DIFFERENTIATION, TISSUE ADAPTATION AND FUNCTION OF MEMORY T CELLS

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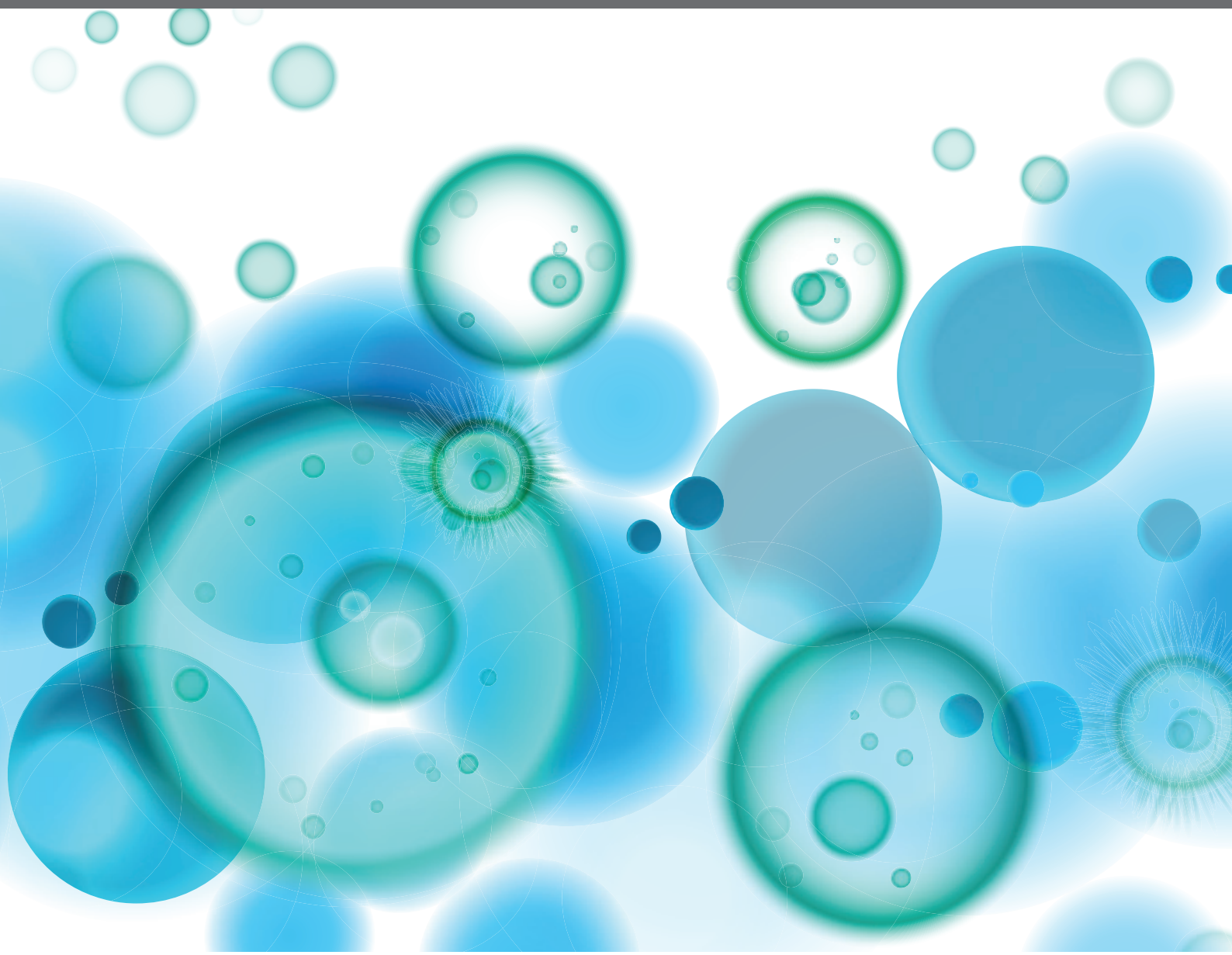

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DIFFERENTIATION, TISSUE ADAPTATION AND FUNCTION OF MEMORY T CELLS

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Upon antigen encounter, naïve T cells differentiate into (i) effectors that combat infected or malignant cells, and at later time points, into (ii) memory cells that provide long-lasting immunity. This differentiation process allows some T cells to leave the confines of secondary lymphoid organs and to enter peripheral tissues in search of pathogens or tumor cells. These different environments pose specific challenges for effector and memory T cells to maintain homeostasis. T cells directed into the lungs are likely to encounter higher levels of oxygen, but lower amounts of nutrients than those directed into the intestinal epithelium. In addition to oxygen tension and nutrient concentrations, other key factors, such as the commensal flora and stromal components, create unique conditions that require tissue-specific adaptations of T cells. These steady state conditions can dramatically change during infection when inflammatory mediators and T cell growth factors are released, requiring the immediate response of T cells. The gradual changes imposed by growing tumors can also be challenging for T cells due to competition with rapidly cycling tumor cells that deplete essential resources of oxygen and glucose.

The strategies that T cells employ to respond to the diverse cues from their surroundings are the focus of current research. It appears that next to circulating memory T cells that are confined to the circulation and those that survey all of the peripheral tissues, dedicated populations of resident memory T cells exist that can optimally adapt to the local circumstances within each tissue. Restrictions on the metabolic requirements of T cells residing in tumor tissue have been found to directly impact on effector functions such as cytokine production. The fundamental principles of how the machinery of T cells can translate local cues into tissue-specific differentiation processes are fascinating and warrant further investigation.

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Short Lifespans of Memory T-cells in Bone Marrow, Blood, and Lymph Nodes Suggest That T-cell Memory Is Maintained by Continuous Self-Renewal of Recirculating Cells

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Memory T-cells are essential to maintain long-term immunological memory. It is widely thought that the bone marrow (BM) plays an important role in the long-term maintenance of memory T-cells. There is controversy however on the longevity and recirculating kinetics of BM memory T-cells. While some have proposed that the BM is a reservoir for long-lived, non-circulating memory T-cells, it has also been suggested to be the preferential site for memory T-cell self-renewal. In this study, we used *in vivo* deuterium labeling in goats to simultaneously quantify the average turnover rates—and thereby expected lifespans—of memory T-cells from BM, blood and lymph nodes (LN). While the fraction of Ki-67 positive cells, a snapshot marker for recent cell division, was higher in memory T-cells from blood compared to BM and LN, *in vivo* deuterium labeling revealed no substantial differences in the expected lifespans of memory T-cells between these compartments. Our results support the view that the majority of memory T-cells in the BM are self-renewing as fast as those in the periphery, and are continuously recirculating between the blood, BM, and LN.

Keywords: bone marrow, memory T-cells, lymphocyte turnover, lifespan, stable isotope labeling, deuterium, mathematical modeling

INTRODUCTION

Immunological memory, the ability of the immune system to respond more quickly and strongly upon repeated antigen exposure, is the hallmark of the adaptive immune system. T-cell memory generated after the first antigen encounter can last for decades, and provides long-lasting immune protection (1, 2). It has convincingly been shown that T-cells with a memory phenotype in human blood renew quite often and are not maintained by a long cellular lifespan (3–11). It is important to realize, however, that most insights into memory T-cell maintenance in humans have been based on cells from peripheral blood. At any given moment in time only a very small fraction of the total body lymphocyte pool is present in the blood (12, 13), whereas the vast majority of memory T-cells are located in lymphoid and non-lymphoid tissues (14). This raises the question whether T-cell lifespan estimates based on cells from peripheral blood are also representative for T-cells located in tissues.

Physiological T-cell niches are an important factor in the maintenance of T-cell memory (15, 16). The bone marrow (BM) has recently attracted a lot of attention as a reservoir for memory T-cells (17–20). Although it is well established that memory T-cells are abundantly present in the BM, preferentially home there following infection (19, 21), and are able to expand in the BM following antigen re-challenge (22), the exact role of BM in the maintenance of T-cell memory is less clear (16, 23). BM has been shown to be a niche for memory T-cells that rest in terms of proliferation, transcription, and migration (18, 24). Hence, BM has been proposed as the place where memory T-cells with long lifespans reside. Other studies have suggested however that memory T-cells in BM are more actively proliferating than those in lymph nodes (LN) (17, 25), suggesting that BM provides the appropriate environment for memory T-cells to self-renew. In an attempt to reconcile the conflicting literature, it has been proposed that BM might provide two distinct niches for recirculating memory T-cells, one which supports cycling of memory T-cells, and another that provides a niche for quiescent memory T-cells (16, 26).

Studies addressing the dynamics of BM memory T-cells have used different models and techniques. In mouse studies, both kinetic markers, such as bromodeoxyuridine (BrdU) and carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling (17, 25, 27), and static markers, such as Ki-67 expression (19, 24), have been used to determine the proliferative status of BM memory T-cells. Dynamic markers provide rich information on the division history of the cells, but BrdU labeling has been linked to cellular toxicity (24, 28) and CFSE labeling requires *ex vivo* cell manipulation, which may interfere with cell homeostasis. A static marker like Ki-67 describes the division status of a cell at a given moment and location, but provides no information about cellular lifespans, and does not take into account that a cell may have proliferated previously, or elsewhere. In human studies, only static markers have been used to assess memory T-cell proliferation in organs other than blood (18). Another point to consider is that in mouse experiments, cell dynamics in BM have typically been compared to those in lymphoid organs, while human studies have based their comparisons on blood-derived cells. The debate in the literature together with the array of different approaches used to estimate the lifespan of BM memory T-cells highlights the difficulty of assessing how memory T-cell populations are maintained, in particular those located outside the blood.

In this study, we simultaneously quantified the dynamics of memory $CD4^+$ and $CD8^+$ T-cells in BM, blood, and lymphoid organs using *in vivo* stable isotope labeling, the state of the art technique to study lymphocyte dynamics *in vivo*. One of the great advantages of this technique is that the turnover of a cell population is traced regardless of time and space, allowing us to reliably follow the division history of a population. In addition, *in vivo* deuterium labeling is non-toxic and does not require *ex vivo* cell manipulation, enabling the study of an unperturbed system. To simultaneously quantify the lifespans of memory $CD4^+$ and $CD8^+$ T-cells in blood, BM and lymphoid organs we made use of the goat as animal model, taking advantage of its relatively large size to obtain enough T-lymphocytes from paired samples of blood, BM, and LNs.

MATERIALS AND METHODS

Goats

Female adult goats ($N = 34$) were purchased from commercial farms and housed at Wageningen Bioveterinary Research, Lelystad, The Netherlands. Additional one-off surplus material from single blood samples taken for mandatory routine diagnostic tests were obtained from 8 adult female goats housed at the Department of Farm Animal Health, Faculty of Veterinary Medicine of the Utrecht University were used for IFN-ɣ ELISA assay.

Ethics

This study was carried out in accordance with national regulations on animal experimentation. The protocol was approved by the animal experiment commissions of Wageningen Bioveterinary Research (permit number AVD401002016580).

In vivo Stable Isotope Labeling

Deuterated water (^2H_2O) (99.8%; Cambridge Isotope Laboratories) was diluted to 4% in drinking water and administered *ad libitum* for 28 days. To determine deuterium enrichment in the body water, heparin plasma was collected during the up- and down-labeling phase, and was frozen and stored at −20°C until analysis.

Sampling and Cell Preparation

Randomly selected animals were sacrificed by intravenous injection of a lethal dose of pentobarbital (Euthasol, AST Farma, Oudewater, The Netherlands) at 17 different time points after start of label administration. During necropsy, the left and right pre-scapular LNs and the middle part of the sternum were isolated. Venous blood was collected from the jugular vein in heparinized Vacutainer (BD Biosciences) tubes prior to injection with pentobarbital. Single cell suspensions from LN were obtained by mechanical disruption, and from BM by flushing the sternum. BM cell suspensions were lysed with lysis buffer (155 mM ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM Na2-EDTA, pH = 7.0). Peripheral blood mononuclear cells (PBMCs) were isolated from blood using SepMate-50 tubes (Stemcell Technologies) and Ficoll-Paque Premium (GE Healthcare) following the manufacturer's protocol. The SepMAte-50 tubes were centrifuged at 1,400 g for 20 min. PBMCs were collected, spun down, and washed prior to cell staining and sorting.

Flow Cytometry and Cell Sorting

BM and LN cell suspensions and PBMCs were stained for extracellular markers using CD4-AF647 (clone 44.38, AbD Serotec), CD8-PE (clone 38.65, AbD Serotec), CD62L (clone DUI-29, WSU) conjugated with pacific blue (PB) (Zenon PB mouse-IgG1 labeling kit, Life Technologies), CCR7-PeCy7 (clone 3D12, BD Biosciences), and CD14-Viogreen (clone TÜK4, Miltenyi Biotec) monoclonal antibodies.

