Title

Fucosyltransferase Induction during Influenza Virus Infection Is Required for the Generation of Functional Memory CD4+ T Cells

Journal

The Journal of Immunology, 200(8)

ISSN

0022-1767

Authors

Tinoco, Roberto
Carrette, Florent
Henriquez, Monique L
et al.

Publication Date

2018-04-15

DOI

10.4049/jimmunol.1701251

Peer reviewed

Fucosyltransferase Induction during Influenza Virus Infection Is Required for the Generation of Functional Memory CD4$^+$ T Cells

Roberto Tinoco, Florent Carrette, Monique L. Henriquez, Yu Fujita, and Linda M. Bradley

T cells mediating influenza viral control are instructed in lymphoid and nonlymphoid tissues to differentiate into memory T cells that confer protective immunity. The mechanisms by which influenza virus–specific memory CD4$^+$ T cells arise have been attributed to changes in transcription factors, cytokines and cytokine receptors, and metabolic programming. The molecules involved in these biosynthetic pathways, including proteins and lipids, are modified to varying degrees of glycosylation, fucosylation, sialation, and sulfation, which can alter their function. It is currently unknown how the glycome enzymatic machinery regulates CD4$^+$ T cell effector and memory differentiation. In a murine model of influenza virus infection, we found that fucosyltransferase enzymatic activity was induced in effector and memory CD4$^+$ T cells. Using CD4$^+$ T cells deficient in the *Fut4/7* enzymes that are expressed only in hematopoietic cells, we found decreased frequencies of effector cells with reduced expression of T-bet and NKG2A/C/E in the lungs during primary infection. Furthermore, *Fut4/7*$^{-/-}$ effector CD4$^+$ T cells had reduced survival with no difference in proliferation or capacity for effector function. Although *Fut4/7*$^{-/-}$ CD4$^+$ T cells seeded the memory pool after primary infection, they failed to form tissue-resident cells, were dysfunctional, and were unable to re-expand after secondary infection. Our findings highlight an important regulatory axis mediated by cell-intrinsic fucosyltransferase activity in CD4$^+$ T cell effectors that ensure the development of functional memory CD4$^+$ T cells. ***The Journal of Immunology*****, 2018, 200: 2690–2702.**

The control of influenza viral infections depends on the ability of virus-specific T cells that are activated in the lymphoid compartment to migrate into the infected lungs where viral replication occurs in epithelial cells (1). Although CD8$^+$ T cells ultimately control these viruses by eliminating infected cells, CD4$^+$ cells play key roles by providing help for CD8$^+$ T cells, by the production of cytokines that control viral replication and elicit death of infected cells, and by direct cytotoxic activity (2). As shown for CD8$^+$ T cells, once the virus is cleared, CD4$^+$ T cells with the capacity to mediate protective memory responses are generated (2). Although many aspects of the regulation of effector and memory CD4$^+$ T cell development in the response to influenza viruses have been delineated, much less is known regarding the role of adhesion mechanisms in these processes, including the roles of the enzymatic machinery that modify adhesion molecules engaging the endothelial expressed selectins, E and P. Selectins are initiators of leukocyte extravasation into tissue in response to infection or injury. In response to the production of inflammatory cytokines, P-selectin, which is stored constitutively in Weibel–Palade bodies of endothelial cells, rapidly redistributes to the surface of vascular endothelium, whereas E-selectin is induced de novo (3). The role of endothelial selectins in the recruitment of leukocytes from blood into tissue has been well studied. However, whether the selectins and the enzymes that modify their ligands instruct the differentiation of virus-specific CD4$^+$ T cells toward memory during influenza virus infection has not been established.

The binding of T cells to the endothelial selectins requires their expression of ligands bearing terminal glycosylation that forms the sialyl Lewis x (sLe^x) determinant that can be induced on PSGL-1 in response to TCR activation (4). Regulation of glycosylation of T cell–expressed E-selectin ligands that include CD44, CD43, and E-selectin ligand-1, which are also modified with sLe^x, has not been studied as extensively. Previous studies have demonstrated that naive T cells lack selectin-binding activity, but that inflammation (5) and proinflammatory cytokines (6) can elicit selectin binding by activated CD4$^+$ T cells, regulated in part by transcription factors T-bet and STAT4 (5, 7). These conditions and TCR stimulation in conjunction with IL-12 (8) or TGF-β_1 (9) in vitro can contribute to the induction of fucosyltransferase 7 (*Fut7*), which mediates the addition of the terminal fucose of sLe^x to PSGL-1 on CD4$^+$ T cells that is required for selectin binding (10). The *Fut7* gene is expressed in hematopoietic cells and in high endothelial venules (11). It is constitutively expressed in myeloid cells and its germline deletion, together with the *Fut4* gene, which adds the terminal fucose of sLe^x to glycolypids (12)

Infectious and Inflammatory Disease Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA 92037

ORCID: 0000-0001-5420-9692 (R.T.).

Received for publication September 1, 2017. Accepted for publication February 4, 2018.

This work was supported by National Cancer Institute Cancer Center Grant P30 CA030199 to the animal, flow cytometry, and histology cores at the Sanford Burnham Prebys La Jolla campus; National Institutes of Health Grants R01 AI106895, R21 AI083606, and P01 AI04653 to L.M.B.; and National Institutes of Health Institutional Research and Academic Career Development Award K12 GM068524 and a University of California San Diego Chancellor's postdoctoral fellowship to R.T.

R.T., F.C., M.L.H., and Y.F. performed experiments in the L.M.B. laboratory. R.T. and L.M.B. designed, analyzed and interpreted experiments, and wrote the manuscript.

Address correspondence and reprint requests to Dr. Roberto Tinoco and Dr. Linda M. Bradley, Infectious and Inflammatory Disease Center, Sanford Burnham Prebys Medical Discovery Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037. E-mail addresses: rtinoco@sbpdiscovery.org (R.T.) and lbradley@sbpdiscovery.org (L.M.B.)

Abbreviations used in this article: dLN, draining lymph node; dpi, day postinfection; *Fut7*, fucosyltransferase 7; IAV, influenza A virus; i.n., intranasally; RT, room temperature; sLe^x, sialyl Lewis x; T_{CM}, T central memory; T_{EM}, T effector memory; Tg, transgenic; T_{RM}, T resident memory; WT, wild type.

in addition to selectin ligands, completely abolishes selectin binding by all cells in mice (13). In vivo, selectin binding by $CD4^+$ T cells is primarily induced on naive cells responding in peripheral lymph nodes (LN) (14) and has been previously shown to regulate the migration of $CD4^+$ and $CD8^+$ effector cells into inflamed skin (15). Although selectin binding is expressed by a large fraction of $CD4^+$ T cells in the lungs of influenza virus–infected mice (16), whether selectins play a prominent role in the recruitment of $CD4^+$ effector T cell to the lungs or selectin binding defines $CD4^+$ effector cells that differ in functional capacity has not been studied with influenza virus infection. In addition, although it is known that selectin-binding $CD4^+$ T cells can persist as memory cells (17), it is not known whether induction of the capacity for selectin binding distinguishes subsets of $CD4^+$ cells with the capacity to form memory.

In this study we show that P-selectin is highly expressed in the lungs after influenza virus infection, and use an adoptive-transfer approach to demonstrate that selectin-binding capacity, as indicated by binding of P-selectin to PSGL-1, is induced on a subset of virus-specific $CD4^+$ T cells in the draining mediastinal LN, and distinguishes the majority of $CD4^+$ T cells in the lungs. However, P-selectin binding was not required for the migration of $CD4^+$ T cells into the lungs. Because both E- and P-selectin could contribute to the development of responses of $CD4^+$ effector cells in the lungs, we analyzed the responses of $CD4^+$ cells deficient in *Fut4* and *Fut7* that are unable to bind selectins. Although these T cells generated primary responses to influenza virus infection, with comparable proliferation and cytokine production, they were recovered in lower numbers due to increased apoptosis, and displayed lower levels of T-bet. Although these $CD4^+$ effector cells persisted as memory cells, they failed to form tissue-resident memory cells and were unable to generate secondary responses upon influenza virus challenge. Our results show that induction of fucosyltransferase enzymes is necessary for the differentiation of $CD4^+$ effector cells with the capacity to form functional memory to influenza viruses.

Materials and Methods

Mice

C57BL/6J mice were purchased from the Jackson Laboratory then bred in specific pathogen-free facilities and maintained in biosafety level 2 facilities postinfection in the Sanford Burnham Prebys Institute vivarium. C57BL/6J mice were bred to Ly5.1 (B6.SJL-Ptprc^a Pepc^b/BoyJ) and OT-II TCR transgenic (Tg) mice to generate wild-type (WT) CD45.1 OT-II Tg animals. *Fut4/7*$^{-/-}$ were obtained from J.B. Lowe and bred with Thy1.1 (B6.PL-Thy 1^a/CyJ) and OT-II Tg mice to generate *Fut4/7*$^{-/-}$ Thy1.1 OT-II Tg animals. All mice used were males between 6 and 8 wk of age. All experiments were approved by the Sanford Burnham Prebys's institutional animal care and use committee.

T cell enrichments and sorting

$CD4^+$ T cells were negatively enriched (Stemcell Technologies) from spleens of WT OT-II and *Fut4/7*$^{-/-}$ OT-II Tg mice. T cells were coinjected i.v. (3.5×10^5 each) into WT recipients at 1:1 ratios and mice infected with influenza viruses 1 d later. For secondary challenge experiments, PR8-OVA_{II}–infected mice were rested to 30 d postinfection (dpi) and spleens were isolated and negatively enriched for $CD4^+$ T cells. WT (CD45.1) and *Fut4/7*$^{-/-}$ (Thy1.1) Tg T cells were stained and live cells sorted (propidium iodide negative). WT and *Fut4/7*$^{-/-}$ Tg T cells were coinjected i.v. at a 1:1 ratio (5000 cells each) into WT recipients.

Infections

Influenza viruses were grown in chicken eggs (10 d of embryonation) and titrated with Madin-Darby canine kidney cells for PFUs. An infective dose that elicited optimal T cell responses without weight loss was used. The viruses were administered intranasally (i.n.) (50 μl) after mice were anesthetized with ketamine (i.p. injection). The engineered influenza A viruses (IAV) Puerto Rico/8/34-OVA_{II} (PR8-OVA_{II}, H1N1) and Hong Kong Aichi/2/68 (HKx31-OVA_{II}, H3N2) that express the $OVA_{323–339}$ peptide recognized by OT-II $CD4^+$ T cells (18, 19). WT mice that received purified OT-II $CD4^+$ T cells were infected with the indicated viruses and T cell numbers enumerated at the indicated time points during the primary and secondary response.

Flow cytometry

Lungs, draining LN (dLN), and spleens were isolated, processed by mechanical disruption, and counted. The lungs were further processed using the mouse lung dissociation kit and gentleMACS (Miltenyi Biotec) before counting. For cell surface staining, 2×10^6 cells were incubated with Abs in staining buffer (PBS, 2% FBS, and 0.01% NaN_3) for 20 min at 4°C. Transcription factor staining and detection of cleaved caspase-3 were performed with protocols from eBioscience. For P-selectin staining, cells were incubated with surface Abs and purified mouse P-selectin IgG fusion protein (555294; BD) in P-selectin binding buffer (DMEM with calcium, 0.1% NaN_3, 0.5% BSA), followed by secondary staining with goat anti-human IgG (Jackson ImmunoResearch). All staining was done in 96-well plates. LSRFortessa and LSRFortessa ×20 flow cytometers (BD) were used for analysis. The following Abs were used from BioLegend: anti-CD4 (GK1.5), anti-Thy1.1 (OX-7), anti-CD45.1 (A20), anti–T-bet (4B10), anti-sLe^x (FH6), anti–IFN-γ (HMG1.2), anti–TNF-α (MP6-XT22), anti-CD29 (HMB1-1), anti-CD11a/CD18 (H155-78); from BD: anti-NKG2A/C/E (20d5), anti-Vβ5.1.2 (MR9-4), anti-CD49e (HMalpha5-1), anti-CD49a (Hα31/8); from eBioscience anti-BrdU (Bu20a), anti-CD127 (A7R34); and from R&D Systems: anti–P-selectin (AF737).

