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Smad4 Promotes Differentiation of Effector and Circulating Memory CD8 T Cells but Is Dispensable for Tissue-Resident Memory CD8 T Cells

Yinghong Hu,* Young-Tae Lee,† Susan M. Kaech,‡ Beth Garvy,* and Linda S. Cauley*

Tissue-resident memory CD8 T cells are a unique subset of virus-specific CTLs that bolster local immune responses after becoming lodged in previously infected tissues. These cells provide enhanced protection by intercepting returning pathogens before a new infection gets established. In contrast, central memory CD8 T cells circulate in the bloodstream and proliferate in secondary lymphoid organs before replenishing effector and memory CD8 T cell populations in remote parts of the body. Both populations of virus-specific memory CD8 T cells participate in immunity to influenza virus infection; however, the signaling pathways that instruct development memory CD8 T cells to distribute to specific tissues are poorly defined. We show that TGF-β promotes the development of pulmonary tissue-resident memory T cells via a signaling pathway that does not require the downstream signaling intermediate Sma- and Mad-related protein (Smad4). In contrast, circulating memory CD8 T cells have no requirement for TGF-β but show signs of arrested development in the absence of Smad4, including aberrant CD103 expression. These signaling pathways alter the distribution of virus-specific CTLs in the lungs but do not prevent robust cytokine responses. Our data show that Smad4 is required for normal differentiation of multiple subsets of virus-specific CD8 T cells. In normal circumstances, Smad4 may be activated via a pathway that bypasses the TGF-β receptor. Improved understanding of these signaling pathways could be used to augment vaccine-induced immunity. The Journal of Immunology, 2015, 194: 000–000.

Vaccines augment immunity to infectious pathogens by stimulating long-lived populations of Ag-specific memory T and/or B cells. During recent decades inactivated vaccines have been widely used to combat seasonal influenza A virus (IAV) epidemics (1). These vaccines induce high concentrations of serum Abs that provide lasting immunity to specific viruses but are not broadly reactive with other strains, and the protection expires as new variants emerge. Other less common approaches include the use of live viral vectors for the production of virus-specific memory CD8 T cells that respond to many different serotypes (1, 2). We recently showed that the combined activities of several distinct CTL populations were required for robust heterosubtypic immunity in the lungs, including some noncirculating tissue-resident memory CD8 T (TRM) cells that are adapted for prolonged survival in peripheral tissues (3, 4). The immunity was less effective when live IAV was delivered outside of the lungs largely because TRM cells did not develop in the correct location (3). Rare cross-reactive Abs also contributed to the immunity (3, 5) by a mechanism that may involve enhanced Ag presentation to CD8 T cells (6). In clinical settings inactivated vaccines are mostly given by i.m. injection and induce high concentrations of serum Abs, but cross-protection is limited by a weak cellular response (1). Similar immunizations with whole virus produced variable results in animal models (7, 8) with a report of robust cell-mediated immunity when the membrane-binding activity of the inactivated virus was preserved (9). The mechanism of early viral clearance from the immunized mice was not entirely clear, as protective CTLs were not analyzed in situ. Limited understanding of the signaling pathways that control homing receptor expression on different subsets of virus-specific memory CD8 T cells is a major impediment in the quest to develop vaccines for pathogens that enter the body from mucosal tissues.

Neuraminidase is a viral coat protein with enzymatic activity, which activates large quantities of latent TGF-β in the lungs during infection with some strains of IAV (10). This suppressive cytokine is a master regulator of diverse cell populations and controls a complex array of integrated signaling pathways that coordinate cytokine expression and the cellular response to infection (11, 12). In immune cells the most clearly defined signaling pathways downstream of the TGF-β receptor are mediated by a cascade of Sma- and Mad-related proteins (Smad), which participate in the development of Th17 cells and IgA Abs (13–15). Recent studies have shown that TRM cells use TGF-β–dependent integrins to interact with epithelial cells that express E-cadherin (16) during long-term residence in the mucosa (17, 18) and cytolyis (19). In other models, highly activated effector CD8 T (Teff) cells that expressed killer cell lectin–like receptor G1 (KLRG1) were sensitive to TGF-β–induced apoptosis (20). Because TGF-β is an important regulatory molecule in the lungs, we investigated how virus-specific CTLs respond to IAV infection when they lack the TGF-β receptor, or Smad4, which serves as an adaptor for multiple Smad-related signaling proteins (21) during activation of the receptors for TGF-β and bone morphogenic proteins (22).

