve B6 mice and infected with LCMV. At >60 days after infection, LCMV-immune mice were challenged with VacV expressing the $GP_{33–41}$ epitope of LCMV (VacV-GP33) on the skin of the left ear and VacV expressing $ovalbumin_{257–264}$ (VacV-OVA) on the right ear. GP33-specific memory P14 CD8$^+$ T cells trafficked into the skin of both VacV infections in an E- and P-selectin–dependent manner, further demonstrating that local inflammation is responsible for recruiting memory CD8$^+$ T cells into a site of infection (Fig. 1, F and G, and fig. S3). However, memory P14 CD8$^+$ T cells reduced the viral burden of the VacV-GP33 infection but provided no protective immunity against VacV-OVA (Fig. 1H). Therefore, these data demonstrate that memory CD8$^+$ T cells of diverse specificities, when present, are a major component of early inflammation after a viral skin infection, and antigen-independent trafficking is required for these cells to provide antigen-specific protective immunity.

IL-15 stimulates core 2 O-glycan synthesis in memory CD8$^+$ T cells

Our previous work revealed that memory CD8$^+$ T cells, but not naïve CD8$^+$ T cells, synthesize core 2 O-glycans and generate functional ligands for E- and P-selectins in response to stimulation with IL-15 (*7*). Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADG), which blocks the incorporation of *N*-acetylglucosamine into O-glycans, prevented IL-15–stimulated binding to recombinant E- and P-selectin proteins (Fig. 2A). Furthermore, removing sialic acids with neuraminidase also eliminated the IL-15–stimulated E- and P-selectin binding (Fig. 2B). Synthesis of mature, core 2 O-glycans bearing sLe^x requires the collective action of a number of enzymes (Fig. 2C). To determine whether a specific set of glycosyltransferase genes that facilitates O-glycan synthesis was transcriptionally regulated by IL-15, we purified memory P14 CD8$^+$ T cells, stimulated them in vitro, and analyzed the expression of candidate genes that could potentially contribute to mature O-glycan synthesis. Expression of the core 2 O-glycan initiation enzyme *Gcnt1* was significantly increased (Fig. 2D). However, we also detected expression of a number of other glycosyltransferases that have been previously implicated in contributing to the formation of E- and P-selectin ligands. IL-15 caused memory CD8$^+$ T cells to increase expression of *B3gnt3* and *B4galt5*, which could function cooperatively to extend lactosamine repeats on core 2 (and/or core 1) O-glycans (*21*, *22*). α1,3-Fucosyltransferase-7 (*Fut7*) and ST3 β-galactoside α-2,3-sialyltransferase-4 (*St3gal4*) were also found to be expressed after IL-15 stimulation. There was no change in expression of ST3 β-galactoside α-2,3-sialyltransferase 1 (*St3gal1*), the enzyme responsible for "capping" core 1 O-glycans with sialic acid and preventing core 2 O-glycan synthesis (*23*). Expression of *Fut4* and *St3gal6*, which have been shown to contribute to sLe^x formation in other leukocyte populations, were also not increased by IL-15 (fig. S4A). Collectively, these data demonstrate that IL-15 stimulates expression of glycosyltransferase enzymes in memory CD8$^+$ T cells that facilitate synthesis of sLe^x containing O-linked glycans.

Addition of the α2,3-linked sialic acid to the core 1 O-glycan substrate inhibits core 2 O-glycan synthesis by *Gcnt1*. The lectin MAL II (*Maackia amurensis* lectin II) binds directly to this sialic acid (*24*), and PNA (peanut agglutinin) binds only to unsialylated core 1 O-glycans (Fig. 2E). IL-15–stimulated memory CD8$^+$ T cells decreased MAL II binding and increased PNA binding compared with those cultured in media alone, demonstrating a loss of sialylated core 1 O-glycans (Fig. 2F). Because *St3gal1* expression was not decreased by IL-15 (Fig. 2D), this suggests that *Gcnt1* is able to "outcompete" *St3gal1* for the core 1 O-glycan substrate. IL-15 also increased expression of the 1B11 epitope (Fig. 2F), which requires *Gcnt1* activity. This analysis demonstrates that IL-15 alters the core 1 status of O-glycans and stimulates core 2 O-glycan synthesis in memory CD8$^+$ T cells.

IL-15 is a homeostatic cytokine that is important for the long-term maintenance of memory CD8$^+$ T cell populations and also functions to stimulate effector functions of T cells (*25*, *26*). To investigate whether steady-state exposure to IL-15 maintained the