T cell stimulation

For cytokine production, $CD4^+$ T cells were assessed after culture with PMA (10 ng/ml) and ionomycin (0.5 μg/ml) for 5 h at 37°C. The culture medium was RPMI 1640 containing 10 mM HEPES, 1% nonessential amino acids and L-glutamine, 1 mM sodium pyruvate, 10% heat-inactivated FBS, antibiotics, 50 U/ml IL-2 (National Cancer Institute), and 1 μg/ml Brefeldin A (Sigma). For intracellular staining in lungs, APCs were first prepared from spleens from uninfected mice. Splenocytes were incubated with dextran sulfate (25 μg/ml; Sigma) and LPS (25 μg/ml; Sigma) for 48 h at 37°C. APCs were washed and treated with mitomycin C (25 μg/ml; Sigma) for 30 min at 37°C, washed twice, and resuspended in culture medium. Lungs from infected mice were then incubated with $OVA_{323–339}$ (2 μg/ml), APCs, 50 U/ml IL-2 (National Cancer Institute), and 1 μg/ml Brefeldin A for 24 h. Cells were surface stained, fixed, permeabilized, and then stained for cytokines (protocol from BD Biosciences).

In vivo proliferation

Mice were injected i.p. with 2 mg BrdU (Sigma-Aldrich) 16 hr before removing the lungs, dLN, and spleen. Proliferation of $CD4^+$ T cells was measured after negative enrichment (Stemcell Technology) followed by coinjection of T cells (3.5×10^5 cells of each) i.v. into WT mice that were infected with PR8-OVA_{II} i.n. CFSE dilution was analyzed in the dLN at 6 dpi.

In vivo labeling

WT or *Fut4/7*$^{-/-}$ OT-II T cells were coinjected into WT hosts and infected with PR8-OVA_{II} i.n. 1 d later. At 8 dpi mice were injected with 3 μg (200 μl) APC-labeled anti-CD4 (RM4-5) Ab i.v. for 4 min, lungs were harvested, processed, and stained with BV605 anti-CD4 (GK1.5 clone). Cells were acquired by flow cytometry and $CD4^+$ T cells in the vasculature (APC^+BV605^+), and parenchyma (APC^-BV605^+) were quantified.

Histology

Lungs were isolated from uninfected and PR8-OVA_{II}–infected mice. Tissues were fixed in zinc formalin (Z-fix; Anatech), embedded in paraffin, and sectioned. For staining, tissues were deparafinized, blocked with avidin for 15 min at room temperature (RT) washed, blocked with biotin for 15 min at RT, then washed and blocked with 2% rabbit blocking serum for 30 min at RT. Without washing, primary anti–P-selectin Ab (AF737; R&D Systems) was added at 1:200 dilution for 1 h at RT then washed with PBS. Secondary biotinylated anti-goat IgG (Vector) was added at 1:300 for 30 min at RT, then washed with PBS. Avidin/biotin complex (peroxidase substrate from Vector) was added for 20 min RT, washed with PBS, and then incubated with diaminobenzidine (Broun Chromogen from Vector) for 5 min at RT. Sections were finally counterstained with hematoxylin. Slides were digitally scanned with an Aperio ScanScope. When conducting the lung inflammation scoring, to quantify the lung inflammation five sections across the main bronchus of each animal were randomly selected and given scores ranging from 0 to 3 based on the level of peribronchial and perivascular inflammation. The values were given according to the

following inflammatory parameters: 0, when no inflammation was detectable; 1, for occasional cuffing with inflammatory cells; 2, for most bronchi or vessels surrounded by a thin layer (one to five cells) of inflammatory cells; and 3, when most bronchi or vessels were surrounded by a thick layer (more than five cells) of inflammatory cells (20).

Data analysis

Flow cytometry data were analyzed with FlowJo software (Tree Star). Graphs were prepared with GraphPad Prism software. GraphPad Prism was used for statistical analysis to compare outcomes using a two-tailed unpaired Student *t* test; significance was set to $p < 0.05$ and represented as $*p < 0.05$, $**p < 0.005$, $***p < 0.001$. Error bars show SEM.

Results

P-selectin levels are increased in lungs during influenza viral infection

To determine if cellular recruitment was associated with the expression of selectins in response to influenza virus infection, we analyzed inflammatory infiltrates and the expression of P-selectin in the lungs of uninfected mice and mice infected with PR8-OVA_{II} IAV (18) at 6 and 8 dpi. As previously described for human lungs (21), P-selectin was expressed in much of the bronchial microvasculature as well as the arterioles and venules in uninfected mice (Fig. 1A, 1B). At 6 dpi, staining for P-selectin was widespread rather than confined to the vasculature (Fig. 1A, 1B). The diffuse staining pattern could reflect association with soluble P-selectin, which is released in response to inflammation (22), and with platelet-expressed P-selectin following their activation (23). Mononuclear infiltrates were a prominent feature with cells surrounding the airways and within the interstitium. By 8 dpi, when viral clearance is occurring (24), P-selectin expression remained detectable on the airway vasculature, and infiltrates remained present (Fig. 1A, 1B). These data indicated that P-selectin expression in the lung was sustained during the T cell effector phase of the response to IAV, and support the potential for selectins to regulate the $CD4^+$ T cell response to this infection.

P-selectin binding distinguished distinct populations of effector and memory $CD4^+$ T cells

To evaluate whether virus-specific $CD4^+$ T cells acquired the capacity to bind selectins during the development of influenza virus–specific effectors, we performed adoptive transfer of naive OT-II

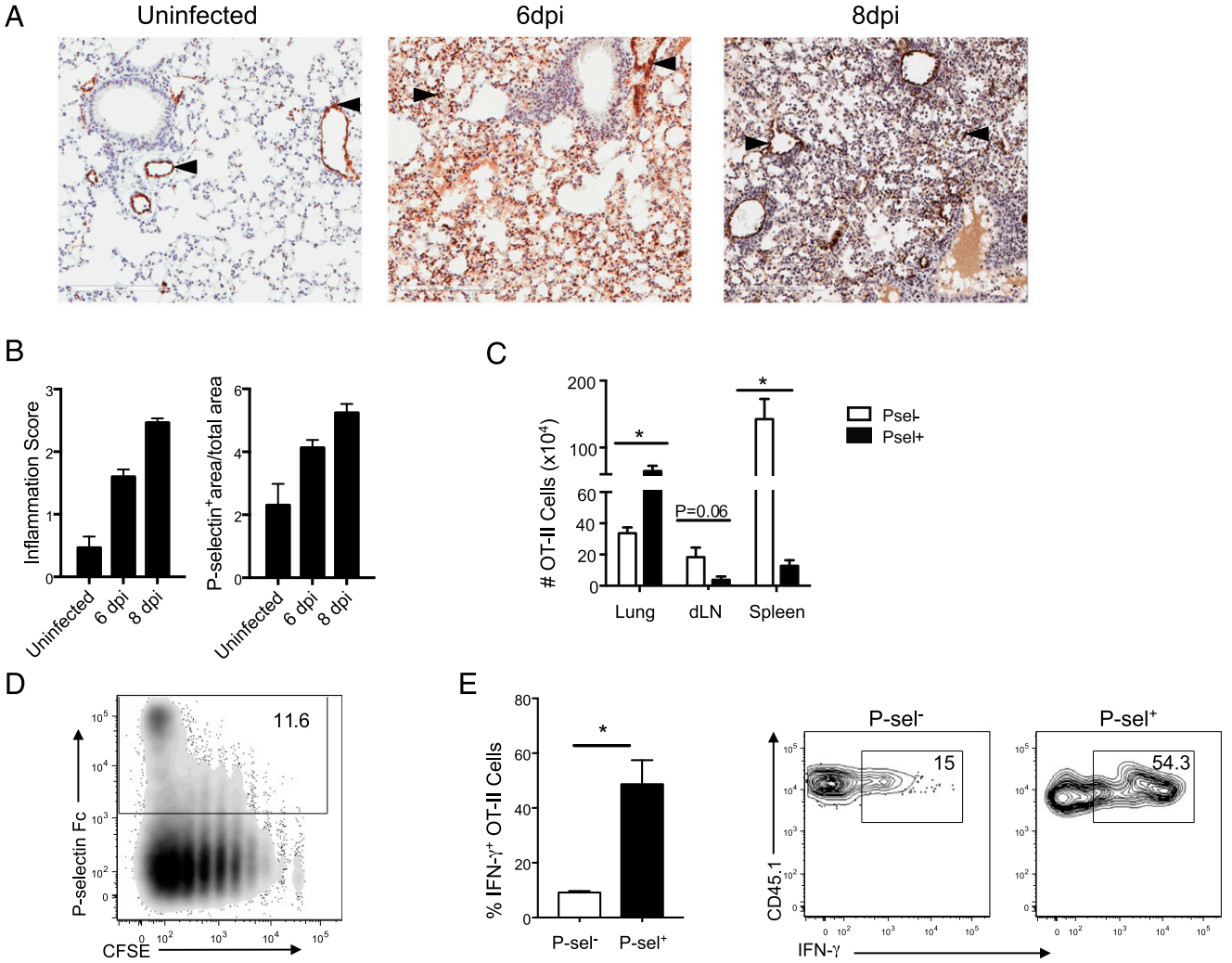

FIGURE 1. P-selectin expression and P-selectin binding are increased during influenza viral infection in the lung. (**A**) WT mice were infected with PR8-OVA_{II} i.n. and their lungs isolated at the indicated time points. Representative sections shown are at original magnification ×20 and black arrows indicate P-selectin staining (brown). (**B**) Inflammation score and P-selectin staining in lungs. (**C**) Number of P-selectin binding ($Psel^+$) and nonbinding ($Psel^-$) OT-II T cells at 8 dpi. (**D**) P-selectin binding by dividing cells in dLN at 6 dpi, gated on $CD4^+CD45.1^+$ cells. (**E**) Cytokine production by $Psel^-$ and $Psel^+$ OT-II T cells in dLN at 8 dpi, gated on $CD4^+CD45.1^+Psel^-$ or $CD4^+CD45.1^+Psel^-$. Data are representative of three independent experiments with ≥5 mice per group. Error bars indicate SEM. A two-tailed unpaired Student *t* test statistical analysis was used to compare outcomes. Significance was set to $p < 0.05$ and represented as $*p < 0.05$.

TCR Tg CD4$^+$ cells marked by CD45.1 into WT (CD45.2) recipients at the time of infection with PR8-OVA$_{II}$ IAV (19). To detect selectin binding, we used a recombinant murine P-selectin human Fc fusion protein, which binds only to PSGL-1 that expresses sLe^x. As shown in Fig. 1C, P-selectin binding is acquired by a subset of responding CD4$^+$ cells in both the lymphoid compartment and in the lungs, where the majority of effector cells were found to bind P-selectin (P-sel$^+$). A comparison of the absolute numbers of P-sel$^+$ cells at the peak of the effector response at 8 dpi in the lung, dLN, and spleen (Fig. 1C) shows these cells are most abundant in the lungs, and supports the concept that these cells represent a major component of the more differentiated CD4$^+$ effector cells that become established in the lungs (25). To test this prediction, we analyzed the induction of P-selectin binding with respect to division by OT-II cells in the dLN post-infection with PR8-OVA$_{II}$. Consistent with previous studies (14, 26), at 6 dpi the capacity for P-selectin binding was acquired by a subset of OT-II cells that had undergone division, with a progressive increase in binding by those CD4$^+$ effector cells that had undergone multiple rounds of division (Fig. 1D). These cells also displayed a greater capacity for IFN-γ production (Fig. 1E). These results showed that acquisition of P-selectin binding capacity distinguishes CD4$^+$ T cells that have acquired greater effector function during the response to IAV.