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Abbreviations used in this article: dpi, days postinfection; EpCAM, epithelial cell adhesion molecule; IAV, influenza A virus; KLRG1, killer cell lectin–like receptor G1; NP, nucleoprotein; SKO, Sma- and Mad-related protein knockout; Smad, Sma- and Mad-related protein; TCM, central memory T; T Eff , effector T; TKO, TGF-BRII knockout; TRM, tissue-resident memory T; WT, wild-type.

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independent of Smad proteins (11), and it is not known which signaling pathways are required for antiviral immunity in the lungs.

In contrast to other pathogens, relatively small numbers of virus-specific CTLs expressed KLRG1 in the lungs during IAV infection (23, 24). Not surprisingly, the size of the KLRG1+ CTL population increased when TGF-βRII was not expressed, whereas CD103+ TRM cells were completely absent (17, 18, 20). In contrast, very few virus-specific CTLs expressed KLRG1 when Smad4 was not expressed, whereas normal numbers of CD103+CD69+ TRM cells accumulated in the lungs. An unusual population of long-lived virus-specific CTLs developed in the absence of Smad4, which exhibited signs of arrested development including aberrant CD103 expression. These unusual CTLs had limited ability to enter encapsulated lymph nodes after viral clearance, whereas most long-lived KLRG1+ CTLs were located in the blood vessels (25). Overall, our data suggest that integrated responses to TGF-β-dependent and TGF-β-independent signaling pathways contribute to the functional heterogeneity of virus-specific memory CD8 T cell populations in the lungs during IAV infection.

Materials and Methods

Mice and reagents

Mice were housed at the University of Connecticut Health Center in accordance with institutional guidelines. Smad4<sup>fl/fl</sup> mice (26) were crossed with mice that express Cre-recombinase under the control of the distal Lck promoter (27), as well as OT1 TcR transgenic mice (28). The distal Lck–Cre TGF-βRII<sup>fl/fl</sup> mice were supplied by Dr. M. Bevan (University of Washington). Virus stocks were grown in fertilized chicken eggs, and titers were determined as described previously (29). Anesthetized mice were infected with 10<sup>5</sup> PFU X31-OVA by intranasal inoculation (30).

Mixed bone marrow chimeras and transfer experiments

Lethally irradiated mice (1000 rad) were reconstituted with 5 × 10<sup>6</sup> bone marrow cells from congenically marked mutant and control mice (1:1 ratio). To eliminate radio-resistant cells, 200 µg anti-CD8 Abs (2.43) were given by i.v. injection 2 d after transfer. Mice were maintained for 6–8 wk before infection. For transfer studies recipient mice received 10<sup>6</sup> congenically marked donor cells from mutant and/or wild-type (WT) donor mice (mixed 1:1).

Sample preparation for flow analysis

Lungs were digested with 150 U/ml collagenase (Life Technologies) in RPMI 1640 medium (1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 10% FBS) and enriched on 44/67% Percoll gradients. Washed lymphocytes were stained with anti-CD8 and nucleoprotein (NP<sub>355/370</sub>) tetramers (31) for 1 h at room temperature. For cytokine analysis, lymphocytes were incubated with SIINFEKL peptide (1 µg/ml) in the presence of brefeldin A for 5 h at 37°C and analyzed with a Cytofix/Cytoperm kit (BD Pharmingen). CCR7 expression was analyzed with CCL19 conjugated to human Ig and Alexa Fluor 488–conjugated anti-human IgG (Life Technologies). BrdU was given by i.p. injection (1 mg in 200 µl PBS), and lymphocytes were analyzed 3–6 h later using a BrdU kit (BD Pharmingen). For intravascular staining mice were injected with 3 µg anti-CD8<sub>β</sub> in 300 µl PBS and sacrificed 5 min later (32).