For intracellular markers, cells were subsequently fixed, permeabilized (Cytofix/Cytoperm; BD Biosciences), and stained intracellularly with Ki-67-FITC monoclonal antibody (clone B56, BD Biosciences). Washing steps for intracellular staining were performed using Perm/Wash buffer (BD Biosciences). Double positive ($CD4^+CD8^+$) thymocytes were used to determine the positive gate for Ki-67, since double positive thymocytes have a clear population of cycling cells (**Supplementary Figure 5A**). Cells were analyzed on an LSR-Fortessa flow cytometer using FACS Diva software (BD Biosciences). Cells were sorted with a purity >93% on a FACSAria III cell sorter (BD Biosciences) using FACS Diva software (BD Biosciences). $CD62L^+CCR7^+$ (double positive naive, DP-N) and $CD62L^-CCR7^-$ (double negative memory, DN-M) $CD4^+$ and $CD8^+$ T-cells were sorted for transcriptome analysis (**Supplementary Figure 1A**). For deuterium enrichment analysis, cells were stained for CCR7 and CD62L and were sorted based only on CCR7 expression; $CCR7^+$ (naive) and $CCR7^-$ (memory) $CD4^+$ and $CD8^+$ lymphocytes were sorted from blood, BM and LN (**Supplementary Figures 1B,C**). Granulocytes were sorted from lysed whole blood based on their FSC and SSC characteristics and used for deuterium enrichment analysis (**Supplementary Figure 1D**). $CD62L^+CCR7^+$ (DP-N), $CD62L^-CCR7^-$ (DN-M), $CD62L^+CCR7^-$, $CD62L^-CCR7^+$, total $CCR7^-$ (memory) and total $CCR7^+$ (naive) $CD4^+$ and $CD8^+$ T-cells were sorted for functional assays.

RNA Isolation

For RNA isolation, FACS sorted $CD62L^-CCR7^-$ (DN-M), $CD62L^+CCR7^+$ (DP-N) $CD4^+$, and $CD8^+$ T-cells from blood and $CD62L^-CCR7^-$ (DN-M) $CD8^+$ T-cells from BM were sorted, spun down, and stored at −80°C prior to RNA extraction. Before thawing, cells were immersed in QIAzol Lysis Reagent (Qiagen). RNA was isolated and purified using the RNeasy kit (Qiagen). The concentration was measured on a NanoDrop ND-2000 (Thermo Scientific) and RNA integrity was examined using the 2200 TapeStation System with Agilent RNA ScreenTapes (Agilent Technologies).

Microarray

Total RNA (50 ng) combined with Spike A was used for amplification and labeling according to the Two-Color Microarray-Based Gene Expression Analysis guide using the Low Input Quick Amp Labeling Kit (Agilent Technologies). For the common reference an equimolar pool of all samples was made and amplified similarly as the test samples with the exception that Spike B was used. Synthesized aRNA was purified with the E.Z.N.A. MicroElute RNA Clean Up Kit (OMEGA bio-tek). The yields of aRNA and CyDye incorporation were measured on the NanoDrop ND-2000. An Agilent microarray (8 × 60 k) was custom-designed using the Agilent e-array microarray design tool v.7.6. The array contains 2,726 negative control probes, 1,319 Agilent control probes and 47,151 probes designed on transcripts from the goat (*Capra hircus*) genome GenBank assembly GCF_001704415.1_ARS1_rna transcripts (July 5, 2017) NCBI repository. Hybridization, washing, and scanning were performed according to manufacturer's instructions with an Agilent G2565CA scanner (Agilent Technologies).

Microarray Data Processing and Normalization

Raw data was read and normalized in R (Version 3.4.0) using the Limma package of Bioconductor. The "Normexp" method with offset = 16, was used for background correction and the resulting data was Quantile Normalized. Empirical Bayes statistics with Benjamini-Hochberg (BH) false discovery rate (FDR) correction was used to obtain statistical output for all to all comparisons.

IFN-γ ELISA

Sorted $CD62L^+CCR7^+$ (DP-N), $CD62L^-CCR7^-$ (DN-M), $CD62L^+CCR7^-$, $CD62L^-CCR7^+$, $CCR7^-$ (memory), and $CCR7^+$ (naive) $CD4^+$ and $CD8^+$ T-cells from blood and LN were cultured and stimulated with PMA (20 ng/ml) and ionomycin (1 ng/ml) for 70 h. Supernatant was collected at 20 and 70 h after stimulation, IFN-γ production was measured using the BOVIGAM TB IFN-γ ELISA kit (Bovigam). Samples were tested in triplicates. IFN-γ optical density (OD) from unstimulated samples (background) was subtracted from the OD of stimulated samples.

DNA Isolation

Genomic DNA was isolated from $CD4^+CCR7^+$ (naive), $CD4^+CCR7^-$ (memory), $CD8^+CCR7^+$ (naive), and $CD8^+CCR7^-$ (memory) T-cells sorted from blood, LN and BM, and granulocytes using the ReliaPrep Blood gDNA Miniprep System (Promega, Madison, WI, USA) and stored at −20°C before processing for gas chromatography/mass spectrometry (GC/MS).

Measurement of ^2H_2O Enrichment in Body Water and DNA

Deuterium enrichment in plasma and DNA was measured by GC/MS using an Agilent 5973/6890 GC/MS system (Agilent Technologies). Plasma was derivatized to acetylene (C_2H_2, M = 26) as previously described (29). The derivative was injected into the GC/MS equipped with a PoraPLOT Q 25 × 0.32 column (Varian), and measured in SIM mode monitoring ions m/z 26 (M+0) and m/z 27 (M+1). From the ratio of ions, plasma deuterium enrichment was calculated by calibration against $^2H_20$ standards of known enrichment. DNA obtained from sorted lymphocytes and granulocytes was hydrolyzed to deoxy-ribonucleotides and derivatized to penta-fluoro-triacetate (PFTA, M = 435) (29). The derivative was injected into the GC/MS equipped with a DB-17 column (Agilent Technologies) and measured in SIM mode monitoring ions m/z 435 (M+0), and m/z 436 (M+1). From the ratio of ions, we calculated the deuterium enrichment in the DNA by calibration against deoxyadenosine standards of known enrichment as previously described (6).

Mathematical Modeling of Plasma and DNA Enrichment Data

To control for changing levels of ^2H in body water over the course of the experiment, a simple label enrichment/decay curve was fitted to ^2H enrichment in plasma:

$$\underline{\text{during label intake } (t \leq \tau)}: S(t) = f(1 - e^{-\delta t}) \quad (1a)$$

$$\underline{\text{after label intake } (t > \tau)}: S(t) = [f\left(1 - e^{-\delta t}\right)]e^{-\delta(t-\tau)} \quad (1b)$$

as described previously (6) (with minor modification because we did not give an initial boost of label), where *S(t)* represents the fraction of ^2H_2O in plasma at time t (in days), f is the fraction of ^2H_2O in the drinking water, labeling was stopped at $t = \tau$ days, and δ represents the turnover rate of body water per day. The best fit for *S(t)* was used in the labeling equations for the different cell populations (see below). Up- and down-labeling of the granulocyte population was analyzed as previously described (6), to estimate the maximum level of label intake that cells could possibly attain (**Supplementary Figure 4** and **Supplementary Tables 1**, **2**). The label enrichment data of all cell subsets were subsequently scaled by the granulocyte asymptote (6).

A mathematical model that allowed for kinetic heterogeneity between cells of the same population was fitted to the labeling data of the different leukocyte subsets. Each kinetic sub-population i was modeled to contain a fraction α_i of cells with turnover rate p_i. Because we observed that the population sizes hardly changed during the labeling and de-labeling phases of our study (data not shown), we considered a steady state for each kinetic sub-population (i.e., production equals loss), and label enrichment of adenosine in the DNA of each sub-population i was modeled by the following differential equation:

$$\frac{dl_i}{dt} = p_i c S(t)\, \alpha_i A - p_i l_i \quad (2a)$$

where l_i is the total amount of labeled adenosine deoxyribose (dR) in the DNA of sub-population i and A is the total amount of adenosine in the cell population under investigation, c is an amplification factor that needs to be introduced because the adenosine dR moiety contains multiple hydrogen atoms that can be replaced by deuterium (6), and p_i is the average turnover rate of sub-population i. Basically, labeled adenosines in sub-population i are gained when a deuterium atom is incorporated with probability $cS(t)$ in the DNA of cells that replicate at rate p_i, and labeled adenosine is lost when cells of sub-population i are lost at rate p_i. For naive T-cells this replication may occur both in the periphery and in the thymus. Scaling this equation by the total amount of adenosine in the DNA of sub-population i, i.e., defining $L_i = l_i/(\alpha_i A)$, yields

$$\frac{dL_i}{dt} = p_i c S(t) - p_i L_i \quad (2b)$$

throughout the up- and down-labeling period, where L_i represents the fraction of labeled adenosine dR moieties in the DNA of sub-population i. The corresponding analytical solutions are

$$L_i(t) = \frac{c}{\delta - p_i}[\delta f\left(1 - e^{-p_i t}\right) - p_i f\left(1 - e^{-\delta t}\right)] \quad (3a)$$

during label intake ($t \leq \tau$), and

$$L_i(t) = \frac{c}{\delta - p_i}[\delta f\left(e^{-p_i(t-\tau)} - e^{-p_i t}\right) - p_i f\left(e^{-\delta(t-\tau)} - e^{-\delta t}\right)] \quad (3b)$$

after label intake ($t > \tau$).

The fraction of labeled DNA in the total T-cell population under investigation was subsequently derived from $L(t) = \sum \alpha_i L_i(t)$, and the average turnover rate p was calculated from $p = \sum \alpha_i p_i$. Average lifespans were calculated as $1/p$.

Because all enrichment data were expressed as fractions, labeling data were arcsin(sqrt) transformed before the mathematical model was fitted to the data. We followed a stepwise selection procedure to determine the number of kinetically different subpopulations to include in the model, adding a new kinetically different subpopulation into the model until the average turnover rate no longer significantly changed (4). For populations that appeared to behave kinetically homogeneously, the fitting procedure set the contribution of the extra subpopulation(s) to zero. The labeling curves of $CD4^+$ $CCR7^-$ (memory) T-cells in blood, LN, and BM as well as $CD8^+$ $CCR7^-$ (memory) T-cells in blood were significantly better described by a model including two kinetically different subpopulations while the other populations required only one.

Statistical Analysis

Differences between groups were assessed using Wilcoxon signed-rank test (GraphPad, software, Inc., La Jolla, CA, USA). Deuterium-enrichment data were fitted with the function nlm in R. The 95% confidence intervals were determined using a bootstrap method where the residuals to the optimal fit were resampled 500 times. Differences with a p-value <0.05 were considered significant.

RESULTS

Flow Cytometric Characterization of Goat Blood, BM, and LN-Derived T-cells

To study the *in vivo* dynamics of memory T-cells simultaneously in blood, BM and LN, we made use of adult female goats as animal model. Immunophenotypic analysis showed that, in goats, BM lymphocytes include on average 1.4% $CD4^+$ and 6.7% $CD8^+$ T-cells, much lower percentages than in blood (18.2% $CD4^+$ and 24.4% $CD8^+$) and LN (31.9% $CD4^+$ and 16.4% $CD8^+$). BM also presented a lower CD4/CD8 ratio compared to blood and LN (**Figure 1A**, **Table 1**). In addition, $CD4^+$ and $CD8^+$ T-cells from BM consistently expressed lower levels of selectin-L (CD62L) and CC chemokine receptor 7 (CCR7), molecules that facilitate T cell homing to lymphoid tissues, than T-cells from blood and LN, with the majority of BM T-cells being $CCR7^-CD62L^-$ (**Figure 1B**, **Table 1**). This flow cytometric characterization suggests that T-cells obtained by flushing the