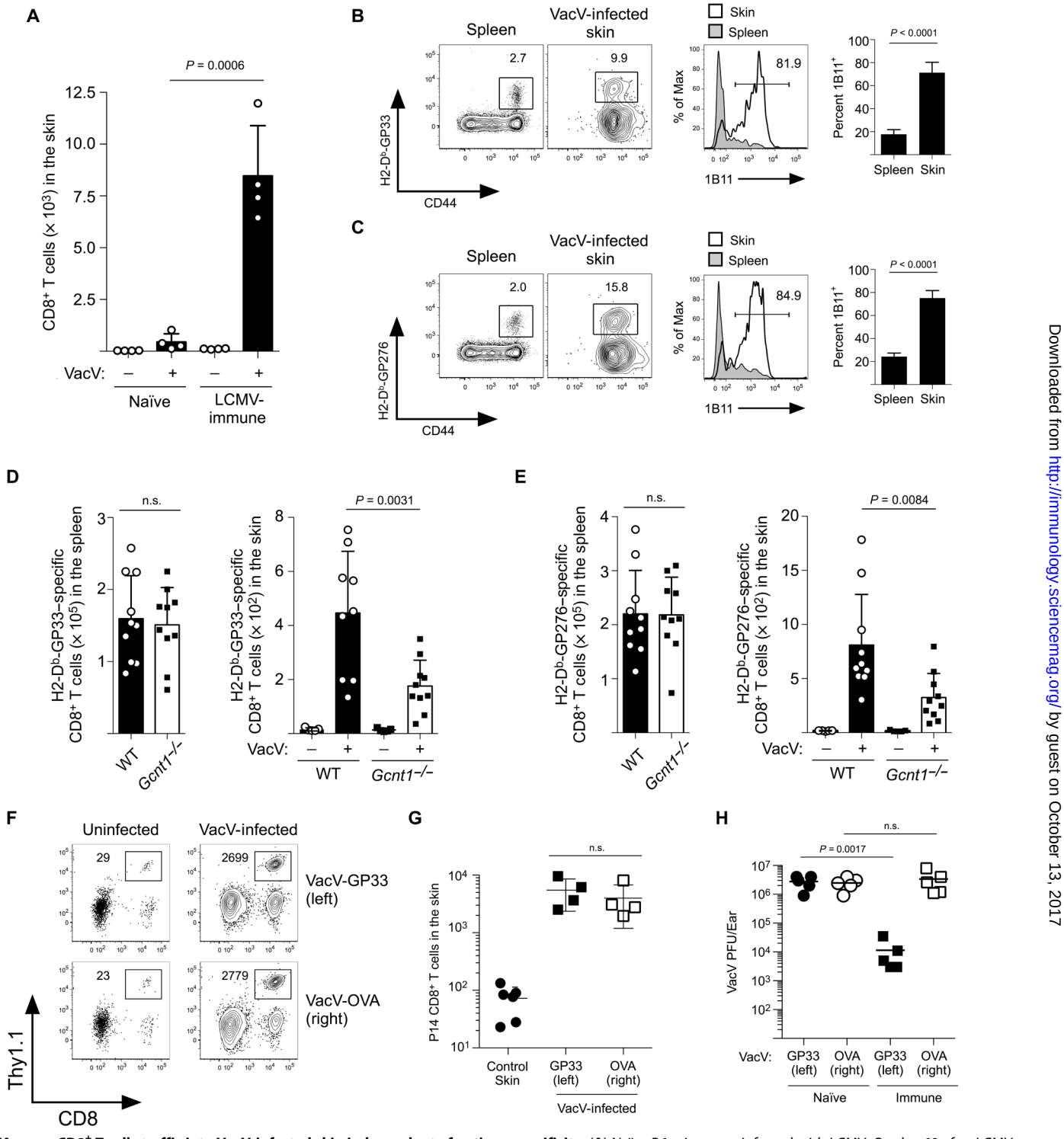

Fig. 1. Memory CD8$^+$ T cells traffic into VacV-infected skin independent of antigen specificity. (**A**) Naïve B6 mice were infected with LCMV. On day 60 after LCMV infection, LCMV-immune or naïve age-matched controls were infected with VacV on the left ear skin by scarification. On day 3 after infection with VacV, trafficking of CD8$^+$ T cells into the skin was quantified. (**B** and **C**) LCMV-specific CD8$^+$ T cells (H2-D^b-GP$_{33–41}$ and H2-D^b-GP$_{276–284}$) were identified in the VacV-infected skin and spleen. Core 2 O-glycosylated CD43 was measured with the 1B11 antibody. (**D** and **E**) WT or *Gcnt1*$^{-/-}$ B6 mice were infected with LCMV. On day 60 after LCMV infection, mice were infected with VacV on the left ear skin. Trafficking of GP33-specific (D) and GP276-specific (E) CD8$^+$ T cells was quantified on day 3 after infection. n.s., not significant. (**F**) Naïve P14 CD8$^+$ T cells were transferred into naïve B6 mice and infected with LCMV-Armstrong. On day 75 after infection, LCMV-immune mice were challenged with VacV-GP33 on the left ear skin and VacV-OVA on the right ear skin. Trafficking of P14 CD8$^+$ T cells into the skin was quantified on day 3 after VacV infection. (**G**) Quantification of (F). (**H**) Same as (F), except VacV was quantified from naïve or LCMV-immune mice on day 4 after infection of the skin.

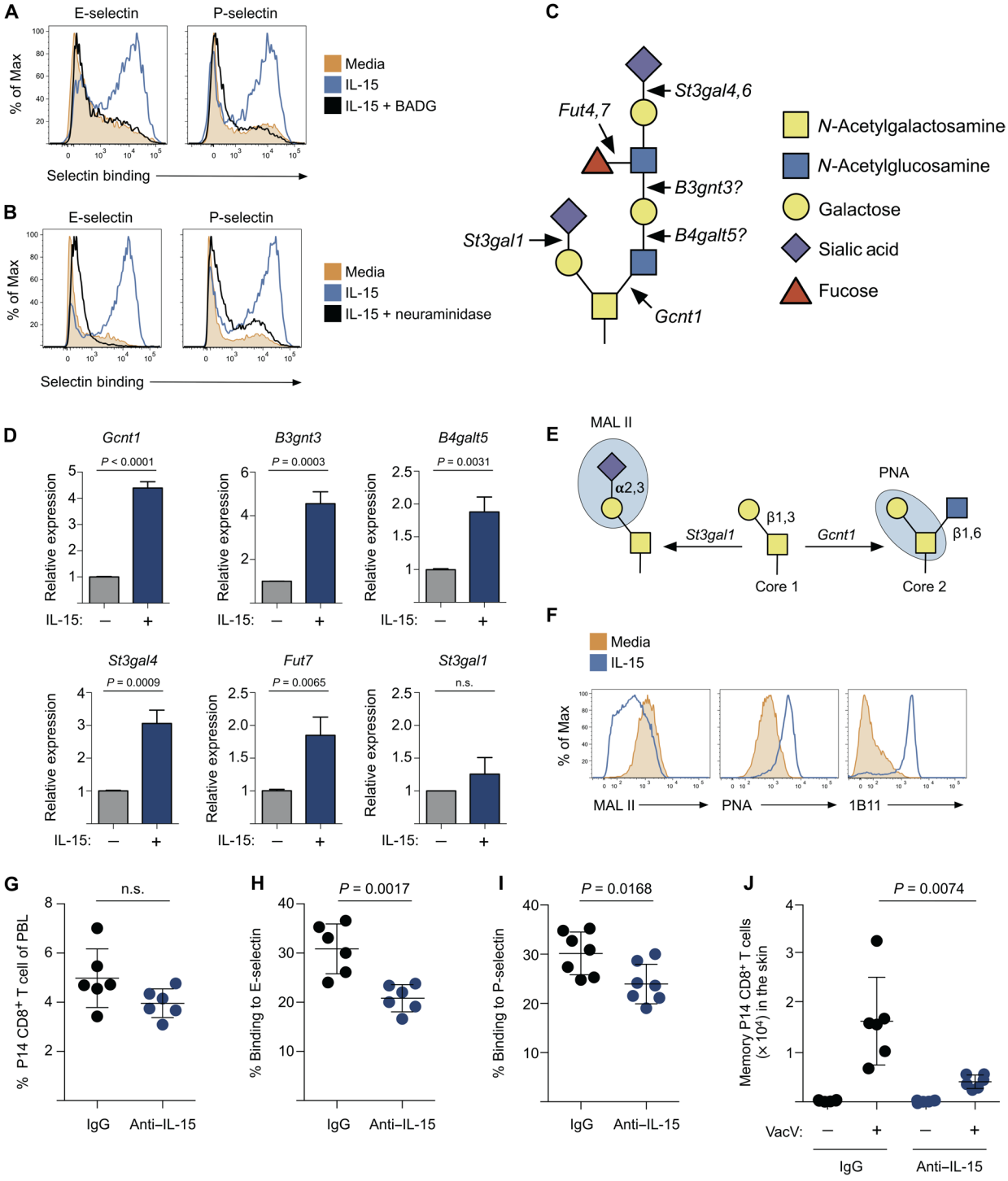

Fig. 2. Core 2 O-glycan synthesis is stimulated by IL-15 signaling. (**A**) Memory P14 CD8$^+$ T cells were purified and stimulated with IL-15 (250 ng/ml) or IL-15 + 5 mM BADG for 3 days. Binding of E- and P-selectin chimeric proteins was quantified. (**B**) Same as (A), except after IL-15 stimulation, memory P14 CD8$^+$ T cells were treated with neuraminidase before analyzing E- and P-selectin binding. (**C**) The synthesis of complex core 2 O-glycans requires the collective action of a number of glycosyltransferases. (**D**) Memory P14 CD8$^+$ T cells were purified and stimulated in vitro with IL-15 (500 ng/ml) for 3 days. Changes in gene expression of *Gcnt1*, *B3gnt3*, *B4galt5*, *St3gal4*, *Fut7*, and *St3gal1* were determined by quantitative polymerase chain reaction. (**E**) *St3gal1* caps the core 1 O-glycan substrate with α2,3-linked sialic acid, which prevents core 2 O-glycan synthesis (by *Gcnt1*) and can be detected with the lectin MAL II. PNA binds to core 1 O-glycans only if they do not contain an α2,3-linked sialic acid. (**F**) Memory P14 CD8$^+$ T cells were stimulated as in (A), and binding of MAL II, PNA, or the 1B11 antibody was analyzed. (**G**) LCMV-immune mice (90 days after infection) were given control IgG or IL-15–neutralizing antibody for 10 days. PBL, peripheral blood lymphocyte. The frequency, E-selectin binding (**H**), and P-selectin binding (**I**) of the memory P14 CD8$^+$ T cell population in the circulation were quantified. (**J**) Mice from (G) to (I) were infected with VacV, and memory P14 CD8$^+$ T cells were quantified in the skin on day 3 after VacV infection.

synthesis of selectin ligands on memory $CD8^+$ T cells, we used an IL-15–neutralizing antibody (*27*). Neutralizing IL-15 caused circulating memory $CD8^+$ T cells to reduce binding to E- and P-selectins but did not affect the circulating frequency (Fig. 2, G to I). When IL-15 was neutralized, trafficking of memory $CD8^+$ T cells into VacV-infected skin was reduced compared with control-treated animals (Fig. 2J). VacV infection caused memory $CD8^+$ T cells in the circulation to increase binding to E- and P-selectins, which was also dependent on IL-15 (fig. S4, B to D). Collectively, these data demonstrate that IL-15 is a critical regulator of core 2 O-glycan synthesis during both homeostasis and after infection and that interfering with this signaling axis alters the trafficking potential of memory $CD8^+$ T cells.