Confocal microscopy

Tissues were fixed with 1% paraformaldehyde and stained with biotinylated Abs to epithelial cell adhesion molecule (EpCAM) or B220 followed by streptavidin–Pacific Orange (Life Technologies), PE-conjugated anti-CD45.1 (eBioscience), anti-CD103 Alexa Fluor 647 (BioLegend), KLRG1 Alexa Fluor 488 (BD Pharmingen), and CD31 Pacific Blue (Life Technologies). Images were recorded on a Zeiss LSM 780 confocal microscope with the Zeiss ZEN 2012 digital imaging suite and an inverted Axio Observer.Z1 with argon, diode, and HeNe lasers using Plan-Neofluar ×10/numerical aperture 0.5 and ×20/numerical aperture 0.5 objectives. Imaris suite (Bitplane) was used for background subtraction and colocalization.

Statistical analysis

Experiments were repeated two to three times using three to four animals per group. Statistical significance was determined using an unpaired two-tailed Student t test. Comparisons that were used to generate p values are indicated by horizontal lines in the figures. A p value <0.05 was considered statistically significant.

Results

To determine how Smad4 and TGF-β regulate the CTL response to IAV infection, we made chimeric mice with mixed bone marrow cells from Smad4 knockout (SKO) mice and littermates lacking Cre or flox sites as WT controls. For comparison, other animals were reconstituted with bone marrow cells from TGF-βRII knockout (TKO) mice (33) and littermates lacking Cre or flox sites. This approach ensures that mutant and WT CTLs are exposed to identical concentrations of Ag and inflammation during

![FIGURE 1.](http://www.jimmunol.org/) CTLs have reciprocal phenotypes in the absence of TGF-βRII and Smad4. Two groups of chimeric mice were made with mixed bone marrow cells from either TKO mice and WT littermates or SKO mice and WT littermates. Results are representative of three independent experiments (three to four animals per group). (A) CD44 and CD62L expression on CD8 T cells in peripheral blood. (B) KLRG1 expression on CD8 T cells in peripheral blood, with percentages of cells in the marked region. TKO or SKO (dashed line) and WT (gray fill) are shown. (C) Chimeric mice were infected with X31-OVA. Total numbers of NP-specific CTLs are shown. TKO (hatched), SKO (black), and littermates (white) are shown. Error bars are means ± SD for three animals per group. *p ≤ 0.05, ***p ≤ 0.005. (D) Gated populations of NP-specific CTLs were analyzed for KLRG1 and CD103 expression.
infection. The secondary lymphoid organs contained approximately equal numbers of WT and TKO or SKO and WT cells. Large percentages of the TKO cells expressed CD44 at high levels 8 wk after transfer (Fig. 1A), including some KLRG1+ CTLs (Fig. 1B). This phenotype was consistent with enhanced homeostatic proliferation after radiation (33). In contrast, the donor cells from SKO and WT donors expressed CD44 at low levels and lacked KLRG1, indicating that they were mostly naive CD8 T cells.

To analyze the CTL response to viral infection, chimeric mice were infected with X31-OVA and analyzed for NP-specific CTLs by MHC class I tetramer analysis. There were large numbers of TKO cells in the lungs and spleens 10 d postinfection (dpi), whereas SKO cells were similar to the controls (Fig. 1C). Phenotypic data showed that high frequencies of TKO cells expressed KLRG1, whereas CD103+ cells were almost completely absent (Fig. 1D). In contrast, very few SKO CTLs expressed KLRG1, whereas large percentages of CTLs expressed CD103 in the lungs.