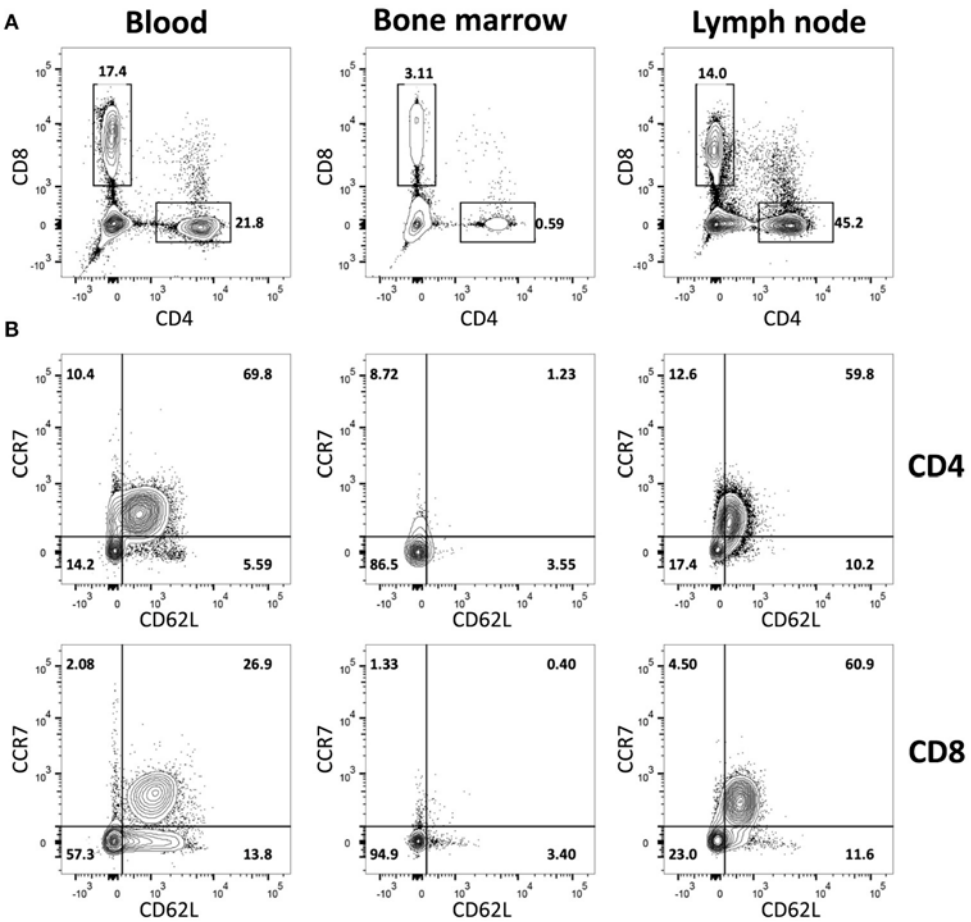

FIGURE 1 | Immunophenotypic characterization of BM-derived T-cells. Blood-, BM- and LN-derived mononuclear cells were obtained from healthy goats. **(A)** Staining of $CD4^+$ and $CD8^+$ lymphocytes and **(B)** of $CD62L^+$ and $CCR7^+$ within $CD4^+$ *(top row)* and $CD8^+$ *(bottom row)* T-cells isolated from blood, BM, and LN of a representative goat.

sternum are a phenotypically distinct population from T-cells found in peripheral blood. CD62L and CCR7 are predominantly expressed by naive T-cells cells and their lack of expression is a hallmark of memory T-cells in both human (30) and mouse (31, 32). We focused on these markers, because of the limited availability of monoclonal antibodies for studies in goats, to separately analyse the $CCR7^+CD62L^+$ and the $CCR7^-CD62L^-$ T-cell populations assuming these to be enriched in naive and memory T-cells, respectively.

$CCR7^-CD62L^-$ T-cells Present Transcriptional and Functional Features of Memory T-cells

To validate the memory and naive phenotype of goat T-cells, we performed microarray based gene expression analysis and IFN-γ release analysis on $CCR7^-CD62L^-$ (double negative memory, in short DN-M), cell subset likely enriched for memory T-cells, and $CCR7^+CD62L^+$ (double positive naive, in short DP-N), cell subset likely enriched for naive T-cells, T-cells. *Transcriptome* analysis on DN-M and DP-N $CD4^+$ T-cells from blood and $CD8^+$ T-cells from blood and BM confirmed at transcriptional level the expression of CD4, CD8A, CD8B, CCR7, and CD62L, and showed high expression of CD3E in all the samples despite the fact that $CD4^+$ and $CD8^+$ T-cells were not sorted based on CD3, because anti CD3 antibody is not available for goat (**Figure 2A**). Multidimensional scaling (MDS) was used to visualize all the expression data. In an MDS geometrical plot, distance between points reflects similarity between samples. In the MDS plot, samples segregated by cell type and organ of origin. Within $CD4^+$ T-cells, the DP-N and DN-M populations clustered separately; within the $CD8^+$ T-cell subset, DN-M cells also clustered together and separately from DP-N cells. $CD8^+$ DN-M T-cells from BM origin clustered together and showed more similarity to blood $CD8^+$ DN-M than to $CD8^+$ DP-N T-cells (**Figure 2B**). Taken together, these results suggest that CCR7 and CD62L expression define transcriptionally distinct T-cell subsets.

Differential gene expression analysis showed, in agreement with gene expression profiles from human and mouse (34),

TABLE 1 | Immunophenotypic characterization of BM-derived T-cells.

	Blood ($N = 18$)		BM ($N = 20$)		LN ($N = 18$)	
Lymphocytes	59.0 (17.6)		23.6 (6.7)		69.9 (12.7)	
$CD4^+$	18.2 (7.0)		1.4 (1.9)		31.9 (12.2)	
$CD8^+$	24.4 (8.1)		6.7 (6.3)		16.4 (4.6)	
CD4/CD8 ratio	0.9 (0.6)		0.2 (0.1)		2.1 (1.0)	
	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
$CCR7^-$ (memory)	40.0 (17.5)	80.2 (13.3)	88.3 (5.7)	97.8 (1.3)	30.2 (7.7)	46.0 (16.1)
$CD62L^-$	45.5 (17.1)	64.6 (14.8)	92.7 (4.3)	94.0 (2.6)	41.6 (14.8)	43.0 (13.7)
$CCR7^-CD62L^-$ (DN-M)	26.5 (9.0)	59.8 (13.1)	83.4 (6.7)	92.3 (2.6)	20.7 (6.9)	32.6 (14.0)

Flow cytometric analysis was used to assess the percentage of blood, LN and BM-derived mononuclear cells expressing CD4, CD8, CCR7, and CD62L. Percentage lymphocytes based on FSC/SSC within the total events, percentage of $CD4^+$ and $CD8^+$ cells within total lymphocytes, CD4/CD8 ratios, and percentage of $CCR7^-$ (memory), $CD62L^-$ and $CCR7^-CD62L^-$ (DN-M) cells within the total $CD4^+$ and $CD8^+$ lymphocyte populations are shown. N = number of animals analyzed. We report mean and standard deviation (SD).

that a small percentage of genes were expressed at significantly different levels between memory and naive T-cells. Applying the criteria for significance, for $CD4^+$ samples, we identified 262 differentially expressed genes [adjusted p-value (BH) $<$ 0.05], corresponding to 0.5% of the total (**Figure 2C**). The differentially expressed genes with an absolute $\log_2$ fold change $\geq$ 1 included genes previously identified to play a role in the differentiation from naive to memory, such as *IL17RB*, *CCR4* (35) and *RUNX2* (36), which were up-regulated, and *SOX4* and *Bach2* (36) which were down-regulated in DN-M as compared to DP-N $CD4^+$ T-cells (**Figure 2C**, **Supplementary Table 3**). Memory and naive $CD8^+$ T-cells generally show more differentially expressed genes than $CD4^+$ T-cells (34); in accordance, we identified 984 differentially expressed genes (2% of the total) between DN-M and DP-N $CD8^+$ T-cells (**Figure 2D**). Significantly up-regulated genes ($\log_2$ fold change $\geq$1) in DN-M compared to DP-N $CD8^+$ T-cells included genes promoting T-cell survival and homeostasis, including *TNFS1B* and *IL12RB2*, molecules involved in immune activation, such as CD58, and genes involved in the cytotoxic effector function of T-cells, like *GZMA* and *CD244* (34). *IL7R* and *TCF7*, genes involved in naive T-cell maintenance (37, 38), were down-regulated in DN-M $CD8^+$ T-cells (**Figure 2D**, **Supplementary Table 3**). For a supervised analysis, we used the conserved transcriptional signature of $CD4^+$ and $CD8^+$ memory T-cell differentiation describing the genes that are up-regulated in memory compared to naive T-cells in both human and mice (33). Goat $CD4^+$ DN-M T-cells showed up-regulated expression of all the genes composing the adaptive memory signature. For $CD8^+$ T-cells, we found that the conserved CD8 memory signature described by Haining et al. (33) was enriched in goat $CD8^+$ DN-M T-cells. Twenty out of 36 genes were up-regulated in $CD8^+$ DN-M T-cells in the 3 different goats (**Figure 2E**).

Finally, to *functionally* validate the memory phenotype of $CCR7^-CD62L^-$ T-cells in goats, we analyzed the ability of DN-M and DP-N T-cells to produce IFN-γ after stimulation (see methods). We found that 70 h after stimulation, $CD4^+$ and $CD8^+$ DN-M T-cells were able to produce higher amounts of IFN-γ than their DP-N counterparts. In addition, DN-M T-cells reacted faster to stimulation, as a substantial amount of IFN-γ production was already detected at 20 h (**Figure 2F**). Altogether, these data support the interpretation that in goats $CCR7^-CD62L^-$ (DN-M) T-cells are memory T-cells. In fact, we found the production of IFN-γ to be reliant on the expression of CCR7, not on CD62L, as $CCR7^-CD62L^+$ T-cells produced similar amounts of IFN-γ as $CCR7^-CD62L^-$ and $CCR7^-$ T-cells, and higher amounts than $CCR7^+CD62L^+$ and $CCR7^+$ T-cells (**Supplementary Figure 2**). Given that just a small fraction of the total population of $CCR7^-$ T-cells expressed CD62L, we therefore focused the kinetic analyses on the $CCR7^-$ population (from now on referred to as memory cells).

Memory T-cells in BM and LN Contain Lower Percentage of Ki-67 Positive Cells Than Memory T-cells in Blood

To study the dynamics of memory T-cells in the different compartments, we first measured the percentage of Ki-67 positive cells in paired samples from blood, BM and LN $CD4^+$ and $CD8^+$ memory ($CCR7^-$) T-cells. Ki-67 is a nuclear protein expressed during all phases of the cell cycle except for G_0, thus actively dividing and recently divided cells express high levels of Ki-67. The percentage of Ki-67^+ cells was significantly lower in memory T-cells from BM and LN compared to those in blood (p-values $<$ 0.0001), with an average fraction of Ki-67^+ cells of 3.2% of $CD4^+$ and 3.9% of $CD8^+$ memory T-cells from blood, 1.1% of $CD4^+$ and 1.3% of $CD8^+$ memory T-cells from BM, and 1.1% of $CD4^+$ and 1.3% of $CD8^+$ memory T-cells from LN (**Figures 3A,B** and **Supplementary Figure 5**). These results are in agreement with previous reports suggesting that memory T-cells in BM show less signs of active cell-division than their counterparts in blood (18, 19, 24).

Memory T-cells From Blood, BM and LN Have Similar Turnover Rates

Low percentages of Ki-67 positive cells in memory T-cells from BM have been interpreted as a sign that BM is the place where long-lived memory T-cells reside (18). However, Ki-67 inherently provides no information on the longevity of the cells. We therefore used *in vivo* deuterium labeling to quantify the turnover