Core 2 O-glycan synthesis is active primarily in T_{CM} $CD8^+$ T cells

It has been demonstrated using both functional readouts and gene expression profiles coupled with principal components analysis that memory $CD8^+$ T cells lie within a differentiation continuum. This includes T_{CM} cells, cells of intermediate differentiation, and, last, terminally differentiated T_{EM} cells (*28*, *29*). Using the differentiation markers CD62L and KLRG1, these three types of memory $CD8^+$ T cell populations can be readily identified after an acute LCMV infection (Fig. 3A). Because our studies revealed a critical role for core 2 O-glycan synthesis in controlling the trafficking of memory $CD8^+$ T cells into inflamed tissues, we next investigated whether core 2 O-glycan synthesis was restricted to specific subsets. The T_{CM} $CD8^+$ T cell subset ($CD62L^+/KLRG1^-$) expressed more core 2 O-glycans compared with T_{EM} ($CD62L^-$) populations (Fig. 3, A and B). Memory $CD8^+$ T cells that did not express CD62L (i.e., T_{EM}) could be further classified based on the expression of KLRG1. Essentially no $KLRG1^+$ T_{EM} $CD8^+$ T cells expressed core 2 O-glycans (Fig. 3B). Expression of the IL-2/15Rβ (CD122) within each of the subsets correlated with the percentage of memory $CD8^+$ T cells that expressed core 2 O-glycans (Fig. 3C), suggesting that sensitivity to IL-15 is an important regulator of core 2 O-glycan synthesis within the memory $CD8^+$ T cell compartment. T_{CM} $CD8^+$ T cells from the circulation could bind to both E- and P-selectins, whereas most T_{EM} do not (Fig. 3, D and E).

To determine whether core 2 O-glycan synthesis was fixed or could be stimulated on either T_{CM} or T_{EM}, we sorted memory $CD8^+$ T cells that were not expressing core 2 O-glycans ($1B11^-$) from each of the three subsets and cultured them in vitro with IL-15. T_{CM} strongly increased core 2 O-glycan synthesis as measured by a de novo increase in 1B11 reactivity, but most $KLRG1^+$ T_{EM} $CD8^+$ T cells could not (Fig. 3F). Consistent with a previous finding (*30*), stimulation of T_{CM} with IL-15 did not alter expression of CD62L or KLRG1 but did reduce expression of CCR7 (fig. S5A). Last, T_{CM} isolated from lymph nodes also synthesized core 2 O-glycans and bound to E- and P-selectins after stimulation with IL-15 (fig. S5B). Therefore, these data demonstrate that core 2 O-glycan synthesis is largely restricted to the T_{CM} subset and can be stimulated by IL-15.

It has generally been accepted that T_{EM} are the memory $CD8^+$ T cells that are recruited into nonlymphoid tissues in response to inflammation. However, because we found that expression of core 2 O-glycans was primarily a feature of T_{CM} $CD8^+$ T cells and not T_{EM}, we next tested whether these subsets would traffic differently into nonlymphoid tissue after a sterile inflammatory challenge. Each population (T_{CM}, $KLRG1^-$ T_{EM}, and $KLRG1^+$ T_{EM}) was sorted, transferred into naïve B6 mice (Fig. 3G), and then challenged intranasally with the Toll-like receptor 9 (TLR9) agonist CpG. T_{CM} $CD8^+$ T cells trafficked into the inflamed lung mucosa better than either of the T_{EM} subsets ($KLRG1^{+/-}$) (Fig. 3, G and H). Furthermore, T_{CM} that trafficked into the lung expressed core 2 O-glycans and bound to both P- and E-selectins better than either of the T_{EM} subsets (Fig. 3, I and J). Collectively, these data demonstrate that within the memory $CD8^+$ T cell compartment, core 2 O-glycans expressing T_{CM} are the major subset that infiltrates nonlymphoid tissue in response to inflammatory challenge.

T_{CM} $CD8^+$ T cells have more core 2 O-glycan synthesis activity than terminally differentiated T_{EM} cells

The proportions of T_{CM} and T_{EM} $CD8^+$ T cells that form after infection or vaccination can be influenced by many variables (*31*). For example, high levels of inflammatory cytokines [e.g., IL-12 and interferon-α/β (IFN-α/β)] and multiple rounds of antigen encounter are known to cause memory $CD8^+$ T cells to differentiate largely into $KLRG1^+$ T_{EM}. Using a previously described method of generating primary or tertiary memory $CD8^+$ T cells with a single or three consecutive LCMV infections (fig. S6A) (*10*, *32*), we generated memory P14 $CD8^+$ T cell populations that became predominantly T_{CM} (primary) or $KLRG1^+$ T_{EM} (tertiary) (Fig. 4A and fig. S6, B to D). In agreement with Fig. 3 (D and E), terminally differentiated tertiary T_{EM} $CD8^+$ T cells exhibited limited capacity to bind to E- and P-selectins compared with primary memory $CD8^+$ T cells (Fig. 4, B to D) but expressed similar levels of proteins that function as selectin ligands (e.g., PSGL-1, CD44, and CD43) (fig. S6E), suggesting that the difference in the generation of E- and P-selectin ligands occurs at the level of posttranslational glycosylation. Tertiary memory $CD8^+$ T cells expressed less CD122 and the common γ chain, CD132 (Fig. 4, E to G). Jacalin, a lectin that binds core 1 O-glycans regardless of sialylation status, bound to both primary and tertiary memory $CD8^+$ T cells, demonstrating that similar levels of total core 1 O-glycans were present. In contrast, PNA reacted more strongly on primary memory $CD8^+$ T cells, consistent with our finding that sensitivity to IL-15 can alter the sialylation state of the core 1 O-glycan substrate (Fig. 4, H to J).

Stimulation through the IL-15 receptor activates a canonical Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) signaling pathway, resulting in the activation of the STAT5 transcription factor by tyrosine phosphorylation. Primary memory $CD8^+$ T cells activated STAT5 better than tertiary memory $CD8^+$ T cells when stimulated with IL-15 (Fig. 4K). Furthermore, as we had shown previously (Fig. 2F), IL-15 caused primary memory $CD8^+$ T cells to decrease MAL II and increase PNA and 1B11 reactivity but had no effect on the O-linked glycosylation status of tertiary memory $CD8^+$ T cells (Fig. 4L). PSGL-1 functions as both a P- and E-selectin ligand, and stimulation of primary memory $CD8^+$ T cells with IL-15 caused PSGL-1 to become glycosylated, indicated by slower migration of the glycosylated version of PSGL-1 after separation by SDS–polyacrylamide gel electrophoresis (*33*). PSGL-1 was mostly in a nonglycosylated form in tertiary memory $CD8^+$ T cells, and stimulation with IL-15 did not change its glycosylation state (Fig. 4M). Last, when stimulated with IL-15, primary memory $CD8^+$ T cells significantly increased binding to both E- and P-selectins, but tertiary memory $CD8^+$ T cells did not (Fig. 4, N to Q). Collectively, these findings demonstrate that core 2 O-glycan synthesis is highly active in T_{CM} $CD8^+$ T cells that form after a primary infection but not in terminally differentiated $KLRG1^+$ T_{EM} $CD8^+$ T cells.