To address whether P-sel$^+$ effector cells could be distinguished after the peak of the effector phase at 8 dpi, we examined the kinetics of P-sel$^+$ cells to 30 dpi, a time point by which the virus is cleared and memory cells are formed. As shown in Fig. 2A, P-selectin binding is maintained by the majority of virus-specific CD4$^+$ cells in the lungs. P-sel$^+$ cells were also maintained in the

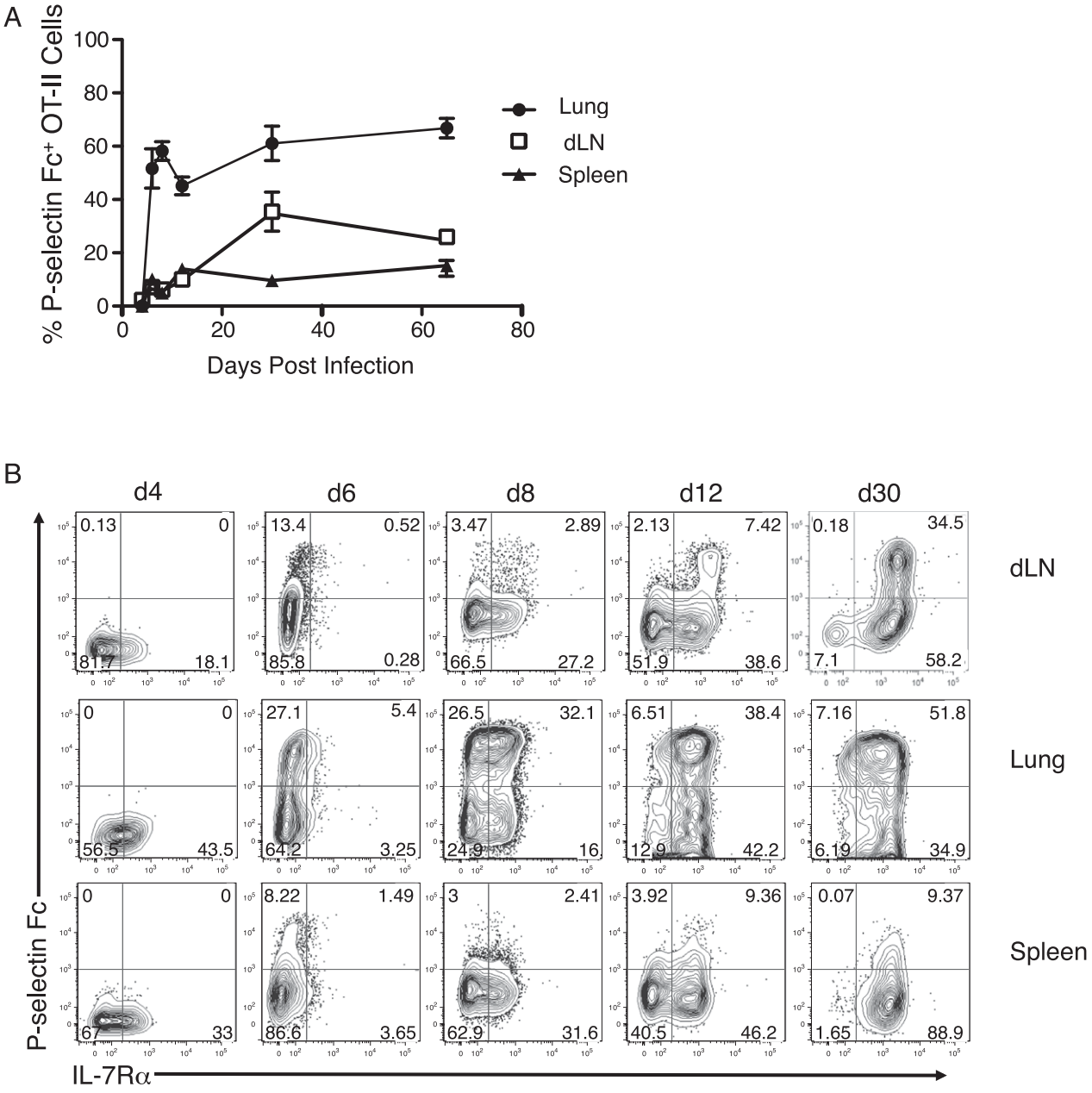

FIGURE 2. Fucosyltransferase activity is upregulated and maintained on subsets of effector and memory CD4$^+$ T cells. (**A**) P-selectin binding in OT-II CD4$^+$ T cells in the indicated organs during PR8-OVA$_{II}$ influenza viral infection. (**B**) Representative FACS plots showing P-selectin binding and IL-7Rα expression in the indicated tissues during influenza viral infection, gated on CD4$^+$CD45.1$^+$ cells. Data are representative of three independent experiments with ≥5 mice per group. Error bars indicate SEM.

lymphoid compartment, and although the frequencies were lower, they were stable over time. In addition, both P-sel$^+$ and P-sel$^-$ subsets of OT-II cells acquired IL-7Rα expression by 8 dpi in the lymphoid tissues and the lungs (Fig. 2B). These data demonstrate that the capacity for P-selectin binding does not determine or alter the ability of CD4$^+$ T cells to persist as memory cells. Because a significant fraction of cells in the lungs did not bind P-selectin, it is unlikely that engagement of P-selectin plays a nonredundant role in CD4$^+$ T cell recruitment into the lungs in response to influenza virus infection or for their retention in the lungs after the virus is cleared (27).

Cell-intrinsic fucosyltransferase induction is required for optimal CD4$^+$ T cell expansion during primary influenza viral infection

Because P-selectin binding is exclusively mediated by PSGL-1, but *Fut7* regulates the addition of fucose to sLe^x on E-selectin binding proteins, it is possible that fucosylation of selectin ligands distinguishes a broader population of highly differentiated CD4$^+$ effectors. Therefore, to address the role of fucosyltransferase involved in selectin-ligand biosynthesis in the regulation of the CD4$^+$ T cell response to IAV, we crossed mice that cannot generate functional selectin ligands due to deletion of the *Fut7* and *Fut4* genes (13) to OT-II Thy 1.1 mice. We cotransferred equal numbers of OT-II cells from *Fut4/7*$^{-/-}$ mice (Thy 1.1, CD45.2) and WT mice (Thy 1.2, CD45.1) and assessed the kinetics of their response to PR8-OVA$_{II}$ infection in naive hosts (Thy 1.2, CD45.2). As seen in Fig. 3A, comparable kinetics of response were observed in the mediastinal dLN and spleen, although the frequency of *Fut4/7*$^{-/-}$ OT-II cells was consistently 2–3 fold lower than that of WT OT-II cells from 8 dpi. However, the recovery of *Fut4/7*$^{-/-}$ OT-II cells from the lungs was significantly lower, and decayed compared with that of WT cells (Fig. 3A). This conclusion is supported by the absolute numbers of WT and *Fut4/7*$^{-/-}$ OT-II cells that are recovered at the peak of the response at 8 dpi (Fig. 3B), and at 21 dpi (Fig. 3C) when the virus Ags are cleared (28).

Fut4/7 activity enhanced CD4$^+$ T cell survival during primary influenza viral infection

To address whether a potential effect on homing of CD4$^+$ cells to the lungs accounted for the decrease in recovery from this site, we analyzed the frequencies of WT and *Fut4/7*$^{-/-}$ OT-II cells at 4 d after cotransfer and PR8-OVA$_{II}$ infection (Fig. 4A). At this time, we did not detect differences in the recovery of WT or *Fut4/7*$^{-/-}$ OT-II cells in the lung, dLN, or spleen; however, by 6 dpi we detected decreased frequencies of *Fut4/7*$^{-/-}$ OT-II T cells in all tissues examined (Fig. 4B). These data suggest a comparable initial response with a modest ability of WT cells to out-compete *Fut4/7*$^{-/-}$ cells in the lymphoid compartment but a dramatic impact on the response in the lungs. To directly test address whether *Fut4/7*$^{-/-}$ deficiency affected proliferation, we analyzed BrdU uptake in WT and *Fut4/7*$^{-/-}$ OT-II cells. We did not detect differences in the frequencies of dividing cells at 8 dpi (Fig. 4C, 4D) in any of the tissues examined, despite the differences in recovery of WT and *Fut4/7*$^{-/-}$ OT-II in the lungs. These results suggest that the survival of *Fut4/7*$^{-/-}$ OT-II cells could be impaired. To test this possibility, we evaluated apoptosis of WT and *Fut4/7*$^{-/-}$ OT-II cells at 8 dpi by measuring the levels of cleaved Caspase 3, together with propidium iodide staining immediately ex vivo, and observed markedly greater cell death in *Fut4/7*$^{-/-}$ than WT OT-II cells in the lungs and lymphoid tissues (Fig. 5A, 5B). These data imply that expression of fucosyltransferase genes in CD4$^+$ effector responses to IAV infection plays a role in maintaining their survival.

To determine if WT and *Fut4/7*$^{-/-}$ OT-II were differentially distributed in the vasculature versus parenchyma lungs, which could potentially account for differences in survival factor usage (29), WT or *Fut4/7*$^{-/-}$ OT-II T cells were coinjected into WT hosts and infected with PR8-OVA$_{II}$. At 8 dpi mice were injected i.v. with APC-labeled anti-CD4 (RM4-5) Ab for 4 min. The lungs were harvested, processed, and stained with BV605-labeled anti-CD4. This approach distinguishes CD4$^+$ T cells in the vasculature

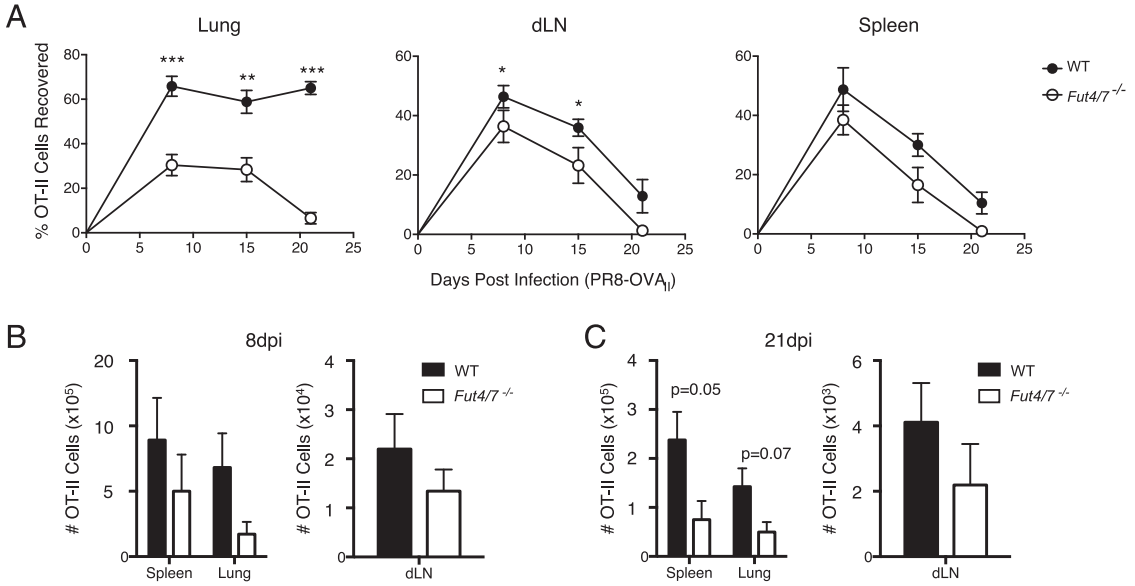

FIGURE 3. Fucosyltransferase-deficient CD4$^+$ T cells can respond during primary influenza infection. (**A**) WT and *Fut4/7*$^{-/-}$ OT-II CD4$^+$ T cells were cotransferred into WT mice and infected with PR8-OVA$_{II}$ i.n. CD4$^+$ T cell frequencies in lung, dLN, and spleen are shown at the indicated time points postinfection. The absolute numbers of WT and *Fut4/7*$^{-/-}$ OT-II T cells are shown at 8 dpi (**B**) and 21 dpi (**C**). WT cells were gated by CD4$^+$CD45.1$^+$ and *Fut4/7*$^{-/-}$ cells by CD4$^+$Thy1.1$^+$ staining. Data are representative of three independent experiments with ≥5 mice per group. Error bars indicate SEM. A two-tailed unpaired Student *t* test statistical analysis was used to compare outcomes. Significance was set to $p < 0.05$ and represented as $*p < 0.05$, $**p < 0.005$, and $***p < 0.001$.