FIGURE 2. Pulmonary T\textsubscript{RM} cells do not require Smad4. Congenically marked OTI-WT (top), OTI-TKO (middle), or OTI-SKO cells (bottom) were sorted for low CD44 expression and transferred to C57BL/6 mice before X31-OVA infection. Error bars are means ± SD from three to four animals per group. Three independent experiments gave consistent results. (A) Numbers of KLRG1+ CTLs (gray shading). (B) Numbers of CD69+ (hatched), CD69+/CD103+ (black shading, T\textsubscript{RM}), CD103+ (gray shading), or no markers (white). Statistical comparisons are shown in Supplemental Table I.

FIGURE 3. TGF-β prevents prolonged cell proliferation. OTI-WT, OTI-TKO, and OTI-SKO cells were sorted for low CD44 expression and transferred to C57BL/6 mice 48 h before infection with X31-OVA. (A) OTI-WT, OTI-TKO, and OTI-SKO cells were analyzed for CFSE dilution 3.5 dpi (three animals per group). OTI-WT (gray fill), TKO or SKO (dashed line), and uninfected (solid line). Two experiments gave similar results. The gating strategy is shown in Supplemental Fig. 1. (B) Percentages of BrdU+ cells within gated populations of OTI-WT (white bars), OTI-TKO (hatched bars), and OTI-SKO (black bars). Means ± SD from three animals per group. Two experiments gave similar results. *p ≤ 0.05. **p ≤ 0.005. (C) Overlaid histograms show gated populations of CD45.1+ CTLs analyzed for activated caspase-3/7. OTI-WT (gray fill), TKO or SKO (dashed line), and unstained (solid line). Pooled data are from three mice; two experiments gave similar results. (D) Gated populations of transferred cells were analyzed for Bcl2 expression. OTI-WT (gray fill), TKO or SKO (dashed line), and isotype control (solid line). Pooled data are from three mice; two experiments gave similar results.
and spleen. The reciprocal phenotypes of the CTLs from TKO and SKO mice show that KLRG1+ T_{EFF} cells and T_{RM} cells respond to TGF-β in very different ways, and that Smad4 is not required for T_{CD103} expression.

Smad4 is not required for the development of pulmonary T_{RM} cells

Because some CD8 T cells were activated in the irradiated mice, we performed similar experiments with donor cells from OTI TeR transgenic mice (34) that express a receptor that is specific for the SIINFEKL peptide from the chicken OVA gene. Donor cells from OTI-TKO, OTI-SKO, and their respective littermates (lacking Cre or flox sites) were sorted for low CD44 expression using the congenic marker. The recipient animals received mixed populations of OTI-TKO and OTI-WT (or OTI-SKO and OTI-WT) cells 48 h after infection. Each population of transferred cells underwent expansion and contraction with similar kinetics (Fig. 2, Supplemental Table I). Large numbers of OTI-TKO cells expressed KLRG1 10 dpi and CD103 was absent 30 dpi, whereas the donor cells from OTI-SKO mice exhibited a reciprocal phenotype (Fig. 2), which was similar to the tetramer+ CTLs from chimeric mice. Only small numbers OTI-TKO cells expressed CD69 in the lungs (Fig. 2B, hatched shading), indicating limited production of pulmonary T_{RM} cells without CD103. Although CD103 expression was dysregulated in the absence of Smad4, similar numbers of OTI-SKO and OTI-WT cells coexpressed CD69 with CD103 in the lungs 30 dpi (Fig. 2B, black shading). These experiments indicate that although TGF-β plays a critical role in the development/maintenance of pulmonary T_{RM} cells (17) Smad4 is not required.