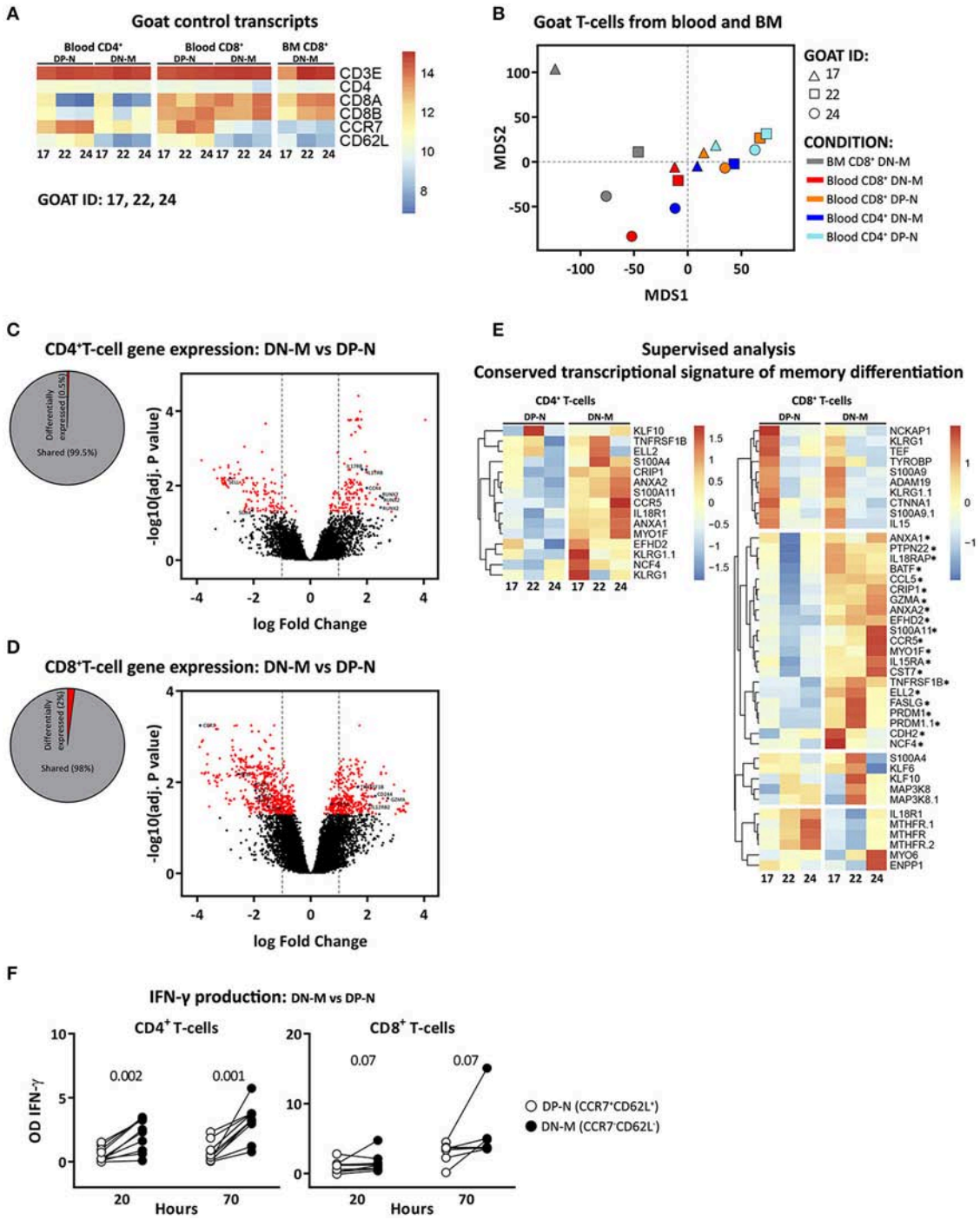

FIGURE 2 | CCR7$^-$CD62L$^-$ (DN-M) T-cells present transcriptional and functional characteristics of memory T-cells. Microarray profiling was performed on DN-M (CCR7$^-$CD62L$^-$) and DP-N (CCR7$^+$CD62L$^+$) CD4$^+$ and CD8$^+$ T-cells from blood and DN-M CD8$^+$ T-cells from BM of 3 goats (goat 17, 22, and 24). **(A)** Heatmap showing normalized expression levels of control genes, CD3E, CD4, CD8A, CD8B, CCR7, and CD62L for all the samples. **(B)** Multidimensional scaling (MDS) of DN-M and DP-N samples from blood and BM for CD4$^+$ and CD8$^+$ T-cell subsets, based on the global transcriptome (~47,151 probes). **(C**,**D)** Diagram showing the percentage significantly differentially expressed genes (adjusted *p*-value (BH) < 0.05) between DN-M and DP-N CD4$^+$ **(C)** or CD8$^+$ **(D)** T-cells from blood, as well as volcano plots illustrating the $\log_2$ fold change differences in gene expression levels between DN-M and DP-N CD4$^+$ **(C)** or CD8$^+$ **(D)** T-cells from blood. Significantly differentially expressed genes (adjusted p-value (BH) < 0.05) are shown in red, blue dots depict genes related to memory differentiation. **(E)** Heatmap showing the normalized expression of genes from the adaptive memory signature (33) in CD4$^+$ T-cells *(left panel)*; and of genes from the conserved CD8 memory signature (33) in CD8$^+$ T-cells *(right panel)*. Genes up-regulated in DN-M compared to DP-N CD8$^+$ T-cells in the 3 different goats are marked with an*. Gene expression is scaled per row. **(F)** DN-M (CCR7$^-$CD62L$^-$) and DP-N (CCR7$^+$CD62L$^+$) CD4$^+$ and CD8$^+$ T-cells sorted from blood were cultured *in vitro* for 70 h in the presence of PMA/ionomycin. Mean IFN-γ production, measured from the supernatant by ELISA, at 20 and 70 h after stimulation is shown as the OD of stimulated samples minus the OD of the background (unstimulated sample). *P*-values obtained using the Wilcoxon signed-rank test are shown.

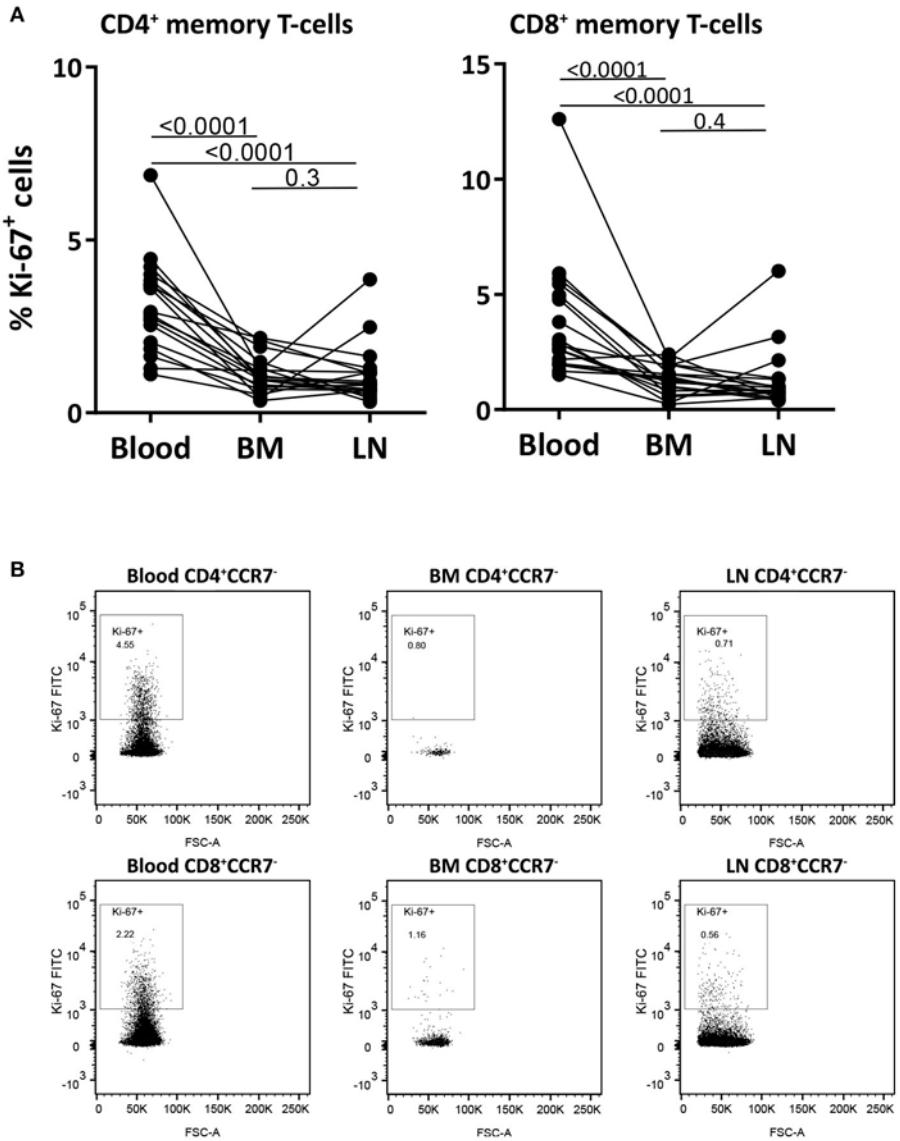

FIGURE 3 | Memory T-cells from blood have higher percentages of Ki-67 positive cells than those from BM and LN. **(A)** The fraction of memory (CCR7$^-$) CD4$^+$ and CD8$^+$ T-cells expressing the proliferation marker Ki-67 was assessed in paired samples from blood, BM, and LN. Paired samples were compared using the Wilcoxon signed-rank test, *p*-values are shown. **(B)** Intracellular Ki-67 staining of CD4$^+$ and CD8$^+$ memory (CCR7$^-$) T-cells isolated from blood, BM, and LN of a representative goat.

of memory T-cells from blood, BM, and LN. Animals received ^2H_2O for 4 weeks and were sacrificed at different time points during the labeling and the subsequent de-labeling period, such that a cross-sectional up- and down-labeling curve of deuterium enrichment could be constructed. Using a mathematical model that takes into account the possible kinetic heterogeneity of a cell population (4), we estimated the average turnover rate (*p)* of the different cell populations, i.e., the fraction of cells replaced by new cells per day, and deduced the corresponding average lifespan (*1/p*) of the cells in that populations (see material and methods).

Despite the observed differences in the percentage of Ki-67 positive cells, deuterium enrichment levels in CD4$^+$ and CD8$^+$ memory (CCR7$^-$) T-cells from blood, BM, and LN were very similar (**Figure 4A**). The fits of the model to the experimental data (**Figure 4A**) and their corresponding estimates revealed no significant differences in the average turnover rates of memory T-cells isolated from BM and blood (**Figure 4B**). The estimated average lifespan of memory T-cells isolated from BM was 50 days [(95% confidence interval (CI) = 21;91] for CD4$^+$ and 54 days (CI = 7;96) for CD8$^+$ cells. Memory T-cells obtained from blood had an estimated average lifespan of 44 days (CI = 27;78) for CD4$^+$ and 32 days (CI = 5;58) for CD8$^+$ (**Figure 4B**). For the LN, we estimated that memory CD4$^+$ T-cells live on average 54 days

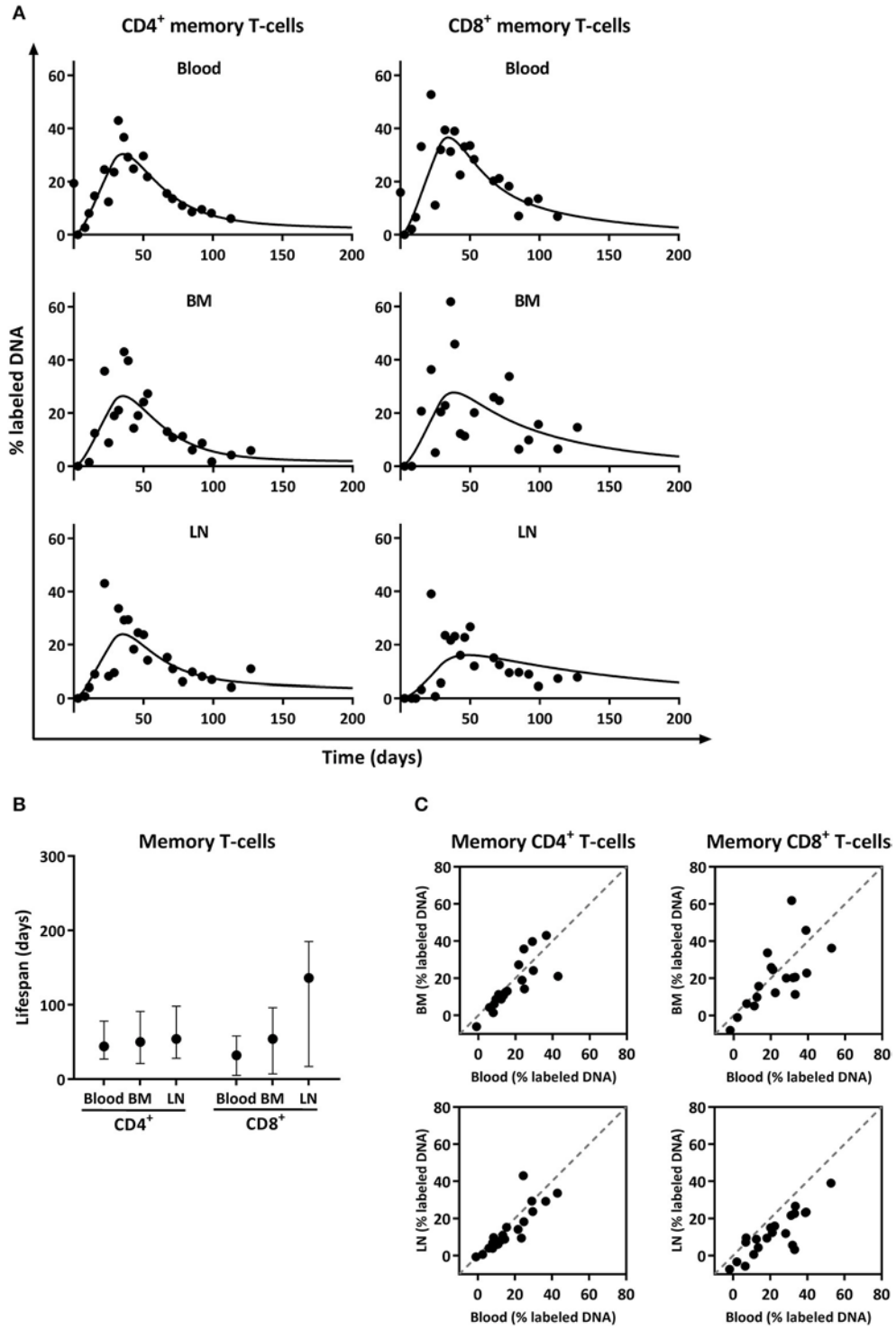

FIGURE 4 | Analysis of deuterium enrichment and summary of the estimated lifespan of $CD4^+$ and $CD8^+$ memory T-cells from blood, BM and LN. **(A)** Best fits to the level of deuterium enrichment measured in the DNA of $CD4^+$ and $CD8^+$ memory ($CCR7^-$) T-cells from blood, BM and LN. Label enrichment in the DNA was scaled between 0 and 100% by normalizing to the maximum enrichment in granulocytes (See material and methods). **(B)** Estimated lifespans of $CD4^+$ and $CD8^+$ memory T-cells in days, and their respective 95% confidence limits. **(C)** Correlation between deuterium enrichment in BM and blood, and LN and blood. The gray dashed line represents the X = Y line.