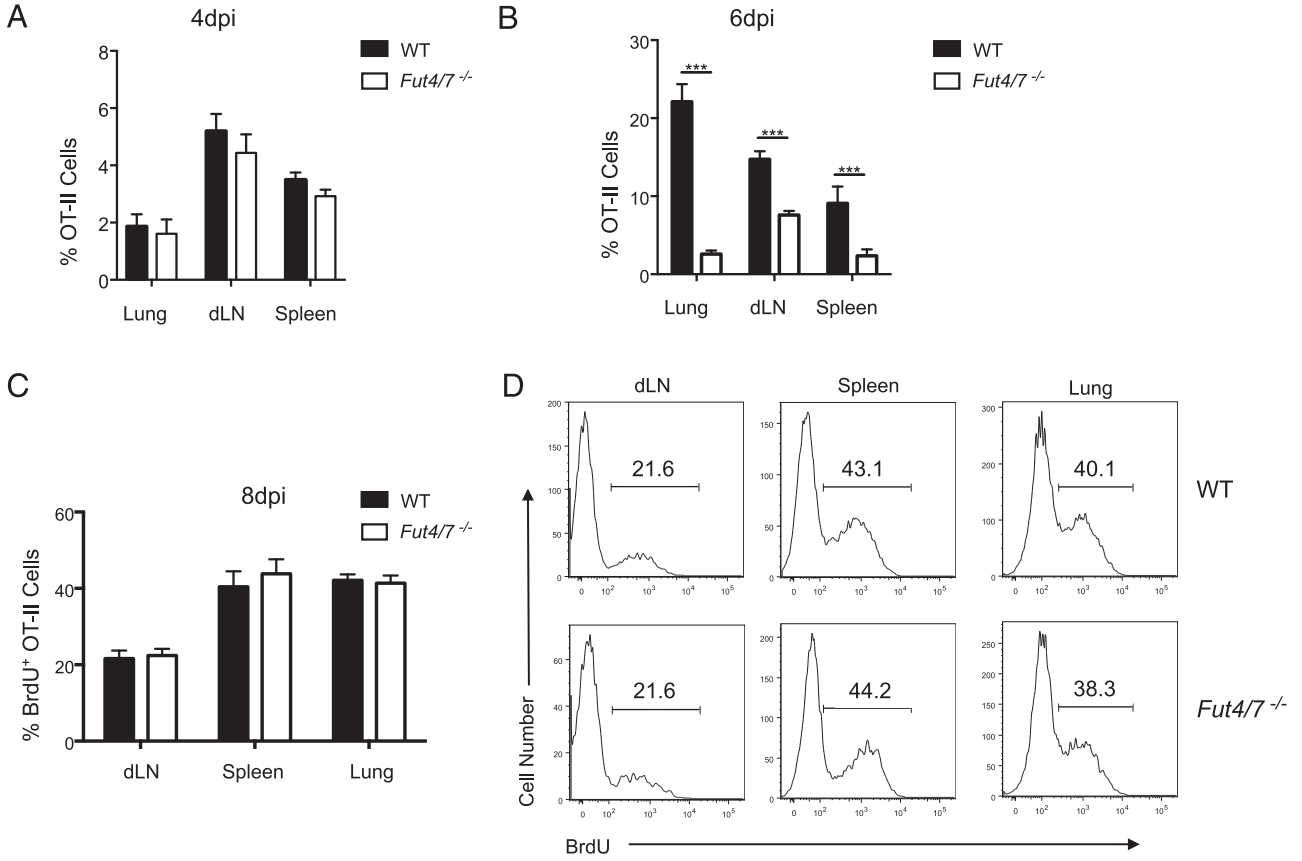

FIGURE 4. $CD4^+$ T cells deficient in fucosyltransferase activity can home to tissues and proliferate like WT T cells during primary influenza viral infection. WT and *Fut4/7*$^{-/-}$ OT-II $CD4^+$ T cells were cotransferred into WT mice and infected with $PR8\text{-}OVA_{II}$ i.n. (**A**) WT and *Fut4/7*$^{-/-}$ OT-II effector T cell infiltration in the indicated tissues 4 dpi and (**B**) 6 dpi. (**C**) BrdU incorporation in WT and *Fut4/7*$^{-/-}$ T cells at 8 dpi and representative FACS plots (**D**). WT cells were gated by $CD4^+CD45.1^+$ and *Fut4/7*$^{-/-}$ cells by $CD4^+Thy1.1^+$ staining. Data are representative of two independent experiments with $\geq$5 mice per group. Error bars indicate SEM. A two-tailed unpaired Student *t* test statistical analysis was used to compare outcomes. Significance was set to $p < 0.05$ and represented as $***p < 0.001$.

(APC^+BV605^+) and parenchyma (APC^-BV605^+). We did not observe differences in the distribution of WT and *Fut4/7*$^{-/-}$ (Fig. 5C) between the two compartments, further supporting the conclusion that the capacity for localization in the lungs is not impaired by *Fut4/7* deficiency.

Altered effector $CD4^+$ T cell differentiation occurred in the absence of Fut4/7 enzymes

To further assess whether effector function was affected by *Fut4/7* deficiency, we analyzed the production of IFN-γ and TNF-α by cotrasferred WT and *Fut4/7*$^{-/-}$ OT-II cells at 8 dpi. We found comparable production of these cytokines in the lungs, dLN, and spleen (Fig. 5D, 5E), suggesting that $CD4^+$ effector T cell function was not compromised with *Fut4/7* deficiency. To further assess the extent of activation and differentiation of $CD4^+$ effector cells in the lungs, we analyzed the expression NKG2A/C/E, which distinguishes the most differentiated Th1 effector cells that coexpress P-selectin binding only in the lungs after IAV infection (30). Whereas a notable fraction of WT OT-II cells induced expression of NKG2A/C/E, which was found only P-selectin binding cells, comparatively few *Fut4/7*$^{-/-}$ OT-II cells expressed NKG2A/C/E (Fig. 6A), supporting the hypothesis that fucosyltransferase induction altered effector differentiation in response to IAV in the lungs. To further assess $CD4^+$ effector T cell differentiation, we analyzed the transcription factor T-bet, whose high expression marks the most differentiated Th1 effector cells (31). As shown in Fig. 6B, at 8 dpi the frequency of T-bet–expressing OT-II cells was significantly lower in *Fut4/7*$^{-/-}$ effector cells compared with WT effector cells in the lungs, where expression was strongly associated with P-sel$^+$ cells. No differences in T-bet levels were found in WT versus *Fut4/7*$^{-/-}$ cells in the lymphoid compartment (data not shown).

Fucosyltransferase activity during primary influenza infection is required for the generation of functional memory $CD4^+$ T cells

Because both WT versus *Fut4/7*$^{-/-}$ OT-II cells were recovered at 21 dpi in the lymphoid compartment and lungs, after viral clearance has occurred (Fig. 3C), *Fut4/7* deficiency does not appear to prevent the persistence of OT-II cells after the contraction of the primary response. However, the extent of $CD4^+$ effector T cell differentiation can impact memory formation, with more differentiated cells becoming terminal, displaying a decreased capacity for response to secondary challenge (32, 33). Therefore, we evaluated the recall responses of WT versus *Fut4/7*$^{-/-}$ OT-II cells in mice that were initially infected with $\times 31\text{-}OVA_{II}$ influenza, a virus that expresses the internal proteins of PR8, but is serotype H3N2 (19). At 28 dpi, these mice were challenged with $PR8\text{-}OVA_{II,}$ which is serotype H1N1. As depicted in Fig. 7A, the kinetics of the primary response were comparable to those seen with $PR8\text{-}OVA_{II}$, (Fig. 3A). However, at 30 dpi after primary infection, *Fut4/7*$^{-/-}$ OT-II cells failed to increase in number during the

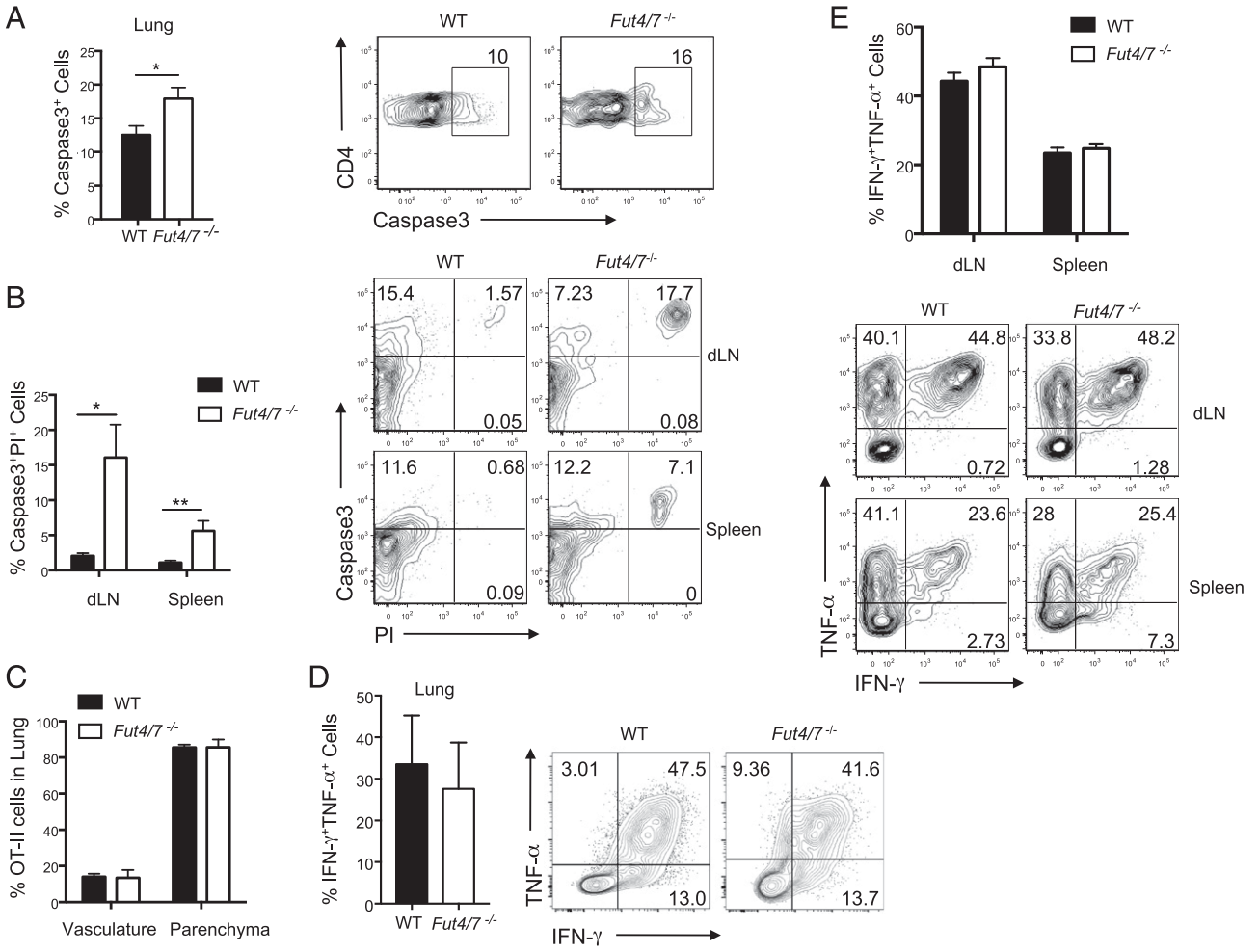

FIGURE 5. Fucosyltransferase-deficient CD4+ T cells had increased apoptosis and similar cytokine production. WT and *Fut4/7*$^{-/-}$ OT-II CD4+ T cells were cotransferred into WT mice and infected with PR8-OVA$_{II}$ i.n. and tissues analyzed at 8 dpi (**A**–**E**). (A) Frequencies of Caspase3+ cells in lung. (B) Frequencies of Caspase3+ and propidium iodide+ cells in dLN and spleen. (C) OT-II T cell positioning in lung vasculature and parenchyma after in vivo Ab labeling. Cytokine production in the lung (D) and dLN and spleen (E). Data are representative of two (A and B) or three (C–E) independent experiments with ≥5 mice per group. WT cells were gated by CD4+CD45.1+ and *Fut4/7*$^{-/-}$ cells by CD4+Thy1.1+ staining. Error bars indicate SEM. A two-tailed unpaired Student *t* test statistical analysis was used to compare outcomes. Significance was set to $p < 0.05$ and represented as $*p < 0.05$ and $**p < 0.005$.

secondary response compared with WT OT-II cells in response to challenge with PR8-OVA$_{II}$ (Fig. 7B). These data suggest the possibility that, as memory cells, *Fut4/7*$^{-/-}$ OT-II cells failed to optimally survive during the secondary effector response.