TGF-β prevents prolonged cell proliferation

The kinetic studies in Fig. 2 show that larger numbers of virus-specific T_{EFF} cells entered the lungs in the absence of TGF-β regulation, whereas the numbers of OTI-SKO cells were very similar to the numbers of OTI-WT cells. To determine whether this variation was due to altered cell proliferation, donor cells from OTI-WT, OTI-SKO, and OTI-TKO mice were labeled with CFSE before transfer and analyzed by flow cytometry 3.5 dpi (Fig. 3A). Other animals were given a single injection of BrdU either 6, 8, or 10 dpi and transferred CTLs were analyzed 3–6 h later (Fig. 3B, Supplemental Fig. 1). The CFSE analysis showed no difference in cell division 3.5 dpi (Fig. 3A), and the CTLs from each group of mice incorporated BrdU at very similar rates 6 dpi (Fig. 3B). At later time points the percentages of BrdU+ CTLs from OTI-TKO mice were much higher than the other groups (Fig. 3B). These studies show that virus-specific CTLs underwent proliferation in the absence of TGF-β regulation. Similar levels of activated caspases-3/7 (Fig. 3C) and prosurvival molecule Bcl-2 (Fig. 3D) indicated that TGF-β did not substantially alter cell survival.

Most long-lived KLRG1+ CTLs remain in the vasculature

Activated CTLs express altered homing receptors in the absence of TGF-β regulation. Because the distribution of virus-specific memory CD8 T cells in the lungs is very important for immunity (3), we used confocal microscopy to compare the distribution of OTI-TKO and OTI-SKO cells during the recovery from IAV infection (Fig. 4). Separate groups of CD45.2+ mice received sorted donor cells from either OTI-TKO or OTI-SKO mice, or their respective littermates as WT controls. The recipient mice were infected with X31-OVA and the lungs were analyzed by confocal microscopy 10 and 30 dpi to compare the distribution of transferred CTLs in each group. The panels on the left side of Fig 4 show the tissue structure, with marked subregions (white boxes) demonstrating KLRG1 and CD103 expression on the transferred CTLs (white shading), as identified using the colocalization function of Imaris software. Abs to EpCAM were used to define the margins of the airways (middle column). In contrast, substantial numbers of CTLs from OTI-WT and OTI-SKO mice expressed CD103 in the airways after the contraction of the T_{EFF} cell response (right columns). Control CTLs from the littermates of OTI-SKO and OTI-TKO mice gave identical results.

**FIGURE 4.** KLRG1+ and CD103+ CTLs maintain different distributions inside the lungs. Congenically marked OTI-WT (top), OTI-TKO (middle), or OTI-SKO cells (bottom) were sorted for low CD44 expression and transferred to C57BL/6 mice before X31-OVA infection. Fragments of lung tissue were stained with Abs to EpCAM for epithelial cells (red), CD31 for blood vessels (yellow), CD45.1 for transferred cells (blue), KLRG1 (green), and CD103 (magenta). Z-stack images were recorded at original magnification $\times 20$ (scale bars, 80 μm). Subregions show (I) colocalization between CD45.1 and KLRG1 10 dpi and (II) colocalization between CD45.1 and CD103 10 and 30 dpi. Representative data are from three animals per group; two experiments gave similar results.
Substantial numbers of OTI-TKO cells expressed KLRG1 30 dpi, indicating that some terminally differentiated CTLs maintain the capacity for long-term survival (Fig. 2). We further examined where these phenotypically distinct subsets of CTLs were located after infection using injected Abs for intravascular staining (35). Congenically marked donor cells were transferred 48 h before infection with X31-OVA and Abs to CD8β were given by i.v. injection 5 min before sacrifice (n = 3/group). Some KLRG1+ CTLs from OTI-TKO and OTI-WT mice were protected from the injected Abs 8 dpi (Fig. 5A, top row), indicating that they entered the lung parenchyma during acute viral infection (36). In contrast, very few KLRG1+ CTLs were protected from the injected Abs 40 dpi (Fig. 5A, bottom row), indicating that “terminally differentiated” CTLs were poorly equipped for long-term survival in peripheral tissues. In contrast, the blood-resident memory CD8 T cells maintained KLRG1 >40 dpi. These long-lived KLRG1+ CTLs universally expressed CD11a at high levels (Fig. 5B), showing that they did not reach the lumen of the airways where high concentrations of host proteases cleave CD11a (37).