(CI = 28;98), and $CD8^+$ T-cells 136 days (CI = 17;185) (**Figure 4B**). The labeling curves of memory $CD4^+$ T-cells isolated from the blood, LN, and BM and of memory $CD8^+$ T-cells isolated from the blood were significantly better described by a model including two kinetically different subpopulations, while the labeling curves of memory $CD8^+$ T-cells from the LN and BM were well described by a kinetically homogeneous model.

Deuterium enrichment in the DNA of memory $CD4^+$ and $CD8^+$ T-cells highly correlated between BM and blood, as well as between LN and blood (both $R^2 > 0.70$) and all data points were close to the x = y line (**Figure 4C**). These results suggest that, despite the observed differences in the fraction of Ki-67 positive cells, the turnover rates of $CD4^+$ and $CD8^+$ memory T-cells obtained from blood, BM, and LN are very similar, and that the vast majority of memory T-cells, even the ones located in BM and LN, are short lived, with an average lifespan of about 50 days.

Of note, both the percentage of Ki-67 positive cells (p-values < 0.0001, **Supplementary Figures 3A,B**), and the level of deuterium incorporation (**Supplementary Figures 3C,D**) were higher in memory ($CCR7^-$) compared to naive ($CCR7^+$) T-cells, in line with the typical observation in mice and humans that memory T-cells express higher levels of Ki-67 and reach higher deuterium enrichment than naive T-cells (4, 39).

Memory T-cells From BM Do Not Share the Tissue Resident Memory (TRM) Transcriptional Signature

The most parsimonious explanation for the opposing Ki-67 and deuterium results would be that memory T-cells are constantly cycling and circulating between BM, blood, and LN (40, 41), and that memory T-cells may pick up deuterium while dividing outside the BM (**Figure 5A**). We hypothesized that, if memory T-cells in BM would belong to a population of circulating T-cells, they would not share the TRM core transcriptional signature defined for human and mouse lymphocytes (42). While we found 2% differentially expressed genes between $CD8^+$ memory T-cells from BM and blood (**Figure 5B**), we did not find any enrichment for genes defining the TRM core transcriptional signature (**Figure 5C**), supporting our hypothesis that the vast majority of BM memory T-cells are not sessile and continuously recirculate.

DISCUSSION

Our 4-week *in vivo* ^2H_2O labeling study suggests that, in goats, memory T-cells from BM are maintained by continuous low-level proliferation. Both for $CD4^+$ and $CD8^+$ T-cells, we found no significant differences in deuterium labeling, and hence in cellular lifespans, between memory T-cells isolated from the blood, BM, and LN, while the percentage of Ki-67 positive cells differed significantly. The finding that the fraction of Ki-67 positive cells was smaller for memory T-cells isolated from the BM compared to memory T-cells from the blood is in line with previous findings in mice and humans (18, 19, 24). Our *in vivo* deuterium labeling data demonstrate, however, that these differences in Ki-67 expression should not be interpreted as a sign that BM memory T-cells are long-lived. Our data support the view that memory T-cells in blood, BM, and LN are part of a dynamic system, in which the vast majority of cells are maintained by self-renewal and continuously recirculate (**Figure 5A**).

The goat as an animal model enabled us to simultaneously compare memory T-cell dynamics in blood, BM, and LN. One major disadvantage of this model is that T-cell subsets in goats are less well characterized than in mice and humans. We here showed that, both for $CD4^+$ and $CD8^+$ T-cells, $CCR7^-$ T-cells present phenotypic (**Figure 1B** and **Table 1**), functional (**Figure 2F**), transcriptional (**Figures 2C–E**) and kinetic (**Figures 3**, **4** and **Supplementary Figure 3**) characteristics of memory T-cells and are distinct from $CCR7^+$ T-cells, which present naive-like features. In line with observations in mice and humans (18–20, 41, 43–46), the BM of goats is composed of lower percentages of $CD4^+$ and $CD8^+$ T-cells than blood and LN, and is enriched in memory T-cells. The differentially expressed genes observed between BM and blood support that $CD8^+$ memory T-cells isolated from BM are a separate population and are not just cells sampled from the small blood vessels in BM (**Figure 5B**). An advantage of this model system is that these outbred animals were routinely vaccinated at young age and exposed to a broad range of pathogens throughout life. It has recently been shown that the memory T-cell compartment of mice exposed to pathogens, unlike that of clean laboratory mice, contains a lot more memory T-cells and thereby resembles that of adult humans (47), and that the BM T-cell composition dramatically changes toward a memory phenotype upon infection and pathogen clearance (19). The conventional environment to which goats were exposed prior to and during the study likely led to a more mature and adult human-like immune system.

Our conclusion that BM memory T-cells must be recirculating through the body is supported by *in situ* BM labeling studies (40) and parabiosis experiments (41), which have shown more directly that memory T-cells migrate in and out of the BM. The fact that short-pulse BrdU labeling resulted in relatively high BrdU incorporation in memory T-cells from BM, while longer BrdU administration led to similar BrdU incorporation in memory T-cells from different organs, also supports the view that memory T-cells recirculate between BM and the other compartments (17). In addition, it has been shown that BM contains highly permeable vessels that are restricted to immature and mature leukocyte migration, suggesting that BM facilitates leukocyte trafficking (48). This is in contrast to studies that have shown that a significant fraction, but not the vast majority, of memory T-cells in BM express CD69, a molecule implicated in tissue retention. Such studies have proposed that memory T-cells reside in BM and do not migrate to other organs (18, 24).

Whether memory T-cells in BM are maintained by continuous cell division or by cellular longevity is also heavily debated (16, 49). Notably, in mouse studies, memory T-cell kinetics in BM are typically compared to those of the spleen and LN, while human studies generally base their comparisons on memory T-cells from blood due to the difficulty in accessing peripheral organs, such as

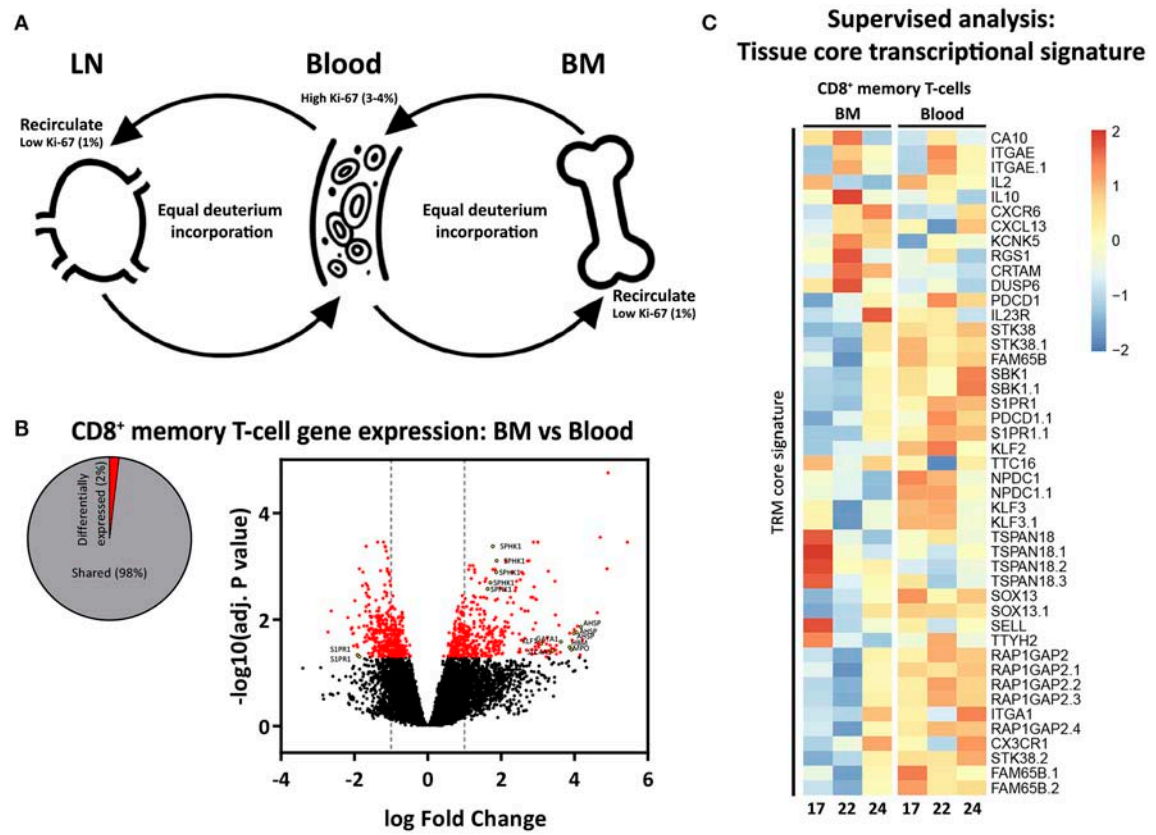

FIGURE 5 | Proposed model of proliferation and recirculation. **(A)** Memory T-cells from BM and LN have a lower fraction of Ki-67 positive cells compared to memory T-cells from blood, but similar deuterium incorporation. We here propose a dynamic model in which memory T-cells are continuously recirculating between BM, blood, and LN. If BM memory T-cells would compose a separate population of resting and resident cells, one would expect to find low percentages of Ki-67 as well as low deuterium incorporation in BM. The fact that memory T-cells isolated from the BM had substantial levels of deuterium enrichment in the DNA shows that memory T-cells from BM do proliferate. The fact that the percentage of Ki-67 positive cells in BM was lower compared to blood while deuterium enrichment curves were very similar strongly suggests that memory T-cells are recirculating. Because of the difficulties in the interpretation of Ki-67 expression (see discussion) it remains unclear where the divisions occur. **(B)** Diagram showing the percentage significantly differentially expressed genes (adjusted p-value (BH) < 0.05) between BM and blood memory ($CCR7^-$) $CD8^+$ T-cells, as well as a volcano plot illustrating the $\log_2$ fold change differences in gene expression. Significantly differentially expressed genes are shown in red, yellow dots depict tissue related genes. **(C)** Heatmap showing normalized expression levels of genes defining the tissue resident memory (TRM) core transcriptional signature (42). Gene expression is scaled per row.

LN and spleen. Our finding that the percentage of Ki-67 positive cells is consistently higher in blood than in BM and LNs is in line with previous studies (18, 50), and illustrates that the source of T-cells taken as a reference has an impact on data interpretation. When comparing Ki-67 expression levels between BM and blood, one could conclude that memory T-cells in BM are resting in terms of proliferation; however, the opposite conclusion would be reached when comparing Ki-67 expression between BM and LN. We overcame this problem by simultaneously comparing the kinetics of memory T-cells from BM, blood and LN.

The dispute in the literature may also be partially due to the different techniques used to measure cell dynamics. For $CD8^+$ memory T-cells, studies based on DNA content analysis (17, 25), BrdU labeling (17, 25, 27, 46, 51, 52) and CFSE labeling (17, 27, 51, 53, 54) have all suggested that in mice the division rate of memory T-cells in BM is greater than in spleen and LN. The fact that all three techniques gave similar results strongly suggests that the BM is the preferential place for memory T-cells to divide. However, separately each technique has its caveats, as outlined in the introduction. Meanwhile, other studies have proposed that memory T-cells from BM are resting in terms of proliferation, as they have shown that $CD8^+$ memory T-cells from BM express lower percentages of Ki-67 than their blood (18) and spleen counterparts (19, 24). Based on such low expression levels of Ki-67 in BM, and the fact that *ex vivo*-isolated BM memory T-cells are transcriptionally less active than the *in vitro*-stimulated memory T-cells (24), it has been proposed that many memory T-cells in BM are resting in G_0 of the cell cycle and are, hence, long lived. This hypothesis has also been supported by a recent study showing that absolute numbers of $CD8^+$ memory lymphocytes in BM are unaffected by cyclophosphamide, an alkylating agent that cross-links DNA leading to apoptosis of cells that attempt to divide, while half of the $CD8^+$ memory T-cells in spleen die during cyclophosphamide treatment (55). Although it remains very puzzling how to reconcile these findings with our own results, the

techniques used in these studies also have some limitations. First, as mentioned before, Ki-67 expression and DNA content analysis are static measurements of cell proliferation. Second, since DNA cross-linking by cyclophosphamide leads to an enormous loss of cells, this could change the dynamics of the remaining cells in response to the lymphopenia that is induced (56–59).

Our current *in vivo* labeling results strongly suggest that memory T-cells from BM, as well as from blood and LN, are relatively short-lived. Mathematical modeling suggested that the memory $CD4^+$ T-cell pools in blood, LN, and BM, and the memory $CD8^+$ T-cell pool in blood are composed of at least two 2 kinetically different subpopulations, as previously reported for memory T-cell populations in mice and humans (4). These kinetically different subpopulations may reflect phenotypically different subsets (e.g., effector memory and central memory T-cells), and/or subsets that differ in the exposure to their cognate antigen. Indeed, it has recently been shown that yellow-fever-virus antigen-specific memory T-cells have longer lifespans than the bulk of memory phenotype T-cells (60). Although we cannot formally exclude the possibility that a subpopulation of memory cells may be composed of very long-lived cells that failed to pick up deuterium during the 4 week labeling period, our data convincingly show that, if present, such a population does not preferentially reside in the BM. To formally prove this, a long-term *in vivo* labeling experiment would have to be designed.