Primary T_{CM} $CD8^+$ T cells traffic into inflamed, nonlymphoid tissues better than terminally differentiated tertiary $KLRG1^+$ T_{EM} cells

Our analysis of IL-15–stimulated core 2 O-glycan synthesis of memory $CD8^+$ T cell subsets suggested that tertiary memory $CD8^+$ T cells

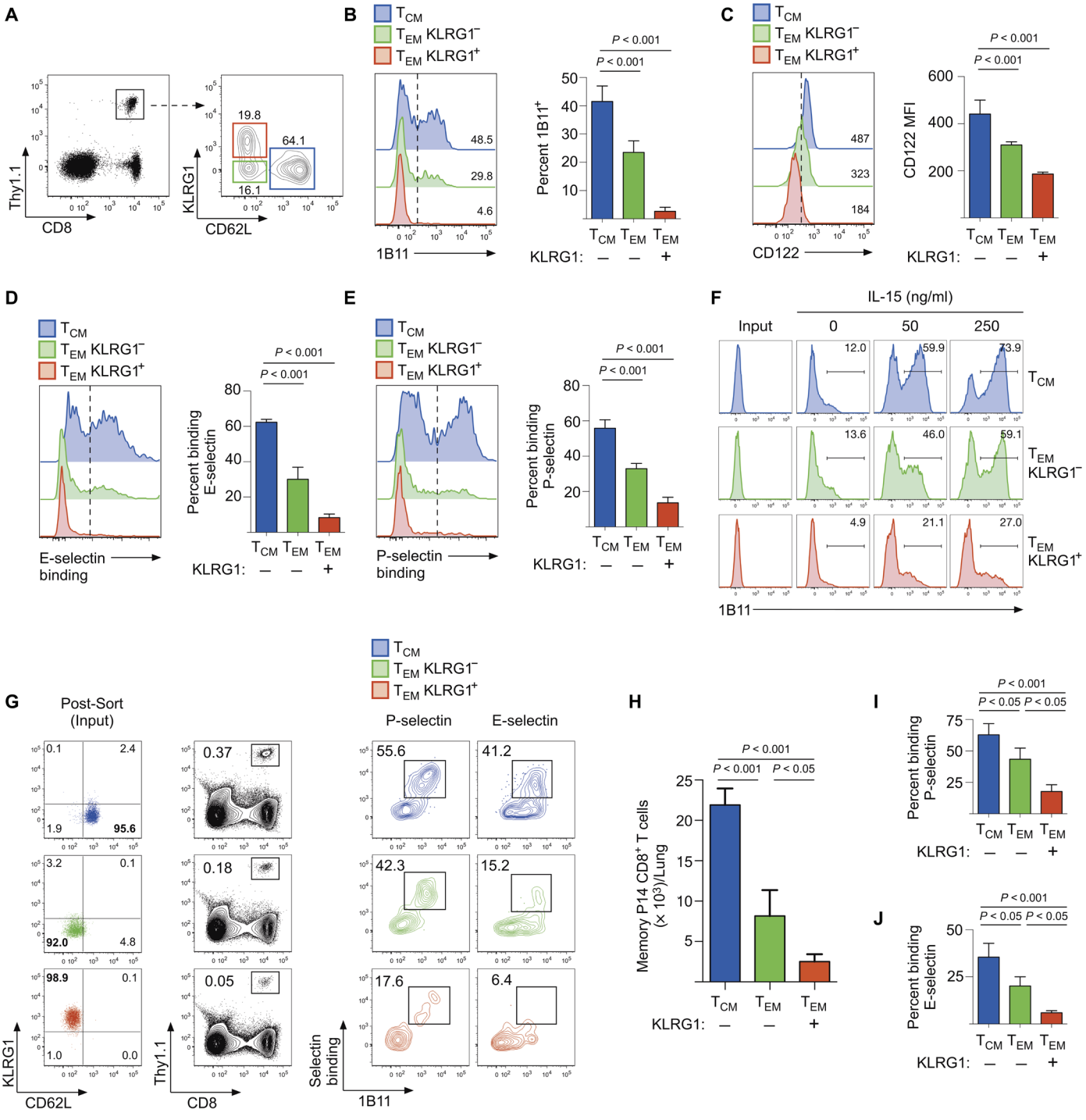

Fig. 3. Core 2 O-glycan synthesis in memory CD8$^+$ T cell subsets. (**A**) Memory P14 CD8$^+$ T cells were analyzed 75 days after LCMV infection for expression of CD62L and KLRG1. (**B** to **E**) Memory P14 CD8$^+$ T cell subsets from (A) were analyzed for expression of core 2 O-glycans (1B11) (B), CD122 (C), E-selectin binding (D), and P-selectin binding (E). MFI, mean fluorescence intensity. (**F**) 1B11$^-$ cells from each subset as shown in (A) were sorted by fluorescence-activated cell sorting and cultured in vitro for 3 days in media alone or supplemented with IL-15 (50 or 250 ng/ml). (**G**) Memory CD8$^+$ T cell subsets were sorted, and 5×10^5 cells were transferred into B6 mice and challenged with CpG intranasally. Recruitment into the challenged lung and binding of E- and P-selectins were analyzed at 60 hours after challenge. (**H** to **J**) Quantification of data shown in (G).

generated through multiple antigen-specific infections would be impaired in trafficking, similar to the small percentage of KLRG1$^+$ T_{EM} that can be detected after a single, acute LCMV infection (Fig. 3A). To directly determine in a competitive manner whether primary or tertiary memory CD8$^+$ T cells trafficked better to the lung mucosa during an inflammatory event, we transferred an equal number of primary (Thy1.1/1.2) and tertiary (Thy1.1/1.1) memory P14 CD8$^+$ T cells into naïve B6 mice (Thy1.2/1.2), which were then challenged