We also analyzed the CTLs in the lung parenchyma for markers of T RM cells 40 dpi. As expected, CD103 was expressed on large numbers of OTI-WT cells in the lung parenchyma (Fig. 5C), but it was not expressed on CTLs in the spleens (Fig. 5D) or blood (Fig. 5C, 5D). In contrast, CD103 was widely expressed on OTI-SKO cells, which were equally distributed inside and outside of the blood vessels (Fig 5C, 5D). CD69 is another marker for T RM cells, which is not regulated by TGF-β. Most OTI-WT CTLs coexpressed CD103 together with CD69 in the lung parenchyma.

**FIGURE 5.** KLRG1+ memory CD8 T cells persist in the vasculature. CD45.1+ donor cells from OTI-TKO, OTI-SKO, and their respective littermates were transferred 48 h before infection with X31-OVA. The mice were injected with Abs to CD8β 5 min before harvest. (A) Gated populations of CD45.1+ and KLRG1+ CTLs in the lungs were analyzed for CD8β+ (blood) and CD8β- (tissue) subsets. The CTLs were analyzed 8 dpi (top) and 40 dpi (bottom); n = 3–4 animals/group. Two experiments gave similar results. (B) Lungs were harvested 30 dpi and gated populations of OTI-TKO and OTI-WT cells were analyzed for CD11a and KLRG1 expression; n = 3 animals/group. Two experiments gave similar results. (C–E) Enriched lymphocytes were recovered 40 dpi. Gated CD45.1+ CTLs were divided into CD8β+ and CD8β− subsets. Representative data are from three to four animals per group. Two experiments gave similar results. CTLs in the (C) lungs and (D) spleens were analyzed for KLRG1 and CD103 expression. (E) CTLs in the lungs were analyzed for CD69 and CD103 expression.
showing that they were T_{RM} cells. Importantly, some OTI-SKO cells coexpressed CD69 with CD103 in the lung parenchyma, indicating that they were also pulmonary T_{RM} cells (Fig. 5E).

Some CTLs from each group of mice expressed CD69 in the bloodstream (Fig. 5E) but universally lacked KLRG1 (not shown). Only small percentages (7%) of OTI-TKO cells were located in the lung parenchyma 40 dpi, but 25% expressed CD69 (i.e., 1.75% of total). These data suggest that some T_{RM} cells entered the tissues when CD103 was not expressed, but were not retained in the airways (Fig. 4A). Taken together, our data show that pulmonary T_{RM} cells require TGF-β, but not Smad4, during prolonged residence in the lungs.

**FIGURE 6.** Reduced numbers of T_{CM} cells accumulate in the resting lymph nodes when Smad4 is not expressed. Congenically marked OTI-WT, OTI-TKO, and OTI-SKO cells were sorted for low CD44 expression and transferred to C57BL/6 mice before X31-OVA infection. (A) CD62L and CD103 expression on gated OTI-WT, OTI-TKO, and OTI-SKO cells 30 dpi. Dot plots show representative data from three to four animals per group. Three independent experiments gave similar results. (B) Numbers of CD62L^{+} T_{CM} (black), CD62L^{-} (white), CD62L^{+} CD103^{+} (hatched), and CD62L^{+} CD103^{-} transition (gray fill) cells 30 dpi. Statistical comparisons are shown in Supplemental Table II. (C) Inguinal lymph node images were imaged 20 dpi. CD31 (yellow), CD45.1 (blue), B220 (red), CD103 (magenta). Subregions (white boxes) show enlarged images of transferred CTLs with total numbers of cells in a single slice (means ± SD; n = 3). Z-stack images were recorded at original magnification ×10. Representative data from three animals per group. Two experiments gave similar results. (D) Overlaid histograms show CCR7 expression on OTI-WT (gray fill), OTI-SKO or OTI-TKO (dashed line), and isotype control (solid line). Representative data are from three groups per experiment. Two experiments gave similar results. (E) Mean fluorescence intensity for CCR7 staining. OTI-TKO (hatched), SKO (black), and littermates (white). *p ≤ 0.05.