All studies based on Ki-67 expression (18, 19, 24), including ours, have reported lower Ki-67 expression of memory T-cells in BM when compared to those in blood and LN. Although the relatively high Ki-67 expression in blood seems to suggest that memory T-cells preferentially divide in circulation rather than in BM or LN, we consider this unlikely. The induction of Ki-67 expression corresponds to the entry of resting cells into the cell cycle, however its expression can be maintained up to 7 days after the completion of mitosis (61–63). This suggests that Ki-67 may be a good indicator of recent cell-cycle activity but may not be a marker for active cell division. We think that blood may be enriched for Ki-67 positive T-cells that have recently divided elsewhere, and may not necessarily have undergone their cell division in blood. One possible explanation is that upon division in the LN, T-cells preferentially egress to blood, which is in line with the observation that recently activated and expanded T-cells egress rapidly from the LN (64). This hypothesis is also supported by the observation that a higher proportion of BrdU labeled cells is found in the lymph nodes immediately after labeling, before BrdU levels in blood and LN reach similar levels (65). Although solving this issue is beyond the scope of the article, this again illustrates the limitations in interpreting data based on snapshot markers such as Ki-67.

Taken together, we here found no evidence for a long lifespan of either $CD4^+$ or $CD8^+$ memory T-cells in BM. Although all reviewed studies convincingly approached the dynamics of BM memory T-cells, the use of different techniques and the comparison to different organs might have led to conflicting results. Because we simultaneously analyzed memory T-cell kinetics in BM, blood and LN using *in vivo* labeling, we conclude that BM memory T-cells do not form a separate population of long-lived cells. In order to translate this fundamental finding to the human situation, a similar *in vivo* deuterium labeling experiment would have to be done in humans. Given that different techniques provide seemingly opposing results, further research is needed not only to address the role of BM in the maintenance of memory T-cells, but also to better understand how to interpret results obtained using different experimental techniques to study lymphocyte turnover and whether clearer insights can be achieved by combining them.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Gene Expression Omnibus (GEO), accession number GSE119116.

AUTHOR CONTRIBUTIONS

MB-P, MV, AK, JD, KT, and JB wrote the manuscript. MB-P, LR, AK, KT, and JB designed the experiments. MB-P, MV, LR, and AK performed the experiments. MB-P and MV analyzed the data. JD, RdB, and JB performed mathematical modeling.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02054/full#supplementary-material

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REVIEW
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CD4+ Memory T Cells at Home in the Tissue: Mechanisms for Health and Disease

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During the last 10 years, a population of clonally expanded T cells that take up permanent residence in non-lymphoid tissues has been identified. The localization of these tissue resident memory (TRM) cells allows them to rapidly respond at the site of antigen exposure, making them an attractive therapeutic target for various immune interventions. Although most studies have focused on understanding the biology underlying CD8 TRMs, CD4 T cells actually far outnumber CD8 T cells in barrier tissues such as lung and skin. Depending on the immune context, CD4 TRM can contribute to immune protection, pathology, or tissue remodeling. Although the ability of CD4 T cells to differentiate into heterogeneous effector and memory subsets has been well-established, how this heterogeneity manifests within the TRM compartment and within different tissues is just beginning to be elucidated. In this review we will discuss our current understanding of how CD4 TRMs are generated and maintained as well as a potential role for CD4 TRM plasticity in mediating the balance between beneficial and pathogenic immune responses.

Keywords: CD4 T cell memory, resident memory, infection, autoimmunity, vaccine

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INTRODUCTION

Following activation, CD4+ T cells have the remarkable ability to differentiate into many different types of effectors. This diversity is required for the generation of effector T cells that are adapted to a particular immune context, as well as the development of long-lived and protective memory T cells. Compared to naïve T cells, memory CD4+ T cells are present at higher numbers, exhibit distinct trafficking behaviors, and generally have more rapid effector function following reinfection (1). Nevertheless, it is unclear how memory CD4 T cells positively versus negatively impact secondary immune responses. In the case of influenza vaccination, memory CD4 T cells have been shown to recognize conserved viral glycoproteins and may be able to provide cross-protective (heterotypic) protection to multiple influenza strains (2). This is in contrast to vaccine elicited antibodies which are directed against highly mutable viral proteins, resulting in the need for new vaccine formulations every year (3). On the other hand, memory CD4+ T cells are generally considered to be a barrier to transplantation tolerance and were recently reported to induce immune pathology in a mouse model of chronic viral infection (4, 5). The capacity of CD4 memory T cells to orchestrate divergent immune outcomes is in part related to their heterogeneity. Distinct types of effector T cells have been shown to give rise to apparently committed memory T cell lineages (6, 7). However, the stability and plasticity of these memory T cell subsets as well as their full impact on secondary immune responses are not yet understood.

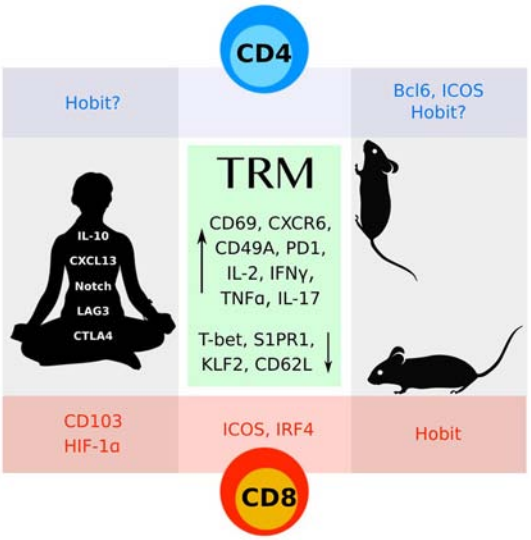

FIGURE 1 | CD4 and CD8 TRM cells identified in mice and humans share many features (green box). More work remains to understand the distinct molecular programs that define these subsets.

More recently, an additional population of memory T cells localized within barrier tissues has been identified (8, 9). Due to their non-circulating status, these tissue resident memory (TRM) cells are uniquely poised to respond to antigen and execute immediate effector functions. While most studies have focused on understanding the cellular requirements and transcriptional basis of CD8 TRM differentiation, our understanding of CD4 TRM biology is less advanced. This review will highlight specific cellular and molecular requirements for CD4 TRM generation and survival within distinct organs, and in response to different pathogens or immune contexts. Since many of the phenotypic characteristics of CD4 TRM are shared with CD8 TRM cells, and are extensively reviewed elsewhere, we will focus our discussion on what sets CD4 TRM cells apart (**Figure 1**). Although much of this review focuses on findings generated in mouse models of infection or autoimmunity, we specifically highlight important observations made on human CD4 TRM throughout the manuscript.

The persistence of memory CD4 T cells in tissues has long been appreciated. In general, CD4 memory T cells appear to preferentially accumulate in mucosal tissues where they outnumber CD8 memory T cells (10, 11). Early work by the Jenkins lab showed that antigen and LPS led to CD4 T cell expansion and migration into tissues including lung, liver, gut, and salivary gland (12). Using whole mouse body imaging to quantify CD4 memory T cells, the authors demonstrated long-term survival of these cells in the tissue as well as their ability to rapidly produce IFNγ following recall activation. The residence status of CD4 TRM cells has been confirmed using parabiosis experiments (13). Similar to CD8 TRM cells, CD4 TRMs are protected from intravascular antibody staining and persist after treatment with FTY720, a sphingosine-1-phosphate receptor 1 (S1PR1) antagonist that prevents lymphocyte egress from lymphoid organs (14, 15). Phenotypically, CD4 TRM express constitutively high levels of PD1 and CD69 (13, 15, 16). CD69 is initially upregulated on activated T cells during priming by antigen presenting cells in the draining lymph node, after which it is downregulated, enabling S1PR1 mediated exit from lymphoid organs (17). T cells then enter the blood circulation en route to the site of inflammation, directed by upregulation of chemokines and adhesion markers as well as homing receptors that are imprinted during T cell interactions with antigen presenting cells in the lymph node (18, 19). Once in the tissue, TRM precursors begin to re-express CD69, although the signals mediating this upregulation are unclear. CD69 can be induced by signals through the T cell receptor (TCR), inflammatory cytokines such as IFNα, and oxygen availability; the independent contribution of these signals to CD4 TRM induction and maintenance is not yet known (20–22). A recent study demonstrated that human CD4 TRM cells isolated from distinct peripheral tissues express high levels of CD69 and are transcriptionally distinct from CD69– CD4 T cells (16). Human CD4 TRM cells share strong homology to mouse TRM cells, with increased expression of genes involved with TCR signaling, adhesion, and cytokines (23, 24).

CD4 TRM GENERATION: CYTOKINE AND ANTIGEN REQUIREMENTS

The common gamma-chain cytokines IL-2, IL-15, and IL-7 have well-established and fundamental roles in CD4 T cell biology (25). While IL-2 drives the initial expansion of activated CD4 T cell effectors, sustained IL-2 signals repress gene programs required for circulating/lymphoid CD4 memory T cell fate (26, 27). In contrast, IL-2 receptor mediated signals are essential for the generation of CD4 TRM cells. In both a Th1 model of viral pulmonary infection as well as a Th2 model of allergic asthma, the absence of IL-2R signaling on activated CD4 T cells resulted in their failure of these T cells to migrate into the lung and establish residence (28, 29). In agreement with these findings, work by the Swain group showed that late antigen recognition by CD4 T cells results in autocrine IL-2 production that supports the maintenance of CD4 TRM cells in the lung following influenza infection (30). An IL-2 independent pathway for CD4 TRM generation has also been identified in influenza infection (31). In this case, IL-15 was required during the priming phase of T cell activation, while late IL-15 signals were unnecessary for long-term CD4 TRM survival. This is in contrast to CD8 TRM cells in the skin and lung which depend on IL-15 for both their generation and maintenance (32). IL-7 signals are essential for the maintenance of both naïve and lymphoid homing CD4 memory cells (33). Lung TRM cells generated after influenza infection express higher levels of IL-7R compared to circulating effectors, and treatment with Fc-fused IL-7 can promote recruitment of circulating CD4 T cells into the lungs where they acquire a TRM-like phenotype and contribute to secondary immune responses (34). Consistent with this, IL-7R blockade in a Th2 allergy model led to decreased numbers of airway resident CD4 T cells (35). Further, in a skin model of

contact hypersensitivity where IL-7 was specifically ablated in the skin, CD4 TRM cells failed to persist long term (36). These data indicate an important role for IL-7 signaling in either the recruitment or survival of CD4 TRM.

In addition to cytokines, antigen recognition by the TCR is required for CD4 T cell diversification into effector and memory subsets. The role of antigen in CD4 TRM maintenance, however, is less clear (37). Transfer of lung derived CD4 TRM cells into naïve recipients demonstrates the ability of these cells to home back to the lungs and survive in an antigen independent manner (13, 38). Consistent with this, CD4 TRM generated after influenza infection do not express Nur77 at late phases of infection, suggesting that they are no longer receiving TCR mediated signals (39). However, these latter data were generated using TCR transgenic OT-II cells which under some settings have been shown to undergo less heterogeneous differentiation compared to polyclonal CD4 T cells (40). Indeed, distinct CD4 T cell receptor clonotypes were recently described to be associated with distinct states of T cell activation following tuberculosis infection or within the tumor microenvironment, suggesting that access to antigen can regulate the extent of T cell heterogeneity (41, 42). It is interesting to note that Nur77 expression is reportedly increased in CD8 TRM cells and is required for long-term TRM survival in multiple tissues (43, 44). Although the survival of Nur77 deficient CD4 TRM cells has not yet been assessed, it is likely that depending on the tissue, intercellular interactions or infection context, CD4 TRM cells are differentially dependent on antigen and T cell receptor signaling.

B CELL REQUIREMENTS FOR CD4 TRM CELLS: RELATIONSHIP TO TFH CELLS?