We next evaluated whether WT and *Fut4/7*$^{-/-}$ OT-II memory T cells differed with respect to their memory phenotype. We categorized T central memory (T_{CM}, CD62L+), T effector memory (T_{EM}, CD62L−), and T resident memory (T_{RM}, CD62L−CD69+ CXCR3+) cells at 30 dpi. We found increased frequencies of *Fut4/7*$^{-/-}$$T_{CM}$ cells compared with WT T_{CM} cells in all tissues examined (Fig. 8A). *Fut4/7*$^{-/-}$ T_{EM} cells were also increased in lungs and dLN, but decreased in the spleen. Significantly, we found decreased frequencies of *Fut4/7*$^{-/-}$ T_{RM} cells compared with WT T_{RM} cells in lungs and dLN and few T_{RM} cells from either population in the spleen (Fig. 8A). These data further support the concept that fucosyltransferase activity alters effector cell differentiation and is critical in the generation of T_{RM} cells after influenza infection. No differences in LFA-1 or β_1-integrin were observed, but the frequencies of *Fut4/7*$^{-/-}$ memory T cells expressing α_1-integrin were decreased in lungs and dLN (Fig. 8B), a surface protein that has previously been linked CD4+ T cell resistance to apoptosis in the lungs (34).

Because greater frequencies of WT than *Fut4/7*$^{-/-}$ OT-II cells persist after resolution of the primary response, it is possible that differences between the recall responses of two donor populations were at least in part due to a lower frequency of *Fut4/7*$^{-/-}$ memory cells at the time of challenge. Because both WT and *Fut4/7*$^{-/-}$ OT-II cells persisted in sufficient numbers at 21 dpi with PR8-OVA$_{II}$ (Fig. 3), for further study at 30 dpi we sorted WT and *Fut4/7*$^{-/-}$ donor populations from spleens based on CD45.1 and Thy 1.1, respectively, and cotransferred them in equal numbers (5×10^3 each) into naive WT recipients that expressed Thy1.2, CD45.2 (Fig. 9A). We assessed their capacity to respond to infection with PR8-OVA$_{II}$. Remarkably, we demonstrate an almost complete failure of *Fut4/7*$^{-/-}$ OT-II memory cells to accumulate in either lymphoid tissues or the lungs (Fig. 9B, 9C) in response IAV infection. The increased numbers of WT OT-II versus *Fut4/7*$^{-/-}$ OT-II memory cells (Fig. 9C) were not due to changes in their proliferation as shown by similar BrdU uptake at 8 dpi (Fig. 9D). These data support the conclusion that induction of fucosyltransferase enzymes contributes to the optimal generation of CD4+ T cell effectors with the capacity to form functional memory cells and re-expand after secondary challenge (33).

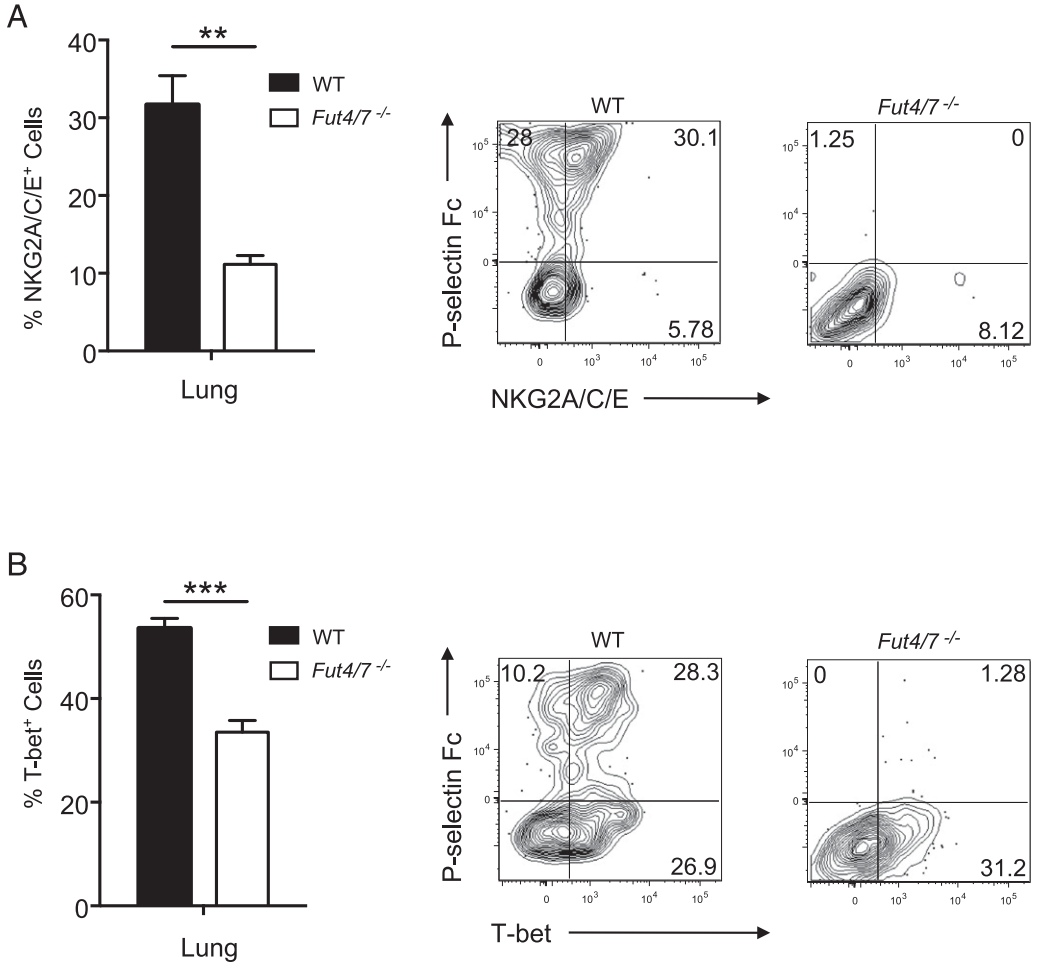

FIGURE 6. Fucosyltransferase-deficient $CD4^+$ T cells cannot engage P-selectin and have reduced T-bet and NKG2A/C/E expression. WT and *Fut4/7*$^{-/-}$ OT-II $CD4^+$ T cells were cotransferred into WT mice and infected with PR8-OVA_{II} i.n. (**A**) Frequencies of NKG2A/C/E and P-selectin binding at 8 dpi. (**B**) Frequencies of T-bet and P-selectin binding at 8 dpi. WT cells were gated by $CD4^+CD45.1^+$ and *Fut4/7*$^{-/-}$ cells by $CD4^+Thy1.1^+$ staining. Data are representative of two independent experiments with ≥5 mice per group. Error bars indicate SEM. A two-tailed unpaired Student *t* test statistical analysis was used to compare outcomes. Significance was set to $p < 0.05$ and represented as $^{**}p < 0.005$ and $^{***}p < 0.001$.

Discussion

In this study we establish that binding of endothelial selectins is not required for the migration of influenza virus–specific $CD4^+$ T cells into the lungs or localization within the lungs postinfection, but that the ability to generate selectin ligands by the induction of fucosyltransferase enzymes that control the addition of terminal fucose residues on glycoproteins and glycolipids is required for optimal effector responses and the development of functional memory cells. These results demonstrate that the ability to synthesize these enzymes is part of the differentiation program that supports effector and memory generation of $CD4^+$ T cells in response to influenza virus infections, potentially irrespective of the generation of functional selectin ligands on selected proteins that include PSGL-1, CD43, and CD44.

Peripheral T cells undergo extensive changes in glycosylation in response to activation and differentiation (35). Terminal fucosylation is a common modification found on many *N*-glycans, mucin *O*-GalNAc glycans, and glycolipids (36). At least 10 fucosyltransferases (*FUT1–7* and *FUT9–11*) can function to add terminal fucose to oligosaccharide chains on these molecules, leading to diverse biological functions and considerable redundancy (37). Studies in mice have shown that *Fut4* and *Fut7* are functionally redundant, and the much weaker contribution of *Fut4* to fucosylation of selectin ligands to form sLe^x was only revealed in the context of *Fut7* deficiency (13). We examined Fut4/7 activity in $CD4^+$ T cells by detecting P-selectin binding by PSGL-1. This receptor, however, is only one of the many molecules that are modified by these enzymes. It is possible that many more proteins and lipids are temporally and spatially fucosylated during T cell differentiation, which ensures the generation of functional memory T cells and secondary effectors.

We considered the possibility that P-selectin could contribute to the regulation of $CD4^+$ effector T cell recruitment into the lungs in the response to influenza viruses because of our finding that it is widely expressed on the vasculature of the airways postinfection. Our analyses of P-selectin binding as a readout of sLe^x expression by PSGL-1 on influenza virus–specific $CD4^+$ T cells revealed that P-selectin binding was highly induced on effector cells that had undergone multiple rounds of division in vivo in the dLN, as previously found in an in vitro analysis that linked increased P-selectin binding to the expression levels of *Fut7* (26). In our study, the $P\text{-sel}^+$ population contained a greater frequency of IFN-γ–producing cells in the lymphoid compartment and lungs after influenza virus infection. In addition, in the lungs, a significant proportion of the $P\text{-sel}^+$ cells coexpressed the NKG2D/A/C/E receptor(s) that were identified as having immunostimulatory functions in NK cells (38), and have recently been shown to distinguish highly differentiated $CD4^+$ T cells that express CTL function in the lung in response to influenza virus infection (30). Given that the highest frequency of $P\text{-sel}^+$ cells was found in the lungs, these findings support the concept that the capacity for

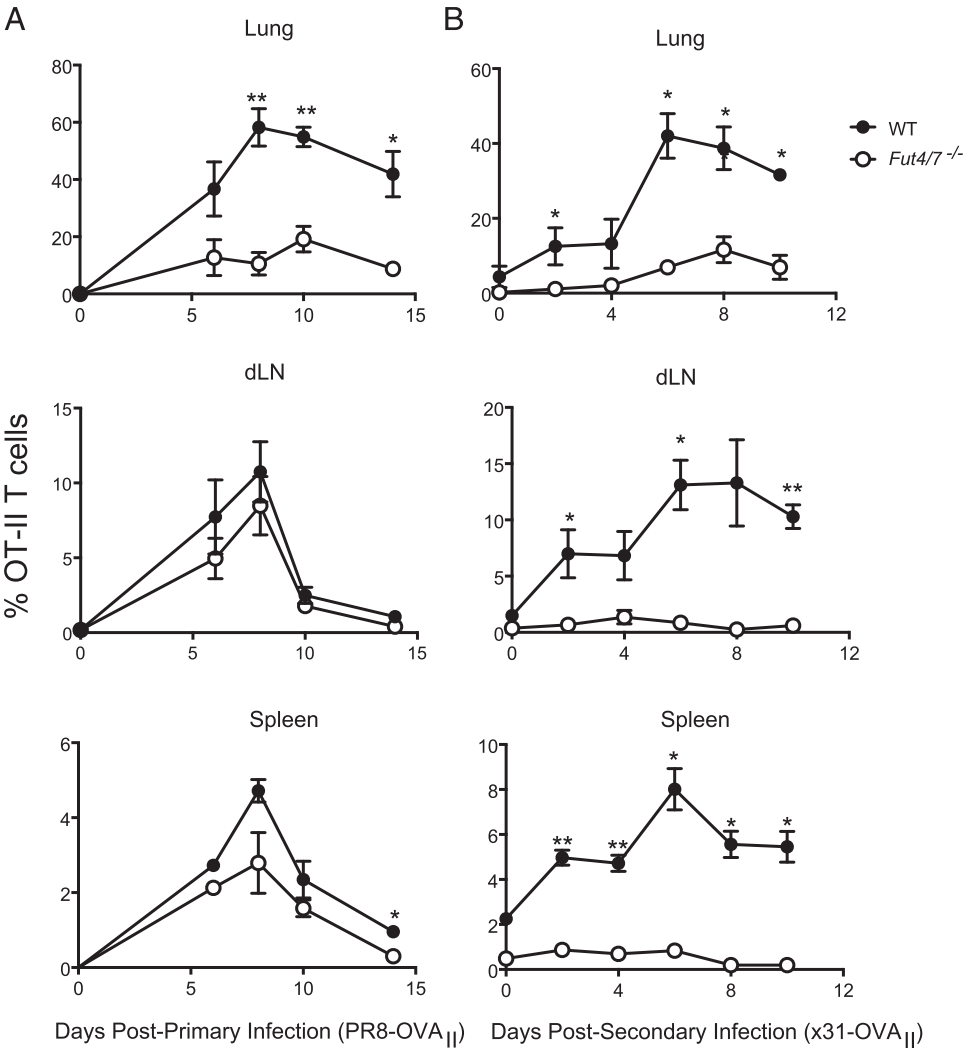

FIGURE 7. Defective secondary expansion of fucosyltransferase-deficient memory CD4+ T cells during secondary influenza infection. WT and *Fut4/7*$^{-/-}$ OT-II CD4+ T cells were cotransferred into WT mice and infected with PR8-OVA$_{II}$ i.n. (**A**) Frequencies of WT and *Fut4/7*$^{-/-}$ OT-II expansion in lung, dLN, and spleen during primary influenza infection at the indicated time points. (**B**) PR8-OVA$_{II}$ immune mice were reinfected with ×31-OVA$_{II}$. Frequencies of WT and *Fut4/7*$^{-/-}$ memory T cell expansion postinfection in lung, dLN, and spleen at the indicated time points. WT cells were gated by CD4+CD45.1+ and *Fut4/7*$^{-/-}$ cells by CD4+Thy1.1+ staining. Data are representative of three independent experiments with ≥5 mice per group. Error bars indicate SEM. A two-tailed unpaired Student *t* test statistical analysis was used to compare outcomes. Significance was set to $p < 0.05$ and represented as $*p < 0.05$ and $**p < 0.005$.