Smad4 is required for differentiation of central memory T cells

Large numbers of cells from OTI-SKO mice expressed CD103 in the spleen 30 dpi. These unusual CTLs mostly lacked CD62L expression, indicating that they were not central memory T (T_{CM}) cells (Fig. 6A). Small numbers of OTI-WT cells also expressed CD103 without CD69 in the mediastinal lymph node (Fig. 2B, gray fill, Supplemental Table I) but were not present in other tissues, indicating a transitional phenotype. Because T_{CM} cells require CD62L to access resting lymph nodes via high endothelial venules (38), we analyzed the distribution of transferred cells inside the secondary lymphoid organs (Fig. 6C). Substantial

**FIGURE 7.** Mice recover from IAV infection with slightly delayed kinetics when Smad4 is not expressed in peripheral T cells. Animals were infected with X31-OVA and weighed daily. Combined data from two independent experiments are shown. (A) Reduction in body weight as percentage of maximum. TGF-βRII KO (○, n = 7) TGF-βRIIflx/flx (●, n = 6), Smad4KO (□, n = 9), Smad4flx/flx (■, n = 6). *p ≤ 0.05, **p ≤ 0.01. (B) Model illustrating the roles of Smad4 and TGFβ during the differentiation of virus-specific CTLs. Left panel, Normal CD8 T cells give rise to mixed populations KLRG1^{+} T_{EFF} cells, CD103^{+} T_{RM} cells, and circulating memory CD8 T cells. Middle panel, TGF-β signaling reduces the numbers of terminally differentiated T_{EFF} cells and is essential for T_{RM} development. Right panel, Smad4 dependent signaling is required for terminal differentiation of virus-specific T_{EFF} cells and T_{CM} cells.
populations of OTI-WT and OTI-TKO cells were distributed in the cortex of the inguinal lymph nodes 30 dpi, whereas there were very few OTI-SKO cells. Small populations of OTI-SKO cells, which lacked CD62L, were found in the inguinal lymph nodes, indicating that CCR7 was sufficient for efficient transendothelial migration in high endothelial venules (Fig. 6D, 6E). The OTI-SKO cells maintained their CD103+CD62L− phenotype >60 dpi (data not shown), showing that Smad4 has a long-term influence on the differentiation program of TCM cells.

Some KLRG1+ CTLs entered the lung parenchyma during acute IAV infection (36) but mostly disappeared during the contraction of the TEff cell response (Fig. 4). Changes in body weight showed that intact TKO mice recovered from primary IAV infection with similar kinetics as WT animals. In contrast, SKO mice recovered from infection with slightly delayed kinetics, indicating that KLRG1+ T Eff cells participate in viral clearance (Fig. 7A). The OTI-WT, OTI-TKO, and OTI-SKO cells all produced large quantities of IFN-γ and TNF-α after peptide stimulation, confirming that they were functional T Eff cells (Supplemental Fig. 2). Taken together, these data support a model whereby Smad4 plays a pivotal role in the fate decisions of developing virus-specific memory CD8 T cells (Fig. 7B). On the one hand, TGF-β-dependent Smad4-independent signaling pathways subdue the T Eff cell response and play an essential role in the differentiation of CD103+ T RM cells in the lungs. On the other hand, the lineage decisions of other virus-specific CTLs require Smad4, which promotes the production of terminally differentiated T Eff cells and circulating memory CD8 T cells, which survey encapsulated lymph nodes between recurrent infections.

Discussion

TGF-β is an important regulatory factor for peripheral CD8 T cells, which suppresses responses to self-antigens and prevents autoimmune disease (33, 39). This cytokine also plays a critical role in cell-mediated immunity in mucosal tissues, where virus-specific CTLs use TGF-β-dependent adhesion molecules for local migration. A variety of signaling intermediates act downstream of the TGF-β receptor, including Smad2 and Smad3, which are chaperoned into the nucleus by Smad4 (21). This pathway is involved in the differentiation of Th1 cells and production of IgA Abs (14, 40), but it is not known to play a major role in CD8 T cell differentiation. In other situations Smad4 chaperones Smads 1, 5, and 8 into the nucleus after activation by the receptor for bone morphogenic protein; however, these pathways have not been fully analyzed in immune cells (41). Additional pathways that respond to the TGF-β receptor without involving Smad proteins use MAPKs, Rho-like GTPases, or PI3K as signaling intermediates leading to phosphorylation of Akt/S6/Foxo1/3a (42–44). At this time it is not clear which pathways support cell-mediated immunity and CD103 expression on pulmonary T RM cells.