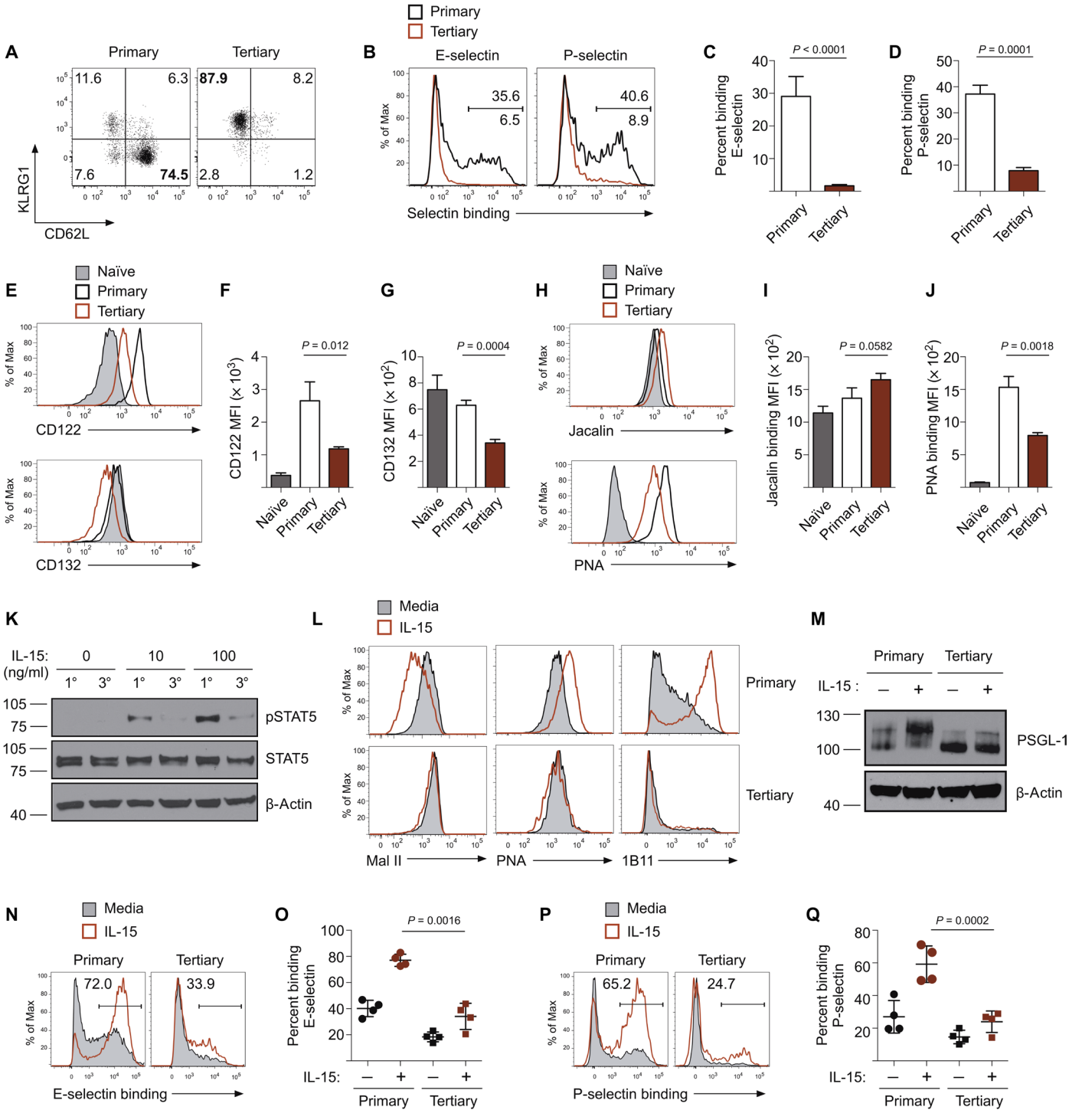

Fig. 4. Memory CD8$^+$ T cells that become terminally differentiated by multiple antigen encounters lose core 2 O-glycan synthesis activity. (**A**) Expression of CD62L and KLRG1 on primary and tertiary memory P14 CD8$^+$ T cells 90 days after LCMV infection and (**B**) binding to E- and P-selectins. (**C** and **D**) Quantification of (B). (**E**) Expression of CD122 and CD132. (**F** and **G**) Quantification of (E). (**H**) Expression of total core 1 O-glycans (Jacalin) and unsialylated core 1 O-glycans (PNA). (**I** and **J**) Quantification of (H). (**K**) Primary and tertiary memory P14 CD8$^+$ T cells were purified and stimulated with the indicated concentration of IL-15 for 15 min, and phosphorylation of STAT5 (Y694) was analyzed by immunoblot. (**L**) Primary and tertiary memory CD8$^+$ T cells were purified and cultured with IL-15 for 3 days. Binding of MAL II, PNA, and the 1B11 antibody was analyzed. (**M**) Same as (L), except the glycosylation of PSGL-1 was analyzed by immunoblot. (**N**) Same as (L), except binding to E-selectin was analyzed by flow cytometry. (**O**) Quantification of (N) from four independent experiments. (**P** and **Q**) Same as (N) and (O), except for P-selectin.

intranasally with CpG (Fig. 5A). Primary memory CD8$^+$ T cells trafficked to the inflamed lung mucosa better than tertiary memory CD8$^+$ T cells (Fig. 5, B to D). In addition, more primary memory CD8$^+$ T cells that trafficked to the CpG-treated lungs expressed core 2 O-glycans and bound to both E- and P-selectins than tertiary memory CD8$^+$ T cells (Fig. 5, E to H). These data agree with Fig. 3 (G to J), demonstrating that T_{CM} CD8$^+$ T cells that are formed after a primary infection are recruited to the inflamed lung mucosa better than tertiary KLRG1$^+$ T_{EM} cells.

Trafficking of memory CD8$^+$ T cells into VacV-infected skin is also dependent on core 2 O-glycan synthesis and E- and P-selectins (*7*). To analyze memory CD8$^+$ T cell trafficking during this infection without antigen influence, we infected mice containing equal frequencies of either primary or tertiary memory P14 CD8$^+$ T cells (Fig. 6A) on the ear skin with VacV-OVA. Primary memory GP33-specific P14 CD8$^+$ T cells rapidly trafficked into the VacV-OVA–infected skin, but significantly fewer tertiary memory CD8$^+$ T cells reached the site of infection

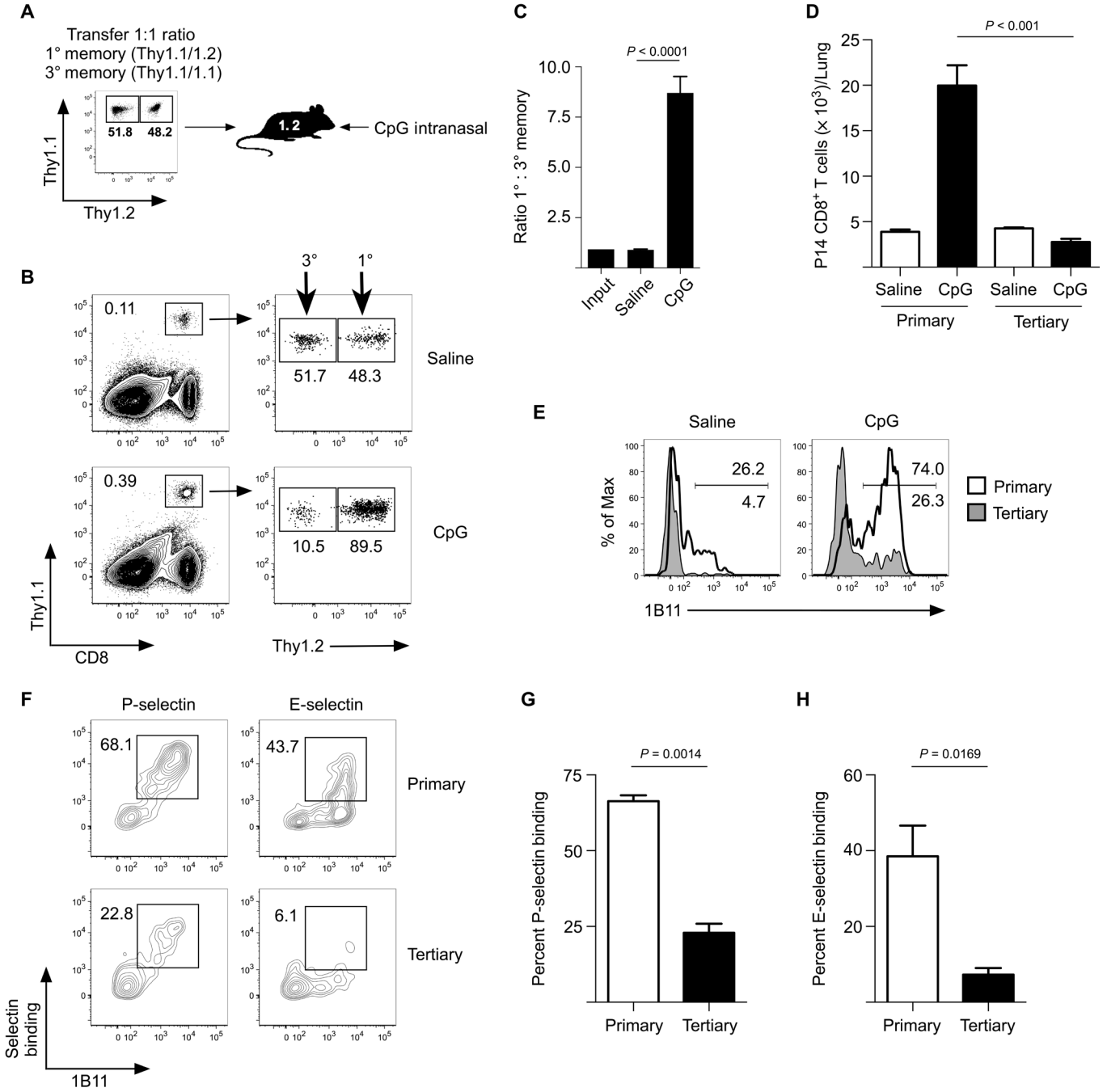

Fig. 5. Primary memory CD8$^+$ T cells traffic into the inflamed lung mucosa better than tertiary memory. (**A**) Primary (Thy1.1/1.2) and tertiary (Thy1.1/1.1) memory P14 CD8$^+$ T cells were purified, and equal numbers of both were transferred into naïve B6 (Thy1.2/1.2) mice. (**B**) Mice from (A) were challenged with saline or CpG, and recruitment to the lung was analyzed 60 hours after challenge. (**C** and **D**) Quantification of (B). (**E**) Expression of core 2 O-glycans (1B11) from (B). (**F**) Cells from (B) were analyzed for binding to E- and P-selectins. (**G** and **H**) Quantification of (F).