T cell interactions with B cells constitute another important aspect of CD4 TRM biology. In peripheral CD4 T cells, B cell interactions with T cells lead to upregulation of the transcription factor Bcl6, which in turn supports the differentiation of follicular helper (TFH) and memory T cells (45). TFH cells are a lymphoid resident population that share many phenotypic and molecular similarities with TRM cells, including high expression of CD69, PD1, ICOS, and P2X7R and a dependency on downregulation of KLF2 and S1PR1 for their induction (23, 46–49). Although these similarities suggest that B cells might be important for CD4 TRM generation, B cell deficiency led to enhanced Th2 TRM generation and maintenance in a house dust mite allergy model (28). Similarly, *Mycobacterium tuberculosis* (Mtb) infection resulted in the generation and maintenance of CD4 TRM in a B cell independent manner (38). In this case, however, CD4 TRM cell survival required T cell intrinsic expression of Bcl6 and ongoing signals through ICOS, both of which are also required to maintain TFH cells at late phases of immune responses in secondary lymphoid organs (50). The authors hypothesized that T cell interactions with ICOS-ligand expressing dendritic cells might be responsible for maintaining CD4 TRM cells. Highlighting the divergent role of B cells in CD4 TRM generation, another report showed that intranasal LCMV infection in the absence of B cells led to impaired Th1 TRM cell survival, despite enhanced initial recruitment of CD4 T cells to the lung (29). Although Bcl6 expression was not explicitly addressed in this model, it is interesting to note that in peripheral CD4 T cells, high levels of T-bet can impair the ability of Bcl6 to repress its target genes (51). Consistent with this idea, high levels of T-bet are associated with decreased generation of both CD4 and CD8 TRM (52, 53). Using a neonatal infection model, the Farber group showed that the susceptibility of infants to respiratory infections is a result of increased T-bet expression in effector T cells which impairs the ability of these cells to stabilize the TRM phenotype (52).

TRM LOCATIONS AND INTERCELLULAR INTERACTIONS

CD4 TRM cells are often observed in cell clusters or ectopic lymphoid structures. The cellular content of these clusters can differ depending on the tissue and cytokine context. Several reports indicate that the presence or absence of these clusters can play a role in CD4 TRM mediated recall responses, protection from host pathology during chronic infection and tissue remodeling or repair during pathogen clearance. In this section we will overview the various tissues where CD4 TRM cells have been identified and discuss the potential of intercellular interactions to modulate local immunity.

Skin

The skin is a barrier tissue home to a large proportion of the memory T cells in the body. Unlike CD8 TRM cells which localize in the epithelium, CD4 T cells are primarily found in the dermis where they demonstrate more motile behavior than their CD8 TRM counterparts (54). Using mice that express the photoconvertible molecule Kaede, a majority of CD4 T cells present in the skin were found to be in equilibrium with the circulation at steady state (55). CD69 expression on these CD4 T cells decreased as they trafficked to the draining lymph node, highlighting the infidelity of CD69 as a marker for CD4 tissue residency (55, 56). Following infection with herpes simplex virus or contact sensitization to induce local inflammation, IFNγ producing CD4 T cells increased in the skin and clustered around hair follicles in association with CCL5 producing CD11b and CD8 T cells (55). Depletion of CD8 T cells led to disruption of these clusters and impaired survival of skin CD4 TRM. The authors noted that the hair follicle is a rich site for chemokine and cytokine production as well as a major site of commensal colonization, both of which might play a role in facilitating the maintenance of immune cell clustering and reactivation of CD4 TRM cells. Skin CD4 TRM have also been identified following *Leishmania major* infection (57, 58). In this case, re-challenge at distal sites from the original infection results in rapid production of IFNγ and recruitment of inflammatory monocytes in a CXCR3 dependent manner. In addition to Th1 TRM cells, Th17 TRM cells have been identified in the skin following infection with *Candida albicans* (59). Although the primary IL-17 producing population in the skin at earlier time points is comprised of γδ T cells, CD4 αβ T cells recruited at later time points localize in the

papillary dermis and upregulate expression of CD69 and CD103. CD103 is a relatively robust marker for CD8 TRM identification, but it is less uniformly expressed on CD4 TRM cells, and may represent a distinct subset that arises in a more limited number of tissues, possibly dependent on environmental TGFβ (60, 61). The skin is also home to resident regulatory T cells which may play a role in the pathogenesis of psoriasis, characterized by the development of TRM dependent skin lesions (62). In this case, CD4 regulatory TRM cells expand and produce low levels of IL-17. Here it is noteworthy that psoriasis can be treated with some success by IL-17 blockade, although the precise mechanism for this has not yet been resolved (63).

Female Reproductive Tract

The female reproductive tract is a prime location for sexually transmitted as well as opportunistic infections, suggesting an important role for CD4 T cells in this tissue. Using a model of genital herpes, the Iwasaki group demonstrated the presence of CD4 TRM localized in clusters with CD11c+ MHC-II+ cells that are disconnected from the circulation (64). These CD4 TRM cells provide superior protection compared to circulating memory cells and are maintained by local interactions with macrophages. In this model, T cell stimulation by macrophages results in T cell production of IFNγ, leading to CCL5 expression by macrophages, thus creating a positive feedback loop for cell clustering. Protection upon re-challenge is also mediated by CD4 T cell production of IFNγ, which acts on stromal cells to prevent viral replication and spread. Although the role of antigen in this system is unclear, a prime and pull immunization strategy where antigen is administered subcutaneously followed by a one-time application of chemokines to the genital mucosa showed that local chemokines are sufficient to recruit but not maintain CD4 TRM (65). It was also reported that CD4 TRM may be reactivated by uninfected local dendritic cells and B cells that acquire antigen from infected epithelial cells (66). Neither dendritic cells nor B cells alone were required for CD4 TRM recall, but depletion of both populations resulted in loss of protection.

Intestines

The intestinal mucosa is a unique barrier tissue that comes into contact with food and environmental antigens as well as commensals and infectious pathogens. Th17 TRM cells specific for segmented filamentous bacteria, a commensal microbe, have been identified in the lamina propria of mice (67). Human Th17 TRM cells that express the C-type lectin-like receptor CD161 have also been identified in the lamina propria of patients experiencing Crohn's disease (68). These cells can be activated to release IL-17 and IFNγ in response to inflammation induced IL-23, which further potentiates their colitogenic potential. Given that pathogenic and protective Th17 cells are regulated by the same environmental cytokines, it is likely that the distinct make-up of the microbiota plays a role in regulating heterogeneity within the gut CD4 TRM compartment. Commensals have also been shown to induce the formation of resident regulatory T cells that produce high amounts of TGFβ which promotes local tolerance (69, 70). In addition, a recent report shows an important contribution by TFH cells residing within Peyer's patches of the gut to maintaining intestinal health (71). Peyer's patches are lymphoid tissue comprised of immune cell sensors that are constantly exposed to luminal antigens and gut bacteria (72). Although the circulating status of these TFH cells has not been addressed, non-circulating TRM cells with high expression of CD69, similar to constitutive expression of CD69 on TFH cells, have been identified in other secondary lymphoid organs (73). Similar to CD8 TRM cells, TFH cells express high levels of the purinergic receptor P2X7R; ATP mediated signals through P2X7R are required to maintain the balance of commensals in this organ (48). In the absence of P2X7R signals, TFH cells expand, providing excessive help to germinal center B cells, ultimately resulting in excessive IgA production and dysregulation of local commensal populations. CD4 TRM cells in the gut can also be induced by oral infection with *Listeria monocytogenes* (74). Th1 TRMs generated in this model accumulate in the lamina propria and epithelium, and are maintained in an IL-15 independent manner. Th2 TRM cells can also be identified in the lamina propria and peritoneal cavity after infection with *Heligmosomoides polygyrus* (75). Re-challenge infection results in TCR dependent production of IL-4, IL-5, and IL-13, although TRM cells in the peritoneal cavity are additionally able to produce cytokines in response to IL-33 and IL-7 signals alone.

Lungs

The lung is a highly vascularized barrier tissue in constant contact with a variety of airborne microbes and environmental pollutants. Infection or inflammation in the lung results in the formation of ectopic lymphoid tissue called inducible bronchus-associated lymphoid tissue (iBALT) (76). Similar to secondary lymphoid tissue, iBALT is characterized by compartmentalized B and T cell areas, follicular dendritic cells, antigen presenting cells, high endothelial venules, stromal cells and limited chemokine networks (77). It is likely that the cellular composition of iBALT plays a role in mediating the balance between protection and pathology in the lung, and may provide a niche for CD4 TRM cell survival.

Respiratory infections such as influenza induce the generation Th1 TRM cells that can be recalled to produce IFNγ and provide protection against heterotypic infections (60, 78). Th1 TRM cells express high levels of the integrins CD11a and VLA-1 (α1β1), the latter of which is required for Th1 TRM generation and survival following recall infection (13, 79). Two recent reports have described a transcriptional signature for Th1 TRM cells isolated from human lung (16, 61). Although Th1 TRM cells were sorted according to either CD69 or CD103 expression, both reports show a strong homology of Th1 TRM cells with CD8 TRM cells described in mice. In addition, CD4+CD103+ TRM cells express high levels of IL-21 receptor, TGFβ and genes associated with Notch signaling. After stimulation with anti-CD3/28 *in vitro*, these lung CD4 TRM demonstrated polyfunctional cytokine production, suggesting heterogeneity within this population. It should be noted, however, that as is normally the case with CD4 T cell cytokine production, ∼50 percent of CD4 TRM cells did not produce any cytokines, suggesting heterogeneity in terms of

CD4 T cell subset or activation state within this compartment. Th1 TRM cells are also generated after chronic infection with tuberculosis and are characterized by high expression of CXCR3 and low expression of KLRG1 (38, 80, 81). Adoptively transferred Th1 TRM cells migrate back to the lung parenchyma where they mediate superior Mtb control but produce less IFNγ compared to CD4 memory T cells in the vasculature. Both influenza and Mtb infection also induce the formation of sustained iBALT. In the case of influenza infection, the presence of iBALT is correlated with accelerated secondary CD4 T cell responses, suggesting that iBALT might provide a survival niche for Th1 TRM (76, 82). The presence of iBALT also plays a role in sustained neutralizing antibody production, indicating a role for ongoing interactions between CD4 TRM and B cells to support resident or memory B cell persistence. Given that the glycoproteins expressed on the surface of influenza are highly variable from year to year, the presence of neutralizing antibodies might not be expected to provide sufficient protection following recall infection. However, since CD4 and CD8 T cells typically recognize conserved epitopes from internal viral proteins, it is possible that prior exposure to influenza results in the accumulation of TRM cells capable of providing cross strain protection (2, 3). Here the maintenance of CD4 TRM cells within immune cell clusters and/or iBALT would provide a starting point for rapid renewal of secondary germinal center responses. Although the specifics of this scenario must still be addressed, a recent retrospective study found that the number of prior influenza exposures can be linked to demographic susceptibility to re-infection, and is associated with conserved antigen epitopes recognized by CD8 T cells (83).

In the case of chronic Mtb infection, iBALT induced by chronic Mtb infection is formed within the granuloma, which is essential for preventing pathogen dissemination (84). iBALT formation is associated with protective immune responses during latent tuberculosis in humans as well as macaque and mouse models of Mtb infection (85). A central component of iBALT in this model is the presence of CXCR5+ CD4 T cells which are initially recruited during the effector phase of infection. Although CXCR5 expression is required for T cell localization within the lung parenchyma and for the long-term persistence of these TRM cells, it is not yet clear how these TRM cells mediate protective immunity. One possibility is that CD4 TRM cells produce cytokines to recruit, organize, or maintain the local immune cell repertoire. If this is the case, it will be particularly important to examine how heterogeneity or division of labor within the CD4 TRM compartment might contribute to distinct aspects of local immunity. For example, do CXCR5+ CD4 TRM cells promote ongoing humoral immunity during Mtb infection? Although the role of antibodies in tuberculosis immunity has been controversial, recent work demonstrates distinct antibody qualities associated with latent vs. active infection (86). It will be interesting to determine whether CD4 T cell help to B cells plays a role in these observations. Another important question is how do CXCR5+ CD4 TRM cells relate to CXCR5- TRM cells or to CD4 TRM cells with the potential to produce IFNγ? One possibility is that CXCR5+ CD4 TRMs can be further recalled to differentiate into IFNγ effectors, similar to the differentiation potential of lymphoid CXCR5+ CD4 memory T cells (50, 87). In this case, CD4 TRMs would self-renew to maintain the presence of long-lived protective memory cells, while simultaneously differentiating into effectors to promote pathogen containment or clearance.

Aside from infections that induce type 1 interferon responses, excessive inflammatory responses in the lung can lead to pathogenic tissue remodeling such as observed in asthma, a chronic inflammatory lung disease triggered by sensitization to inhaled allergens (88). In both asthmatic patients as well as animal models of the disease, Th2 memory T cells generated during inflammatory outbreaks are thought to contribute to pathogenesis (28, 89, 90). Memory Th2 cells are maintained within iBALT, and are supported by Thy1+ IL7 producing lymphatic endothelial cells that express IL-33, CCL21, and CCL19 (91). In agreement with this, iBALT formation is also observed in patients with chronic obstructive pulmonary disease and excessive Th2 TRM responses associated with lung fibrosis (92, 93). Using a mouse model of allergy induced asthma, a recent report identified two distinct populations of tissue resident ST2+ Th2 T cells in the lung: one subset produces IL-5 to recruit eosinophils; the other produces amphiregulin, which programs the induction of inflammatory eosinophils ultimately leading to lung fibrosis (93). Amphiregulin production by regulatory TRM cells was also shown to prevent excessive host pathology following influenza infection, by inducing epithelium proliferation and repair after viral clearance (94). Here, the local production of pro-inflammatory cytokines IL-18 and IL-33 induced amphiregulin production by ST2+ Tregs, to promote tissue repair in a TCR independent manner. In these cases of both Th2 lung fibrosis and post influenza tissue repair, amphiregulin production by distinct TRM subsets promotes distinct types of local immunity. These findings highlight the importance of understanding how heterogeneity within the CD4 TRM compartment impacts the outcome of local immune responses.