Our studies add an important piece to the puzzle by showing that Smad4 is not required for the development of CD103+CD69+ T RM cells in the lungs but is required for normal differentiation of multiple subsets of circulating CD8 T cells that use the blood-stream to move around the body. The Smad4-dependent subsets include KLRG1+ T Eff cells, which proliferate extensively in response to cytokine costimulation (45, 46), as well as central memory CD8 T cells, which use CD62L to access resting lymph nodes between recurrent infections (47). Substantial numbers of Smad4-deficient CTLs survived the contraction of the T Eff cell response and lacked CD62L but expressed CCR7 together with CD103, which are not characteristics of effector memory CD8 T cells. This abnormal phenotype may reflect enhanced use of alternative signaling molecules downstream of the TGF-β receptor, such as MAPKs, Rho-like GTPases, and PI3K, or Smad proteins that can function without Smad4 (48). Because the TGF-β receptor does not have exclusive control over Smad4, the effects of an alternative receptor cannot be excluded. Such signaling could begin with the receptor for bone morphogenetic protein or another unidentified cytokine. We speculate that when all signaling pathways are intact, Smad4 suppresses CD103 expression on Ag-experienced CD8 T cells by a mechanism that can be overridden by high concentrations of TGF-β in mucosal tissues.

Our data provide important insights into the mechanisms that control the distribution of virus-specific CTLs inside the lungs during IAV infection. Epithelial cells are an important site of viral replication and release large quantities of infectious virus into the lungs. Irreparable damage can occur when the virus reaches the alveoli and causes severe pulmonary effusion (49). Although TGF-β and Smad4 play minor roles in viral clearance during primary infection with this mildly pathogenic strain of IAV, dysregulated CTL responses may have a more serious impact on heterosubtypic immunity when pulmonary T RM cells provide frontline defenses in the lungs. We found most T RM cells embedded in the walls of the large airways, which is an ideal position for controlling viral replication. Although pulmonary T RM cells can reduce viral burdens with little assistance from T CM cells (3), diverse memory populations are required for immunity to frequently encountered pathogens, because T CM cells replenish CTL populations in remote parts of the body, including pulmonary T RM cells (3, 50).

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Representative gates for BrdU analysis. Lymphocytes were harvested from the MLN 8dpi and analyzed for; A) live cells; B) lymphocytes; C) Singlets; D) CD8; E) Congenic markers and F) BrdU.
Supplementary Figure 2
Congenically marked OTI-WT, OTI-TKO and OTI-SKO cells were sorted for low CD44 expression and transferred to C57BL/6 mice before X31-OVA infection. Lymphocytes were isolated from the lungs 40 dpi and stimulated with SIINFEKL peptide for 5 hrs in the presence of Brefeldin A. The percentages of donor cells producing IFN\(_\gamma\) and TNF\(_\alpha\) are shown. Two experiments gave similar results.
### Supplemental Table 1. Statistical comparisons for Figure 2.

An unpaired two-tailed Student’s t test was used to compare the numbers of mutant and wild type cells after transfer (ns = not significant; * P < 0.05; ** P <0.01; *** P < 0.005). Red lettering indicates larger numbers of mutant CTLs and blue lettering indicates larger numbers of wild-type CTLs.
### OTI-TKO vs. OTI-WT

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### OTI-SKO vs. OTI-WT

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### Supplemental Table 2. Statistical comparisons for Figure 6B.

An unpaired two-tailed Student's t test was used to compare the numbers of mutant and wild type cells after transfer (ns = not significant; * P < 0.05; ** P <0.01; *** P < 0.005). Red lettering indicates larger numbers of mutant CTLs and blue lettering indicates larger numbers of wild-type CTLs.