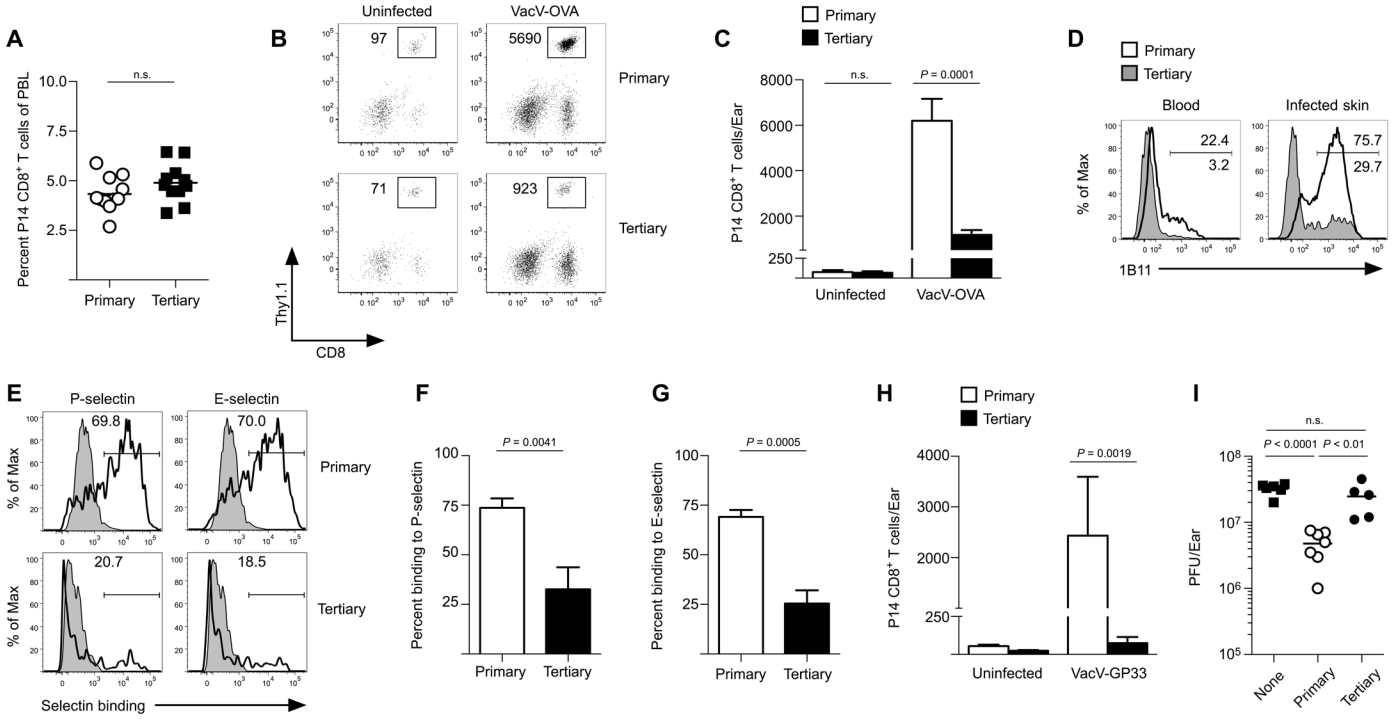

Fig. 6. Primary memory CD8$^+$ T cells traffic into VacV-infected skin and provide protective immunity against viral infection. (**A**) Frequencies of primary and tertiary memory P14 CD8$^+$ T cells in individual B6 mice. (**B**) Mice containing equal frequencies of either primary or tertiary memory CD8$^+$ T cells were infected with VacV-OVA on the left ear skin, and recruitment of memory GP33-specific P14 CD8$^+$ T cells was analyzed on day 3 after infection. (**C**) Quantification of (B). (**D**) Expression of core 2 O-glycans (1B11) on primary and tertiary memory P14 CD8$^+$ T cells in the blood and VacV-infected skin. (**E**) Binding to E- and P-selectins on P14 CD8$^+$ T cells isolated from the skin. (**F** and **G**) Quantification of (E). (**H**) Primary and tertiary memory P14 CD8$^+$ T cells were purified from spleens, 2×10^6 cells were transferred into naïve B6 mice, and the left ear skin was infected with VacV-GP33. Recruitment of memory P14 CD8$^+$ T cells into the infected and control skin was analyzed on day 3 after infection. (**I**) Same as (H), except viral load was measured by standard plaque assay on day 4 after infection.

(Fig. 6, B and C). Expression of core 2 O-glycans was highly enriched on primary memory CD8$^+$ T cells that trafficked into the VacV-OVA–infected skin compared with the same antigen-specific cells in the circulation (Fig. 6D). Fewer tertiary memory CD8$^+$ T cells expressed core 2 O-glycans in the circulation and in the infected ear skin (Fig. 6D), resulting in less binding to P- and E-selectins (Fig. 6, E to G). Next, to determine whether differential trafficking affected protective immunity, we purified primary and tertiary memory P14 CD8$^+$ T cells and transferred them into naïve B6 mice, which were then infected on the skin with VacV-GP33. Primary memory CD8$^+$ T cells rapidly trafficked into the skin and provided protection against the infection (Fig. 6, H and I). In contrast, limited numbers of tertiary memory P14 CD8$^+$ T cells trafficked into the infected skin and were unable to provide significant protective immunity (Fig. 6, H and I). Therefore, these data demonstrate that the capacity to synthesize core 2 O-glycans that function as P- and E-selectin ligands is essential for memory CD8$^+$ T cells to leave the circulation and traffic into skin to provide protective immunity.

Last, we tested whether these findings could be generalized to another form of skin inflammation. Sensitization and subsequent local challenge with the chemical hapten dinitrofluorobenzene (DNFB) causes contact hypersensitivity, a type of inflammation mediated by activated T cells. More memory CD8$^+$ T cells are recruited to DNFB-challenged skin in sensitized mice compared with mice that were not sensitized (fig. S7, A and B). We initiated contact hypersensitivity on LCMV-immune mice containing equal frequencies of circulating primary or tertiary memory P14 CD8$^+$ T cells. Primary memory CD8$^+$ T cells trafficked to the DNFB-challenged skin better than tertiary memory CD8$^+$ T cells and expressed more core 2 O-glycans (fig. S7, C to F). Thus, in three different models of inflammation, primary memory CD8$^+$ T cells traffic better than tertiary memory cells to inflamed nonlymphoid tissue.

Core 2 O-glycan synthesis is active in conventional but not inflationary MCMV-specific memory CD8$^+$ T cells

Murine cytomegalovirus (MCMV) is a β-herpes virus that, after a robust acute infection, establishes lifelong latency in the host. Because our studies using acute LCMV infections demonstrated that T_{CM} CD8$^+$ T cells actively synthesize core 2 O-glycans, whereas KLRG1$^+$ T_{EM} CD8$^+$ T cells did not, we next assessed core 2 O-glycan synthesis and tissue trafficking of endogenous MCMV-specific memory CD8$^+$ T cells. In contrast to most acute infections, two major types of antigen-specific CD8$^+$ T cell responses occur in mice infected with MCMV (*34*). "Conventional" CD8$^+$ T cells (M57- and M45-specific) undergo typical expansion and contraction, similar to what is found after most acute infections, and give rise to a memory population that is primarily T_{CM} and KLRG1$^-$ T_{EM} (Fig. 7, A to C). In contrast, a separate group of antigen-specific CD8$^+$ T cells (M38- and m139-specific) become "inflationary" and continue to expand after MCMV has entered the latent stage of the infection (Fig. 7A) and become predominantly