selectin binding marks highly differentiated effector CD4+ T cells. However, because the P-sel^{-} subset also contained cells that had undergone multiple divisions, induction of P-selectin–binding capacity is not required for CD4+ effector T cell differentiation. In addition, E-selectin binding is detectable on CD4+ T cells responding to influenza virus infection in the lungs, which could contribute to T cell recruitment/responses in this site (16). However, a greater frequency of CD4+ T cells activated in vitro under non–Th subset–polarizing conditions bind P-selectin than E selectin, whose binding is distinctly regulated (8). Thus, it remains possible that P-selectin without E-selectin binding can distinguish a subset of primary CD4+ effector T cells. Moreover, although *Fut7* expression can be induced in the majority of CD4+ T cells cultured under Th1 conditions that favor *Fut7* induction (39), the observed heterogeneity may reflect differences in the extent of effector differentiation or dynamic regulation of expression levels of sLe^{x} in the presence of continuous stimulation.

Our finding that P-selectin–binding cells persist into the memory phase after influenza virus infection demonstrates that, unlike CD8+ memory cells (40), fucosylation can be maintained on CD4+ T cells after the resolution of the response, consistent with a previous report that epigenetic modifications can sustain *Fut7* transcription (26). It has been suggested that maintenance of selectin-binding activity could distinguish effector memory from central memory cells that have a greater capacity for self-renewal in the lymphoid compartment (10). However, it is not yet known whether there are differences in the capacity of selectin binding and nonbinding CD4+ memory T cells to respond to challenge.

Because *Fut4* and *Fut7* are primarily known to regulate cell adhesion and migration, it is notable that deficiency of these enzymes is sufficient to cause CD4+ T cell–intrinsic dysfunction, which was primarily linked to impaired survival rather than apparent differences in proliferation, cytokine responses, or migration. Although the survival defect was relatively modest in the lymphoid compartment, much greater loss of *Fut4/7*$^{-/-}$ compared with WT CD4+ T cells in the lungs is could reflect a more terminal effector phenotype with greater susceptibility to TCR/Fas-mediated activation-induced cell death in the site of infection, or greater susceptibility to apoptosis inducing cytokines such as TNF-α. Because increased apoptosis of *Fut4/7*$^{-/-}$ CD4+ effector T cells also occurred in dLN, this reduction in cell number could also account for decreased *Fut4/7*$^{-/-}$ CD4+ T cells leaving the LN and entering the infected lungs where these cells could encounter additional differentiation signals. Because we observed decreased survival of *Fut4/7*$^{-/-}$ CD4+ T cells during primary infection, it is possible that *Fut4/7* induction also ensures survival of secondary effectors as we did not detect differences in their proliferation. Although fucosyltransferases have not been previously linked to T cell survival, studies of *FUT7*-transfected human hepatocarcinoma cells showed that enzyme expression resulted in decreased susceptibility to radiation-induced apoptosis (41). *FUT7* expression in these cells was also found to upregulate integrin α5

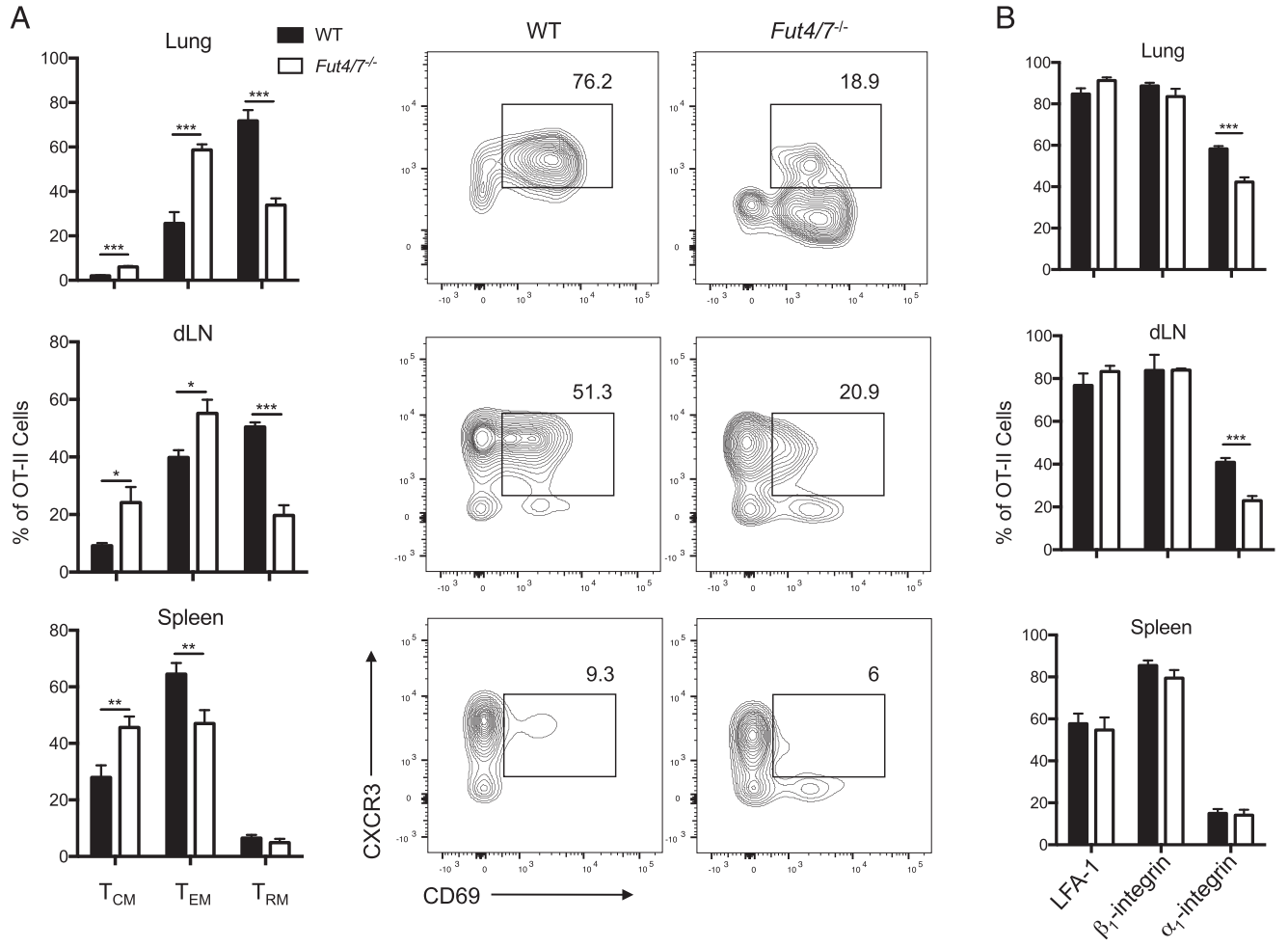

FIGURE 8. $CD4^+$ T cells require fucosyltransferase activity to generate tissue-resident memory cells. WT and *Fut4/7*$^{-/-}$ OT-II $CD4^+$ T cells were cotransferred into WT mice and infected with $PR8\text{-}OVA_{II}$ i.n. to generate memory T cells and tissues analyzed at 30 dpi. (**A**) Memory T cell subsets were quantified based on T_{CM} ($CD62L^+$), T_{EM} ($CD62L^-$), and T_{RM} ($CD62L^-CD69^+CXCR3^+$) phenotypes and representative FACS plots are shown. (**B**) Frequencies of memory T cells expressing LFA-1, β_1-integrin, and α_1-integrin. WT cells were gated by $CD4^+CD45.1^+$ and *Fut4/7*$^{-/-}$ cells by $CD4^+$ $Thy1.1^+$ staining. Data are representative of three independent experiments with ≥5 mice per group. Error bars indicate SEM. A two-tailed unpaired Student *t* test statistical analysis was used to compare outcomes. Significance was set to $p < 0.05$ and represented as $*p < 0.05$, $**p < 0.005$, and $***p < 0.001$.

and their adhesion to its receptor, fibronectin (42). Upregulation of integrin α5 has been implicated in cancer cell survival in several conditions, including hypoxia (43). Importantly, integrin α5 in association with integrin β1 (α5β1) promotes adhesion of Jurkat T cells to fibronectin through the P-selectin ligand, PSGL-1, which requires PI3K activation (44). Whether upregulation of α5β1 is associated with induction of *Fut7* on T cells has not been examined, and although we did not detect differences in α_5 expression with *Fut4/7* deficiency, we did observe decreased expression of integrin α_1, which has previously been linked to $CD4^+$ T cell survival (34), and engagement of the extracellular matrix is a fundamental mechanism that maintains cell survival (45). Expression of *FUT1* and *FUT4* has also been associated with tumor growth and survival (46), providing additional evidence for the role(s) of fucosylation-dependent regulation.

In addition to regulating the functions of multiple adhesion receptors, fucosylation can directly regulate T cell effector responses. In a T cell transfer model of colitis, *Fut8*$^{-/-}$ $CD4^+$ T cells were markedly less pathogenic than WT $CD4^+$ T cells and produced reduced levels of Th1 cytokines, outcomes that were linked to the lack of core 2 *O*-glycan fucosylation of CD3 that resulted in impaired TCR signaling. Like *Fut7*, *Fut8* is induced in effector T cells in vivo (ImmGen database). Our finding that $CD4^+$ T cells from *Fut4/7*$^{-/-}$ mice exhibit decreased survival but not diminished production of Th1 cytokines suggests that fucosylation of core 2 *O*-glycans is not necessary for optimal TCR engagement on $CD4^+$ T cells. In addition, *Fut7* but not *Fut4* is induced in $CD4^+$ T cells by 48 h after TCR signaling via anti-CD3 stimulation in vitro (47), and is inhibited by blocking cell division (26). Thus, contributions of *Fut7* and/or *Fut4* to $CD4^+$ T cell survival occurs after initial TCR signals are received, and the addition of terminal fucose residues to glycans may play a key role regulating the response to survival signals that are necessary for the optimal differentiation of effector T cells, which can give rise to memory cells. In support of the possibility that *Fut7* can regulate signaling through cell surface receptors, is the finding that sLe^x is expressed on the insulin receptor α subunit in human hepatocarcinoma cells, and that induced overexpression of *Fut7* leads to tyrosine phosphorylation of insulin receptor substrate-1 as well as activation of PI3K/Akt, and MAP kinases (48). Thus, *Fut7* expression could, for example, be required for fucosylation of receptors that mediate $CD4^+$ effector T cell survival, such as cytokines [e.g., IL-6 (49)] or molecules that impact cellular metabolism such as glucose transporters and lipids [e.g., Glut-1 (50)].