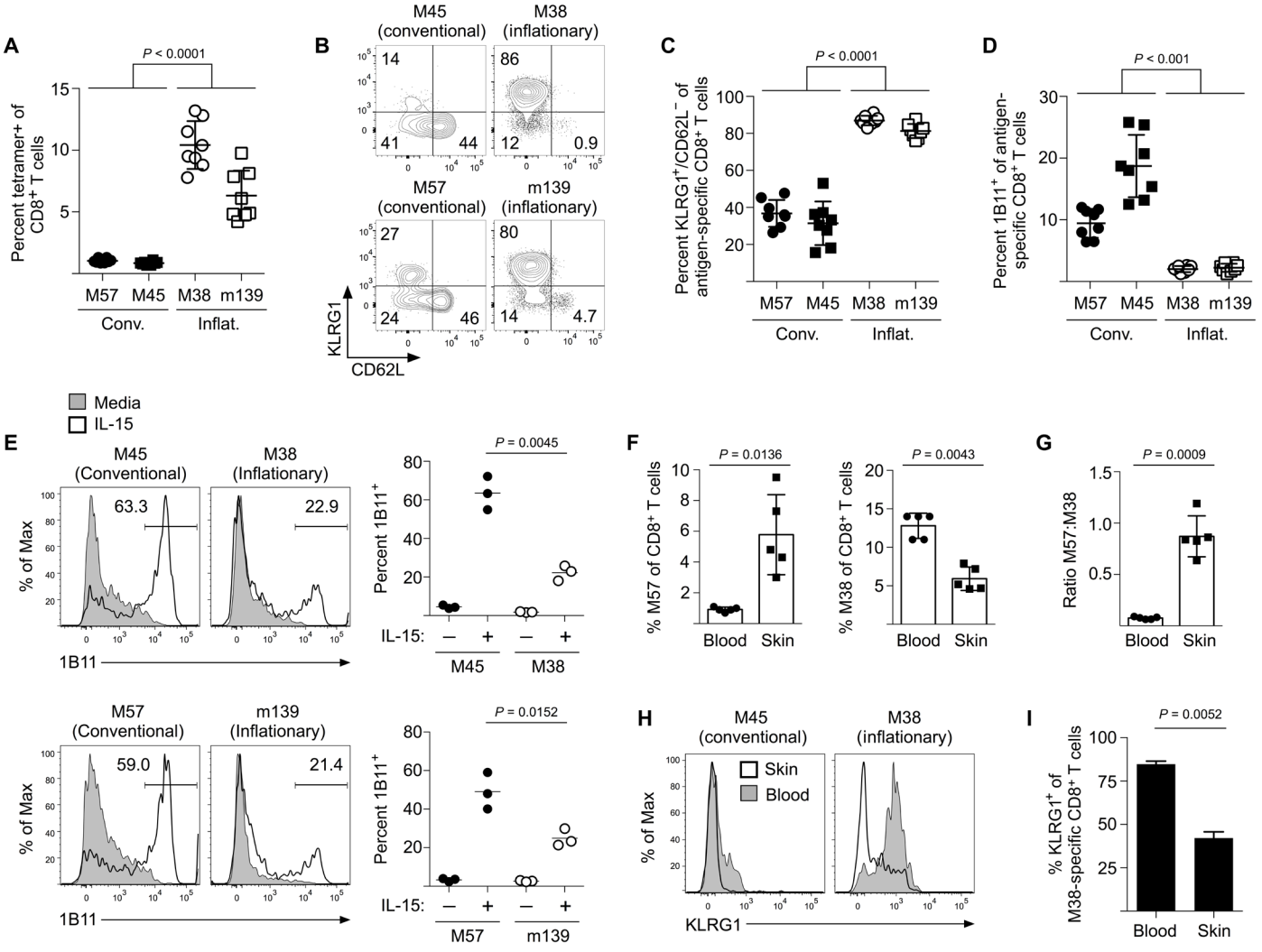

Fig. 7. Inflationary KLRG1$^+$ T_{EM} MCMV-specific CD8$^+$ T cells lose core 2 O-glycan synthesis. (**A**) Naïve B6 mice were infected with MCMV. On day 120 after infection, the frequency of MCMV-specific CD8$^+$ T cells was quantified by MCMV-specific major histocompatibility complex class I tetramers. (**B** and **C**) Expression of CD62L and KLRG1 on MCMV-specific CD8$^+$ T cells from (A). (**D**) Core 2 O-glycan expression (1B11) from (A). (**E**) CD8$^+$ T cells of MCMV-infected mice were purified and stimulated in vitro with IL-15 (250 ng/ml) for 3 days. Expression of core 2 O-glycans (1B11) was analyzed. (**F**) MCMV-infected mice were infected with VacV on the left ear skin. Frequency of M57- and M38-specific CD8$^+$ T in the blood and skin on day 5 after VacV infection was quantified. (**G**) Quantification of trafficking efficiency from (F). (**H**) Same as (F). Expression of KLRG1 on conventional (M45-specific) and inflationary (M38-specific) CD8$^+$ T cells from the blood and skin after VacV infection. (**I**) Quantification of (H).

KLRG1$^+$ T_{EM} (Fig. 7, B and C) (*35*). A larger percentage of conventional memory CD8$^+$ T cells in the circulation expressed core 2 O-glycans compared with the inflationary CD8$^+$ T cells (Fig. 7D). Furthermore, IL-15 stimulation caused the conventional memory CD8$^+$ T cells to synthesize core 2 O-glycans but not the inflationary MCMV-specific CD8$^+$ T cells (Fig. 7E). These data are consistent with our findings using LCMV infections, demonstrating that terminally differentiated KLRG1$^+$ T_{EM} CD8$^+$ T cells lose the capacity to synthesize core 2 O-glycans.

To test whether there were differences in the trafficking of these two types of memory CD8$^+$ T cells, we challenged mice latently infected with MCMV with VacV on the skin. The frequency of conventional, M57-specific memory CD8$^+$ T cell frequency in the skin increased compared with the circulation, whereas the frequency of inflationary M38-specific CD8$^+$ T cells in the skin was lower than in the blood (Fig. 7F). Because M57- and M38-specific CD8$^+$ T cells were analyzed in the same animal, we could quantify the relative frequencies of these two antigen-specific cell types in the circulation compared with those that trafficked into the skin. This revealed that M57-specific CD8$^+$ T cells infiltrated the VacV-infected skin nearly 10 times better than inflationary M38-specific CD8$^+$ T cells (Fig. 7G). Most of the inflationary M38-specific CD8$^+$ T cells that trafficked into the VacV-infected skin were actually KLRG1$^-$, although most of these antigen-specific cells in the circulation were KLRG1$^+$ (Fig. 7, H and I). Collectively, these data demonstrate that inflationary MCMV-specific CD8$^+$ T cells that become KLRG1$^+$ T_{EM} exhibit limited core 2 O-glycan synthesis activity, thereby excluding most of them from entering nonlymphoid tissues during a local inflammatory event.

DISCUSSION

For nearly two decades, the T_{CM} and T_{EM} cell paradigm has been the foundation for describing memory T cell trafficking potentials, although studies directly testing the trafficking of defined memory T cell subsets have not been rigorously performed. To further complicate matters, the recent identification of tissue-resident memory $CD8^+$ T cells (*36*) presents the possibility that previous, classic studies that identified T_{EM} $CD8^+$ T cells in nonlymphoid tissues (*9*, *37*) could have actually been observing this newly described subset, not T_{EM} actively patrolling nonlymphoid tissues. It has recently been reported that circulating T cells exhibiting a T_{CM} phenotype can be identified in the human skin (*38*), demonstrating that T_{CM} are not confined to the circulation and lymph nodes but likely traffic into nonlymphoid tissues. A recent study by Gerlach *et al.* (*39*) found that the T_{CM}/T_{EM} classification also does not accurately predict the homeostatic trafficking patterns of memory $CD8^+$ T cells. The authors found that $CX3CR1^{Hi}$/$CD62L^{Lo}$/$CD27^{Lo}$ T_{EM} $CD8^+$ T cells were not present in the lymph collected from the thoracic duct, suggesting that these cells were confined to the circulation. Here, by using models of infection and inflammation, we show the importance of core 2 O-glycan synthesis activity in regulating the tissue trafficking of memory $CD8^+$ T cells, which we found is predominantly a feature of the T_{CM} subset.

We have recently shown that core 2 O-glycan synthesis is highly dynamic and occurs independent of antigen restimulation in memory $CD8^+$ T cells (*7*). The capacity for memory, but not naïve, $CD8^+$ T cells to synthesize core 2 O-glycans was attributed to epigenetic changes of *Gcnt1*. Essentially, all memory $CD8^+$ T cells are also reactive to the lectin PNA (*40*), which identifies unsialylated core 1 O-glycans. Because sialylation of core 1 O-glycans prevents core 2 O-glycan synthesis, expression or function of *St3gal1* may also be actively suppressed in long-lived memory cells. These findings support the model that after activation, antigen-experienced memory $CD8^+$ T cells "unlock" core 2 O-glycan synthesis through epigenetic mechanisms, which is then ultimately controlled by external inflammatory factors. Although our experiments indicate a role for IL-15 in both the maintenance and the induction of core 2 O-glycans, other activators of STAT5 (e.g., IL-2, IL-7, and IL-21) could certainly stimulate core 2 O-glycan synthesis during some infections or pathological conditions. IL-15 controls not only the homeostatic proliferation of T_{CM} but also the persistence of T_{EM} $CD8^+$ T cells (*10*, *41*). Thus, we cannot conclusively rule out that there may be reduced survival of $KLRG1^+$ T_{EM} compared with T_{CM} after they traffic into nonlymphoid tissues. However, IL-15–driven homeostatic proliferation could be one of the mechanisms that maintain core 2 O-glycans on T_{CM} $CD8^+$ T cells in the circulation, and potentially, these are the memory T cells that infiltrate nonlymphoid tissues in response to local inflammation.

In summary, our studies here show that core 2 O-glycan synthesis is highly active in T_{CM} but diminishes as memory $CD8^+$ T cells become terminally differentiated, which essentially excludes them from entering nonlymphoid tissues in response to inflammation. The term "tertiary memory" in this study refers to antigen-specific memory $CD8^+$ T cells that have been activated using three consecutive LCMV infections, generating a nearly homogeneous $KLRG1^+$ T_{EM} population of memory T cells. As mentioned previously, a number of factors influence the proportions of T_{CM} and T_{EM} $CD8^+$ T cells that form after infection or vaccination. Although most studies have found that repeated, antigen-specific challenges give rise to predominantly $KLRG1^+$ T_{EM} $CD8^+$ T cells (*12*, *32*, *42*), there are certainly exceptions, and in some instances, they can generate memory $CD8^+$ T cells with a lower proportion of $KLRG1^+$ T_{EM} compared with a primary infection (*43*). Nevertheless, our findings demonstrate that memory $CD8^+$ T cell differentiation into T_{CM} and T_{EM} ultimately dictates their trafficking potential, and thus, understanding the mechanisms that shape the composition of memory T cell populations remains critical for improving host defense and vaccine design against specific pathogens.

MATERIALS AND METHODS

Study design

The goal of this study was to identify which memory $CD8^+$ T cells trafficked into nonlymphoid tissues in response to local inflammation. We used adoptive transfers of TCR-tg T cells and LCMV infections to generate diverse populations of memory $CD8^+$ T cells in mice. Phenotyping and ex vivo cytokine stimulation assays were used to investigate the mechanisms regulating core 2 O-glycan synthesis and to identify which T cell subsets could actively synthesize core 2 O-glycans. To quantify trafficking of memory $CD8^+$ T cells in vivo, LCMV-immune mice were (i) infected on the skin with VacV, (ii) challenged intranasally with the TLR9 agonist CpG, or (iii) subjected to contact hypersensitivity with DNFB. All experiments were performed two or more times.

Mice and viral infections

C57BL/6J mice (6 to 10 weeks old) were purchased from the Jackson Laboratory. *Gcnt1*$^{-/-}$ mice (*44*) and P14 TCR-tg mice (*45*) have been previously described. For adoptive transfers, 1.0×10^4 to 2.5×10^4 naïve $Thy1.1^+$ P14 $CD8^+$ T cells from either blood or spleen were injected intravenously in 200 μl of phosphate-buffered saline (PBS). LCMV-Armstrong was injected intraperitoneally [2×10^5 plaque-forming units (PFU)]. VacV-GP33 and VacV-OVA have been previously described (*46*, *47*). Infections with VacV were performed on anesthetized mice by placing 5×10^6 PFU of virus in 10 μl of PBS on the ventral side of the ear pinna and then poking the virus-coated skin 25 times with a 27-gauge needle. MCMV strain MW97.01 was injected intraperitoneally (2×10^5 PFU). All animal experiments and infectious agents were approved by the Oregon Health and Science University Institutional Animal Care and Use Committee and Institutional Biosafety Committee.

Inflammatory challenges and IL-15 neutralization

Intranasal challenge with CpG was performed by applying 50 nmol of CpG ODN 1826 (Integrated DNA Technology) in saline onto the nares of anesthetized mice (*7*). To induce contact hypersensitivity, we sensitized the shaved abdomens of LCMV-immune mice with 0.5% DNFB (Sigma) in 4:1 acetone/olive oil. On day 5 after sensitization, the ear skin was challenged with 20 μl of 0.1% DNFB in acetone/olive oil. IL-15–neutralizing antibody (clone M96) was provided by Amgen. To neutralize IL-15, 200 μg of anti–IL-15 antibody or control rat immunoglobulin G (IgG) (Sigma) was administered every other day for 10 days by intraperitoneal injection in 200 μl of saline. Blocking antibodies against E-selectin (clone 9A9) and P-selectin (clone RB40.34) were purified from hybridomas, and 250 μg of the antibody was injected intraperitoneally.

Leukocyte isolation from skin or lung

Ears from infected mice were removed, and the dorsal and ventral sides of the ear pinna were separated and allowed to incubate for 30 to 60 min at 37°C with 1 to 2 ml of Hanks' balanced salt solution (HBSS) (Gibco)

containing $CaCl_2$ and $MgCl_2$ supplemented with collagenase (125 U/ml) (Invitrogen) and deoxyribonuclease I (60 U/ml) (Sigma-Aldrich) at 37°C. To isolate leukocytes from the lung after CpG challenge, mice were anesthetized with ketamine/xylazine followed by whole-body perfusion with 10 ml of PBS injected into the left ventricle of the heart. For the skin and lung, whole-tissue suspensions were generated by gently forcing the tissue through a wire mesh screen. Leukocytes were then purified from whole-tissue suspensions by resuspending the cells in 10 ml of 35% Percoll (GE Healthcare)/HBSS in 50-ml conical tubes followed by centrifugation (500*g*) for 10 min at room temperature.

Lectin and selectin binding

Fluorescein or biotin-conjugated Jacalin, PNA, and MAL II (Vector Laboratories) were incubated with cells for 30 min in 1% fetal bovine serum (FBS)/PBS at room temperature. Binding of biotin-conjugated lectins was detected with fluorescein isothiocyanate–streptavidin (BioLegend). E-selectin (5 μg/ml) and P-selectin (1.5 μg/ml) human IgG Fc chimeric proteins (R&D Systems) were incubated with cells for 30 min in 1% FBS/ Dulbecco's PBS containing Ca^{2+} and Mg^{2+} (Gibco) at room temperature. Binding of selectins was detected using anti-human IgG Fc phycoerythrin (eBioscience). Cells were then stained with fluorescent antibodies as described in Supplementary Materials and Methods ("Flow cytometry and antibodies" section).

Statistical analysis

Statistical analyses were performed with Prism version 6.0 (GraphPad Software) using either paired or unpaired Student's *t* test or analysis of variance (ANOVA) with Tukey's post-test for significance.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. Trafficking of memory $CD8^+$ T cells into VacV-infected skin.
Fig. S2. $CD8^+$ T cells require *Gcnt1* to synthesize core 2 O-glycans for binding to E- and P-selectins.
Fig. S3. Trafficking of memory $CD8^+$ T cells into VacV-infected skin requires interactions with E- and P-selectins.
Fig. S4. IL-15 regulates E- and P-selectin binding during VacV infection.
Fig. S5. T_{CM} $CD8^+$ T cells synthesize core 2 O-glycans and maintain expression of CD62L.
Fig. S6. Phenotype of terminally differentiated tertiary memory $CD8^+$ T cells using repetitive LCMV-Armstrong infection.
Fig. S7. Primary memory $CD8^+$ T cells are recruited into the skin better than tertiary memory $CD8^+$ T cells during contact hypersensitivity.
Table S1. Primer pairs for gene expression analysis in Fig. 2 and fig. S4.
Table S2. Raw data sets and statistical analyses.

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

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Selecting memory T cells

Recruitment of immune cells to infected tissues relies on interactions between receptors on immune cells and adhesion molecules expressed by vascular endothelial cells, including E- and P-selectins. Here, Osborn *et al.* have compared the trafficking of effector and central memory T (T_{CM}) cells and find that the ability to enter tissues is largely restricted to T_{CM} cells. They found that interleukin-15 –driven transcriptional programming of T_{CM} cells promotes glycosylation of selectin ligands, allowing these cells to bind E- and P-selectins and to enter inflamed tissues. The studies represent a key advance in our understanding of how distinct memory T cell subsets contribute to recall responses.