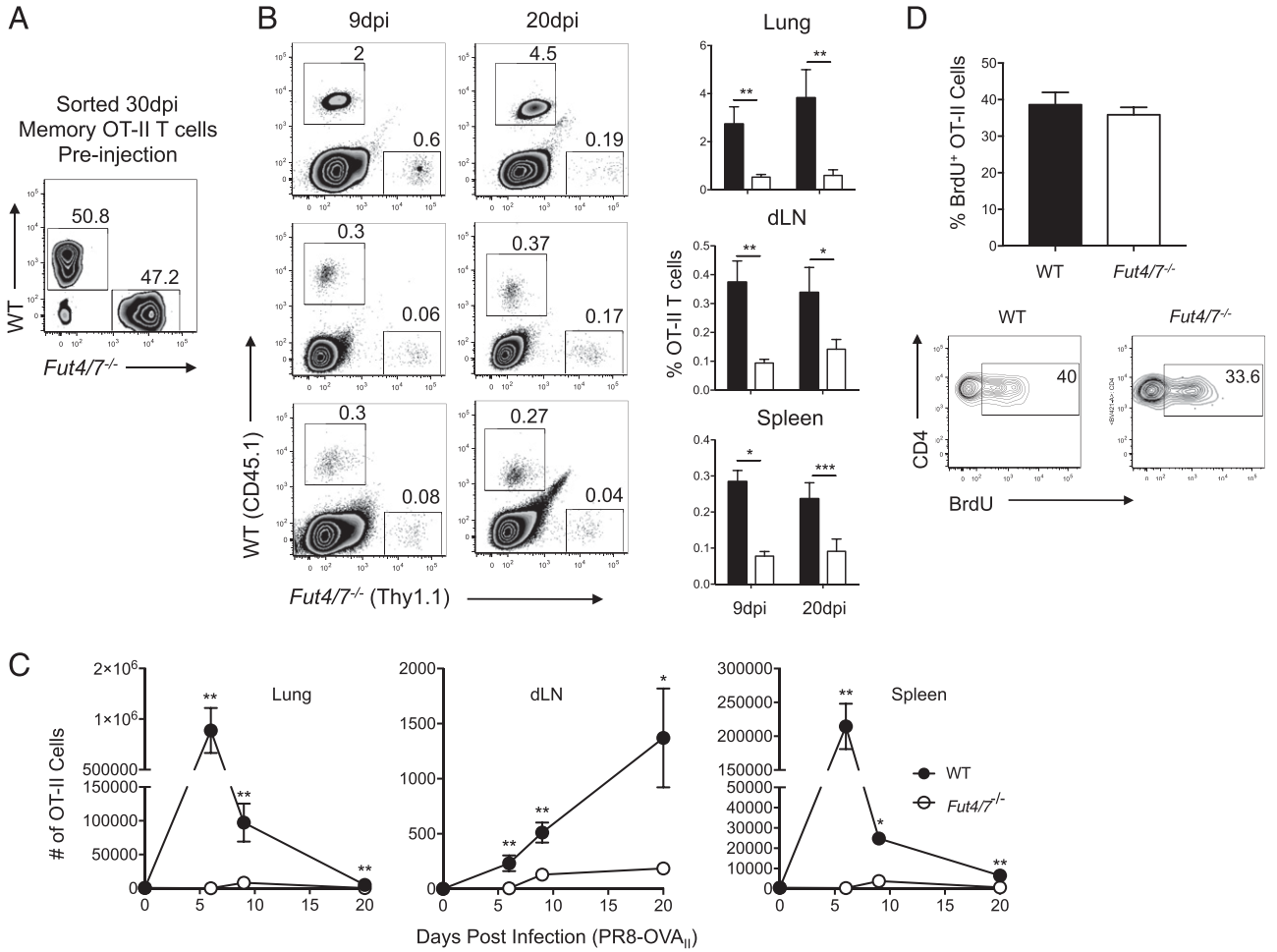

FIGURE 9. *Fut4/7*$^{-/-}$ CD4$^+$ T cells fail to accumulate after secondary infection. WT and *Fut4/7*$^{-/-}$ OT-II CD4$^+$ T cells were cotransferred in WT mice and infected with PR8-OVA$_{II}$ i.n. to generate memory T cells. (**A**) Memory T cells were sorted from the spleen at 30 dpi and cotransferred at 1:1 ratio in naive WT mice that were subsequently infected with PR8-OVA$_{II}$. (**B**) Frequencies of WT or *Fut4/7*$^{-/-}$ OT-II T cells at 9 and 20 dpi in the indicated tissues. (**C**) Absolute numbers of WT and *Fut4/7*$^{-/-}$ OT-II T cells in lung, dLN, and spleen at the indicated time points. (**D**) BrdU incorporation in dLN at 6 dpi. WT cells were gated by CD4$^+$CD45.1$^+$ and *Fut4/7*$^{-/-}$ cells by CD4$^+$Thy1.1$^+$ staining. Data are representative of two independent experiments with ≥5 mice per group. Error bars indicate SEM. A two-tailed unpaired Student *t* test statistical analysis was used to compare outcomes. Significance was set to $p < 0.05$ and represented as $*p < 0.05$, $**p < 0.005$, and $***p < 0.001$.

It is striking that we did not observe changes in CD4$^+$ effector T cell proliferation or cytokine production in *Fut4/7*$^{-/-}$ compared with WT T cells in the lymphoid compartment, which would have suggested the dramatic impairment of secondary effector responses. These results suggest the possibility that the critical temporal requirement for CD4$^+$ T cell regulation by *Fut4/7* occurs at the level of the effector to memory cell transition, which leads to a smaller cohort of persisting memory cells. Furthermore, T_{CM} and T_{EM} cells were significantly more prevalent in the lungs and dLN in the *Fut4/7*$^{-/-}$ population, whereas T_{RM} cells dramatically reduced, suggesting a failure to form memory cells with the capacity to persist in the tissues. Although we did not identify a functional defect, we did observe diminished expression of T-bet by *Fut4/7*$^{-/-}$ CD4$^+$ effector T cells in the lungs where their decreased recovery/survival was most pronounced. Although T-bet expression is associated with IFN-γ production by CD4$^+$ effector T cells, the deficiency of this transcription factor does not necessarily impact IFN-γ production (51), which could account for our finding of comparable responses of primary WT and *Fut4/7*$^{-/-}$ CD4$^+$ effector T cells. Although T-bet and several other transcription factors have been extensively studied in CD8$^+$ T cells, which require T-bet expression for memory formation (52), few studies have addressed its role in CD4$^+$ T cell differentiation. In one study, diminished T-bet expression was found to distinguish CD4$^+$ memory T cell precursors in the acute LCMV infection model (53). However, another study demonstrated that CD4$^+$ T cell memory develops in the absence of T-bet in this model (54). Therefore, our findings of decreased T-bet expression by *Fut4/7*$^{-/-}$ CD4$^+$ effector T cells in the lungs is most likely reflective of a lower extent of effector differentiation that may be linked to the inability of *Fut4/7*$^{-/-}$ CD4$^+$ effector T cells to induce fucosylation of sLe^x in response to IL-12 and STAT-4 signaling. In this regard, it is important to note that effector CD4$^+$ T cells can give rise to persistent, functional memory cells with high efficiency (55, 56). We therefore conclude that differences in T-bet expression are unlikely to account for the profound defect in memory responses by *Fut4/7*$^{-/-}$ CD4$^+$ T cells, but rather that a dynamic interplay of T-bet and fucosylation plays an unexpected role in the regulation of CD4$^+$ T cell responses.

It is not clear why *Fut4/7*$^{-/-}$ CD4$^+$ T cells that persist after the contraction phase of the response to influenza virus infection have such a profound defect in their capacity to increase in numbers after secondary challenge even when transferred in a 1:1 ratio with WT cells into naive hosts. The few T cells detected during secondary challenge engage in the response as measured by

proliferation, indicating that a minor population escaped the fate of what is very likely to be large-scale activation-induced apoptosis. However, our results support a conclusion that the induction of fucosylation as part of remodeling of cell surface proteins and potentially lipids by glycosylation in response to $CD4^+$ T cell activation and effector differentiation can have profound effects on the regulation of memory responses. Moreover, it is important to emphasize that fucosylation per se is a sufficiently minor change in the overall cell surface glycan modifications, so significant effects on the distribution of molecules such as the selectin ligands PSGL-1, CD44, and CD43 are not likely to account for the changes in secondary responses that we observed.

We conclude that the induction of fucosylation, in this study exemplified by *Fut7*, and possibly to a lesser extent *Fut4*, has an unanticipated role in the regulation of $CD4^+$ effector T cell responses to influenza virus infection that is primarily manifested by the support of survival, particularly during the contraction phase of the response, which also governs the fitness of the memory population to respond to secondary challenge. In total, our study underscores the importance of considering posttranslational glycosylation of proteins and possibly lipids as fundamental to determining T cell fate determination and the formation of functional memory cells in the response to microbial infections.

Acknowledgments

We thank Melissa M. Lin for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References

1. Kreijtz, J. H., R. A. Fouchier, and G. F. Rimmelzwaan. 2011. Immune responses to influenza virus infection. *Virus Res.* 162: 19–30.
2. Strutt, T. M., K. K. McKinstry, N. B. Marshall, A. M. Vong, R. W. Dutton, and S. L. Swain. 2013. Multipronged CD4(+) T-cell effector and memory responses cooperate to provide potent immunity against respiratory virus. *Immunol. Rev.* 255: 149–164.
3. McEver, R. P. 2015. Selectins: initiators of leucocyte adhesion and signalling at the vascular wall. *Cardiovasc. Res.* 107: 331–339.
4. Carlow, D. A., K. Gossens, S. Naus, K. M. Veerman, W. Seo, and H. J. Ziltener. 2009. PSGL-1 function in immunity and steady state homeostasis. *Immunol. Rev.* 230: 75–96.
5. Lord, G. M., R. M. Rao, H. Choe, B. M. Sullivan, A. H. Lichtman, F. W. Luscinskas, and L. H. Glimcher. 2005. T-bet is required for optimal proinflammatory CD4+ T-cell trafficking. *Blood* 106: 3432–3439.
6. Ebel, M. E., O. Awe, M. H. Kaplan, and G. S. Kansas. 2015. Diverse inflammatory cytokines induce selectin ligand expression on murine CD4 T cells via p38α MAPK. *J. Immunol.* 194: 5781–5788.
7. Syrbe, U., U. Hoffmann, K. Schlawe, O. Liesenfeld, K. Erb, and A. Hamann. 2006. Microenvironment-dependent requirement of STAT4 for the induction of P-selectin ligands and effector cytokines on CD4+ T cells in healthy and parasite-infected mice. *J. Immunol.* 177: 7673–7679.
8. Schroeter, M. F., B. A. Ratsch, J. Lehmann, R. Baumgrass, A. Hamann, and U. Syrbe. 2012. Differential regulation and impact of fucosyltransferase VII and core 2 β1,6-N-acetyl-glycosaminyltransferase for generation of E-selectin and P-selectin ligands in murine CD4+ T cells. *Immunology* 137: 294–304.
9. Wagers, A. J., and G. S. Kansas. 2000. Potent induction of alpha(1,3)-fucosyltransferase VII in activated CD4+ T cells by TGF-beta 1 through a p38 mitogen-activated protein kinase-dependent pathway. *J. Immunol.* 165: 5011–5016.
10. Ley, K., and G. S. Kansas. 2004. Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation. *Nat. Rev. Immunol.* 4: 325–335.
11. Malý, P., A. Thall, B. Petryniak, C. E. Rogers, P. L. Smith, R. M. Marks, R. J. Kelly, K. M. Gersten, G. Cheng, T. L. Saunders, et al. 1996. The alpha(1,3) fucosyltransferase Fuc-TVII controls leukocyte trafficking through an essential role in L-, E-, and P-selectin ligand biosynthesis. *Cell* 86: 643–653.
12. Huang, M. C., A. Laskowska, D. Vestweber, and M. K. Wild. 2002. The alpha (1,3)-fucosyltransferase Fuc-TIV, but not Fuc-TVII, generates sialyl Lewis X-like epitopes preferentially on glycolipids. *J. Biol. Chem.* 277: 47786–47795.
13. Homeister, J. W., A. D. Thall, B. Petryniak, P. Malý, C. E. Rogers, P. L. Smith, R. J. Kelly, K. M. Gersten, S. W. Askari, G. Cheng, et al. 2001. The alpha(1,3) fucosyltransferases FucT-IV and FucT-VII exert collaborative control over selectin-dependent leukocyte recruitment and lymphocyte homing. *Immunity* 15: 115–126.
14. Campbell, D. J., and E. C. Butcher. 2002. Rapid acquisition of tissue-specific homing phenotypes by CD4(+) T cells activated in cutaneous or mucosal lymphoid tissues. *J. Exp. Med.* 195: 135–141.
15. Smithson, G., C. E. Rogers, P. L. Smith, E. P. Scheidegger, B. Petryniak, J. T. Myers, D. S. Kim, J. W. Homeister, and J. B. Lowe. 2001. Fuc-TVII is required for T helper 1 and T cytotoxic 1 lymphocyte selectin ligand expression and recruitment in inflammation, and together with Fuc-TIV regulates naive T cell trafficking to lymph nodes. *J. Exp. Med.* 194: 601–614.
16. Kretschmer, U., K. Bonhagen, G. F. Debes, H. W. Mittrücker, K. J. Erb, O. Liesenfeld, D. Zaiss, T. Kamradt, U. Syrbe, and A. Hamann. 2004. Expression of selectin ligands on murine effector and IL-10-producing CD4+ T cells from non-infected and infected tissues. *Eur. J. Immunol.* 34: 3070–3081.
17. Jennrich, S., B. A. Ratsch, A. Hamann, and U. Syrbe. 2007. Long-term commitment to inflammation-seeking homing in CD4+ effector cells. *J. Immunol.* 178: 8073–8080.
18. Thomas, P. G., S. A. Brown, M. Y. Morris, W. Yue, J. So, C. Reynolds, R. J. Webby, and P. C. Doherty. 2010. Physiological numbers of CD4+ T cells generate weak recall responses following influenza virus challenge. *J. Immunol.* 184: 1721–1727.
19. Thomas, P. G., S. A. Brown, W. Yue, J. So, R. J. Webby, and P. C. Doherty. 2006. An unexpected antibody response to an engineered influenza virus modifies CD8 + T cell responses. *Proc. Natl. Acad. Sci. USA* 103: 2764–2769.
20. Sun, Y. Q., M. X. Deng, J. He, Q. X. Zeng, W. Wen, D. S. Wong, H. F. Tse, G. Xu, Q. Lian, J. Shi, and Q. L. Fu. 2012. Human pluripotent stem cell-derived mesenchymal stem cells prevent allergic airway inflammation in mice. *Stem Cells* 30: 2692–2699.
21. Feuerhake, F., G. Füchsl, R. Bals, and U. Welsch. 1998. Expression of inducible cell adhesion molecules in the normal human lung: immunohistochemical study of their distribution in pulmonary blood vessels. *Histochem. Cell Biol.* 110: 387–394.
22. Blann, A. D., S. K. Nadar, and G. Y. Lip. 2003. The adhesion molecule P-selectin and cardiovascular disease. *Eur. Heart J.* 24: 2166–2179.
23. Lê, V. B., J. G. Schneider, Y. Boergeling, F. Berri, M. Ducatez, J. L. Guerin, I. Adrian, E. Errazuriz-Cerda, S. Frasquilho, L. Antunes, et al. 2015. Platelet activation and aggregation promote lung inflammation and influenza virus pathogenesis. *Am. J. Respir. Crit. Care Med.* 191: 804–819.
24. Flynn, K. J., J. M. Riberdy, J. P. Christensen, J. D. Altman, and P. C. Doherty. 1999. In vivo proliferation of naïve and memory influenza-specific CD8(+) T cells. *Proc. Natl. Acad. Sci. USA* 96: 8597–8602.
25. Román, E., E. Miller, A. Harmsen, J. Wiley, U. H. Von Andrian, G. Huston, and S. L. Swain. 2002. CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function. *J. Exp. Med.* 196: 957–968.
26. Syrbe, U., S. Jennrich, A. Schottelius, A. Richter, A. Radbruch, and A. Hamann. 2004. Differential regulation of P-selectin ligand expression in naive versus memory CD4+ T cells: evidence for epigenetic regulation of involved glycosyltransferase genes. *Blood* 104: 3243–3248.
27. Cauley, L. S., T. Cookenham, T. B. Miller, P. S. Adams, K. M. Vignali, D. A. Vignali, and D. L. Woodland. 2002. Cutting edge: virus-specific CD4+ memory T cells in nonlymphoid tissues express a highly activated phenotype. *J. Immunol.* 169: 6655–6658.
28. Jelley-Gibbs, D. M., D. M. Brown, J. P. Dibble, L. Haynes, S. M. Eaton, and S. L. Swain. 2005. Unexpected prolonged presentation of influenza antigens promotes CD4 T cell memory generation. *J. Exp. Med.* 202: 697–706.
29. Strutt, T. M., K. Dhume, C. M. Finn, J. H. Hwang, C. Castonguay, S. L. Swain, and K. K. McKinstry. 2017. IL-15 supports the generation of protective lung-resident memory CD4 T cells. *Mucosal Immunol*. DOI: 10.1038/mi.2017.101.
30. Marshall, N. B., A. M. Vong, P. Devarajan, M. D. Brauner, Y. Kuang, R. Nayar, E. A. Schutten, C. H. Castonguay, L. J. Berg, S. L. Nutt, and S. L. Swain. 2017. NKG2C/E marks the unique cytotoxic CD4 T cell subset, ThCTL, generated by influenza infection. *J. Immunol.* 198: 1142–1155.
31. Intlekofer, A. M., N. Takemoto, E. J. Wherry, S. A. Longworth, J. T. Northrup, V. R. Palanivel, A. C. Mullen, C. R. Gasink, S. M. Kaech, J. D. Miller, et al. 2005. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. [Published erratum appears in 2006 *Nat. Immunol.* 7: 113.] *Nat. Immunol.* 6: 1236–1244.
32. Catron, D. M., L. K. Rusch, J. Hataye, A. A. Itano, and M. K. Jenkins. 2006. CD4+ T cells that enter the draining lymph nodes after antigen injection participate in the primary response and become central-memory cells. *J. Exp. Med.* 203: 1045–1054.
33. Wu, C. Y., J. R. Kirman, M. J. Rotte, D. F. Davey, S. P. Perfetto, E. G. Rhee, B. L. Freidag, B. J. Hill, D. C. Douek, and R. A. Seder. 2002. Distinct lineages of T(H)1 cells have differential capacities for memory cell generation in vivo. *Nat. Immunol.* 3: 852–858.
34. Chapman, T. J., and D. J. Topham. 2010. Identification of a unique population of tissue-memory CD4+ T cells in the airways after influenza infection that is dependent on the integrin VLA-1. *J. Immunol.* 184: 3841–3849.
35. Daniels, M. A., K. A. Hogquist, and S. C. Jameson. 2002. Sweet 'n' sour: the impact of differential glycosylation on T cell responses. *Nat. Immunol.* 3: 903–910.
36. Becker, D. J., and J. B. Lowe. 2003. Fucose: biosynthesis and biological function in mammals. *Glycobiology* 13: 41R–53R.
37. Schneider, M., E. Al-Shareffi, and R. S. Haltiwanger. 2017. Biological functions of fucose in mammals. *Glycobiology* 27: 601–618.
38. Raulet, D. H. 2003. Roles of the NKG2D immunoreceptor and its ligands. *Nat. Rev. Immunol.* 3: 781–790.
39. Underhill, G. H., D. G. Zisoulis, K. P. Kolli, L. G. Ellies, J. D. Marth, and G. S. Kansas. 2005. A crucial role for T-bet in selectin ligand expression in T helper 1 (Th1) cells. *Blood* 106: 3867–3873.
40. Nolz, J. C., and J. T. Harty. 2014. IL-15 regulates memory CD8+ T cell O-glycan synthesis and affects trafficking. *J. Clin. Invest.* 124: 1013–1026.

41. Wang, H., Q. Y. Wang, Y. Zhang, Z. H. Shen, and H. L. Chen. 2007. Alpha1,3 Fucosyltransferase-VII modifies the susceptibility of apoptosis induced by ultraviolet and retinoic acid in human hepatocarcinoma cells. *Glycoconj. J.* 24: 207–220.
42. Wang, Q. Y., Y. Zhang, Z. H. Shen, and H. L. Chen. 2008. alpha1,3 fucosyltransferase-VII up-regulates the mRNA of alpha5 integrin and its biological function. *J. Cell. Biochem.* 104: 2078–2090.
43. Spangenberg, C., E. U. Lausch, T. M. Trost, D. Prawitt, A. May, R. Keppler, S. A. Fees, D. Reutzel, C. Bell, S. Schmitt, et al. 2006. ERBB2-mediated transcriptional up-regulation of the alpha5beta1 integrin fibronectin receptor promotes tumor cell survival under adverse conditions. *Cancer Res.* 66: 3715–3725.
44. Luo, J., C. Li, T. Xu, W. Liu, X. Ba, X. Wang, and X. Zeng. 2014. PI3K is involved in β1 integrin clustering by PSGL-1 and promotes β1 integrin-mediated Jurkat cell adhesion to fibronectin. *Mol. Cell. Biochem.* 385: 287–295.
45. Gilmore, A. P. 2005. Anoikis. *Cell Death Differ.* 12(Suppl. 2): 1473–1477.
46. Zhang, Z., P. Sun, J. Liu, L. Fu, J. Yan, Y. Liu, L. Yu, X. Wang, and Q. Yan. 2008. Suppression of FUT1/FUT4 expression by siRNA inhibits tumor growth. *Biochim. Biophys. Acta* 1783: 287–296.
47. Blander, J. M., I. Visintin, C. A. Janeway, Jr., and R. Medzhitov. 1999. Alpha (1,3)-fucosyltransferase VII and alpha(2,3)-sialyltransferase IV are up-regulated in activated CD4 T cells and maintained after their differentiation into Th1 and migration into inflammatory sites. *J. Immunol.* 163: 3746–3752.
48. Wang, Q. Y., Y. Zhang, H. J. Chen, Z. H. Shen, and H. L. Chen. 2007. Alpha 1,3-fucosyltransferase-VII regulates the signaling molecules of the insulin receptor pathway. *FEBS J.* 274: 526–538.
49. Dienz, O., and M. Rincon. 2009. The effects of IL-6 on CD4 T cell responses. *Clin. Immunol.* 130: 27–33.
50. Macintyre, A. N., V. A. Gerriets, A. G. Nichols, R. D. Michalek, M. C. Rudolph, D. Deoliveira, S. M. Anderson, E. D. Abel, B. J. Chen, L. P. Hale, and J. C. Rathmell. 2014. The glucose transporter Glut1 is selectively essential for CD4 T cell activation and effector function. *Cell Metab.* 20: 61–72.
51. Harms Pritchard, G., A. O. Hall, D. A. Christian, S. Wagage, Q. Fang, G. Muallem, B. John, A. Glatman Zaretsky, W. G. Dunn, J. Perrigoue, et al. 2015. Diverse roles for T-bet in the effector responses required for resistance to infection. *J. Immunol.* 194: 1131–1140.
52. Kallies, A. 2008. Distinct regulation of effector and memory T-cell differentiation. *Immunol. Cell Biol.* 86: 325–332.
53. Marshall, H. D., A. Chandele, Y. W. Jung, H. Meng, A. C. Poholek, I. A. Parish, R. Rutishauser, W. Cui, S. H. Kleinstein, J. Craft, and S. M. Kaech. 2011. Differential expression of Ly6C and T-bet distinguish effector and memory Th1 CD4 (+) cell properties during viral infection. *Immunity* 35: 633–646.
54. Mollo, S. B., J. T. Ingram, R. L. Kress, A. J. Zajac, and L. E. Harrington. 2014. Virus-specific CD4 and CD8 T cell responses in the absence of Th1-associated transcription factors. *J. Leukoc. Biol.* 95: 705–713.
55. Harbertson, J., E. Biederman, K. E. Bennett, R. M. Kondrack, and L. M. Bradley. 2002. Withdrawal of stimulation may initiate the transition of effector to memory CD4 cells. *J. Immunol.* 168: 1095–1102.
56. Hu, H., G. Huston, D. Duso, N. Lepak, E. Roman, and S. L. Swain. 2001. CD4(+) T cell effectors can become memory cells with high efficiency and without further division. *Nat. Immunol.* 2: 705–710.