CD4 VS. CD8 TRM CELLS, MOUSE VS. HUMAN TRM CELLS: WHERE DO WE STAND?

As discussed above, most studies have focused on identifying phenotypic and molecular characteristics that discriminate CD8 TRM from circulating or lymphoid homing CD8 memory T cells. CD4 TRM studies have largely been placed within the context of these CD8 TRM findings, with the consensus being that the two cell types have much in common. A recent report by Farber and colleagues identified a core transcription signature shared by both CD4 and CD8 TRMs isolated from human organs (16). This signature, which was also largely shared with mouse TRM cells, included adhesion molecules such as CD103 and CD49a, chemokine receptors such as CXCR6 and CX3CR1, and genes known to be involved in dampening or inhibiting T cell responses, including PD1, DUSP6 and IL-10. On the other hand, CD4 TRM cells exhibited more clonal diversity compared to CD8 TRM, which likely reflects their underlying heterogeneity. In support of this, dimension reduction of RNAseq data revealed a broader spread of CD4 TRM cells compared

to the tight grouping of CD8 TRM cells across the multiple tissues examined. These data are also consistent with a study that examined trafficking markers and cytokine production by human CD4 T cells distributed across several different lymphoid and non-lymphoid tissues at steady state (95). Using mass cytometry, the authors dissected CD4 TRM heterogeneity in terms of tissue specific expression of homing markers, along with the identification of distinct phenotypic and functional (in terms of cytokine production) CD4 T cell clusters within individual tissues. These studies underscore the complexity of CD4 TRM cell biology and lay the groundwork for applying more recently developed single cell technologies to the exploration of CD4 TRM cell heterogeneity.

Another important consideration is how closely the observations made in mouse models correlate with human TRM cell biology. It is important to note that a majority of mouse studies have examined antigen specific CD4 T cell responses, while most human studies have focused on a broader characterization of TRM cells. While many similarities exist, there are also some discrepancies, although whether these differences are a result of mouse-to-human comparison or CD4-to-CD8 comparison is unclear. For example, mouse CD8 TRM generated after LCMV infection express high levels of the transcription factor Hobit. Although Hobit could be identified in circulating human cytolytic CD4 T cells, it was not significantly upregulated in human TRM cells isolated from liver, gut, or skin (16). On the other hand, another study looking at CD103+ CD4 TRM cells isolated from human lung found increased mRNA expression of Hobit, although protein was not expressed (61). Importantly, in this latter study, CD103 was observed on ∼10% of CD4 TRM cells isolated from the human lung, which is in contrast to mouse models either at steady state or after infection (61). Here the authors also identified a prevalent Notch signature in human CD4 TRM cells. Although the role of Notch signaling has not yet been addressed in mouse CD4 TRM cells, it was previously reported to be highly expressed in human CD8 TRM cells and is also required for the maintenance of mouse CD8 TRM cells (96, 97). Given the importance of Notch signaling in the survival of circulating memory CD4 T cells (98, 99), it seems likely that Notch would also play a role in CD4 TRM cells. Going forward, it will be important to connect observations in mouse models, which yield greater flexibility in terms of immune manipulation and organ harvest, with the valuable observations being made in human tissues. In addition, it will be essential to determine whether a minor subset of circulating TRM or pre-TRM cells can be identified, particularly after oral, intranasal or topical immunization leading to TRM induction. The identification of such cells would greatly aid the comparison of mouse and human TRM studies.

CONCLUDING REMARKS

CD4 TRM cells localized within barrier tissues are poised to provide immediate protection from re-challenge infection. In some cases, however, the long-term survival of CD4 TRM cells within inflammatory or autoimmune contexts can lead to host immunopathology. Understanding the cellular requirements and transcriptional basis underlying the acquisition and maintenance of CD4 TRM phenotype, function and heterogeneity is crucial for identifying ways in which CD4 TRM cells could be targeted for human health. One impediment to the detailed characterization of CD4 TRM cells is that the processing steps for isolation of TRM cells result in extremely poor cell recovery and the potential to bias against certain cell subsets (100). However, recent advances in multiplexed single cell imaging and single cell RNA transcriptomics in combination with TCR repertoire analysis will greatly assist in dissecting the relationship of CD4 TRM heterogeneity to cell activation state, function, and intercellular interactions (42, 101). It will additionally be important to examine how CD4 TRM cells respond to re-stimulation, whether they can self-renew, and whether or not they exhibit fate plasticity. Along these lines, secondary effector CD4 T cells responding to influenza in the lung contain both Th17 and TFH subsets, neither of which are present during primary infection (102). It will be of great value to determine whether such cells arise from distinct CD4 TRM precursors or whether they are newly generated in lymphoid organs. Understanding CD4 TRM flexibility during chronic infection or within the tumor microenvironment will also be important for assessing the potential of vaccines to target these populations.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Non-hematopoietic Control of Peripheral Tissue T Cell Responses: Implications for Solid Tumors

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In response to pathological challenge, the host generates rapid, protective adaptive immune responses while simultaneously maintaining tolerance to self and limiting immune pathology. Peripheral tissues (e.g., skin, gut, lung) are simultaneously the first site of pathogen-encounter and also the location of effector function, and mounting evidence indicates that tissues act as scaffolds to facilitate initiation, maintenance, and resolution of local responses. Just as both effector and memory T cells must adapt to their new interstitial environment upon infiltration, tissues are also remodeled in the context of acute inflammation and disease. In this review, we present the biochemical and biophysical mechanisms by which non-hematopoietic stromal cells and extracellular matrix molecules collaborate to regulate T cell behavior in peripheral tissue. Finally, we discuss how tissue remodeling in the context of tumor microenvironments impairs T cell accumulation and function contributing to immune escape and tumor progression.

Keywords: non-hematopoietic cells, T cell, immunotherapy, extravasation, interstitial migration, fluid flow, trafficking

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INTRODUCTION

Immune surveillance and protective immunity is dependent upon sequential, rapid activation, and mobilization of hematopoietic cells that undergo multiple intercellular interactions to mediate immune control. Rather than being stochastic, these interactions are guided by non-hematopoietic cells that generate and maintain tissue scaffolds. The microenvironments through which leukocytes traffic (e.g., blood, lymphoid organs, peripheral tissues) differ significantly with respect to cellular and protein composition and remodel in the context of disease and with age. Thus, in order to perform their protective function, both effector and memory T cells must adapt to continuously changing physical, biochemical, and metabolic tissue environments.

De novo T cell priming is initiated in lymph nodes (LN) that drain peripheral sites of infection, inflammation, and tumors. Within lymphoid organs, non-hematopoietic cells direct cellular interactions and increase the probability of immune activation. Lymph-borne antigen is transported to LNs through afferent lymphatic vessels that connect to the subcapsular sinus allowing delivery of large particulate antigens (>70 kDa) to interfollicular dendritic cells (DC) and subcapsular macrophages (1, 2). Small antigens (<70 kDa) enter fibroblast reticular cell-lined (FRC) conduits and are sampled by resident DCs (3). Both the packing of collagen fibers within FRC-conduits and direct filtration by lymphatic endothelial cells (LEC)-lining the lymphatic sinus

floor determine LN size exclusion properties and thus dictate antigen delivery (3, 4). While lymph flow is constitutive at steady state, lymphatic fluid transport is rapidly reduced following cutaneous infection, indicating that peripheral tissue context and lymphatic vessel function dictate antigen delivery (5).

Within LNs, non-hematopoietic stromal cells generate and maintain chemokine gradients to direct leukocyte recruitment and positioning. Afferent lymphatic vessels direct DC homing and express adhesion molecules that permit transendothelial migration, while specialized blood vessels, high endothelial venules, facilitate naïve lymphocyte entry. FRCs provide a physical scaffold within the paracortex, express homeostatic chemokines that bring mature, antigen-loaded DCs in close proximity with naive T cells (6), and modulate their contractile phenotype to permit LN enlargement and lymphocyte expansion (7). Following activation, T cells egress LNs along shingosine-1-phosphate (S1P) gradients actively maintained by efferent LECs and ultimately recirculate into blood (8). These newly T cell receptor (TCR)-stimulated effector T cells are now proficient to recognize inflamed blood endothelium in peripheral, non-lymphoid tissues and are restricted from re-entry into LNs (9). Importantly, while naïve T cells require TCR stimulation in lymphoid organs for activation, pre-existing memory T cells acquire tissue-homing capability independent of TCR-stimulation and are rapidly mobilized to sites of inflammation where they exert their protective function (5, 10, 11). Thus, though effector and memory cells are subject to the same peripheral tissue microenvironments and barriers upon arrival, the signals required to activate mechanisms of homing and tissue adaptation may be distinct (9).

Just as in secondary lymphoid organs, non-hematopoietic cells in peripheral, non-lymphoid tissue provide a functional scaffold that determines T cell infiltration, motility, effector function, and retention. Tissue remodeling in chronic diseases, such as cancer, significantly alters requirements for T cell behavior and function. Here we discuss the current state of knowledge regarding interaction between T cells and non-hematopoietic stromal components in peripheral, non-lymphoid tissue. How effector and memory T cells adapt within and navigate through these non-hematopoietic barriers is poorly understood, and yet the heterogeneity of tissue structure and function within which T cells impart immune control must necessitate an array of adaptive mechanisms. A more detailed understanding of mechanisms used by effector and memory T cells to adapt to their peripheral tissue environment will provide crucial insight into the ways in which solid tumors inhibit T cell function and mediate immune escape.

GETTING IN: T CELL EXTRAVASATION ACROSS THE VASCULAR ENDOTHELIUM

Inflamed Endothelial Cells Provide Signal Two for Tissue Infiltration

Though activated effector and memory T cells acquire the machinery necessary for homing to inflamed tissue in response to TCR and inflammatory stimuli in circulation and lymphoid organs (9), activated vascular endothelial cells (EC) that line post capillary venules in tissue provide the critical signal 2 necessary for infiltration. Lymphocytes home to sites of inflammation following a cascade of adhesive and signaling events mediated by sequential ligation and activation of selectins, integrins, and chemokines on ECs. EC activation and expression of these necessary adhesive molecules occurs only at sites of inflammation, thus ensuring specific infiltration of inflamed tissue (12) and sparing normal, uninflamed tissues from unnecessary lymphocyte infiltration, such that ECs act as key determinants for the anatomic tissue distribution of stimulated lymphocytes (**Figure 1**).

At steady-state, low levels of lymphocyte adhesion molecule expression (13, 14) is maintained by tonic nitric oxide signaling (15) and lack of inflammatory stimuli. In response to challenge, tissue-resident macrophages, mast cells, and damaged fibroblasts (16) produce tumor necrosis factor α (TNFα) and interleukin-1 (IL-1) (17, 18), which are sufficient to activate local but not systemic ECs (19). Activation of nuclear factor-κB (NF-κB) in ECs by these inflammatory stimuli upregulates P- and E-selectins, intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1), and chemokines, and EC-specific loss of NF-κB is sufficient to prevent lymphocyte infiltration into tissue (17). Selectins bind to carbohydrate moieties on glycoproteins expressed by effector and memory T cells (9). Selectin binding initiates T cell rolling along the inflamed endothelium (20), allowing for subsequent chemokine detection. Chemokines produced by ECs then direct actin-dependent spreading, polarization, and lateral migration of arrested lymphocytes across the endothelial surface, presumably to identify sites permissive to transmigration, marked by clustered cell adhesion molecule (CAM) expression. High-affinity adhesive interactions between ICAM-1 and VCAM-1 and their respective integrins (LFA-1/αLβ2, and VLA-4/α4β1) ultimately lead to lymphocyte arrest (18).

While the endothelium rapidly responds to inflammatory cues to recruit circulating lymphocytes, it may also inhibit T cell adhesion and migration under certain conditions. T cells have decreased adhesion to inflamed ECs co-cultured with dermal fibroblasts, but not fibroblasts isolated from synovial joints of rheumatoid arthritis patients (21), indicating that fibroblasts help to maintain the endothelial barrier to lymphocyte infiltration in healthy tissue while their dysfunction may promote disease. PEPITEM, a small peptide released from adiponectin-stimulated B cells, binds to cadherin-15 on ECs and triggers production and release of sphingosine 1 phosphate (S1P), which reduces T cell trafficking across endothelium (22), and low expression of adiponectin receptor on B cells is associated with chronic lymphocyte infiltration in diseases such as type 1 diabetes, rheumatoid arthritis, and aging (22).

Upon adhesion to inflamed endothelium, lymphocytes next traverse the endothelial barrier. Endothelial cells actively support and guide lymphocytes to sites permissive to transmigration while still maintaining barrier integrity via integrin-dependent mechanisms of actin remodeling (23). At sites of transmigration, ICAM-1/LFA-1 and VCAM-1/VLA-4 clustering forms an immunological synapse-like interaction between ECs and T cells

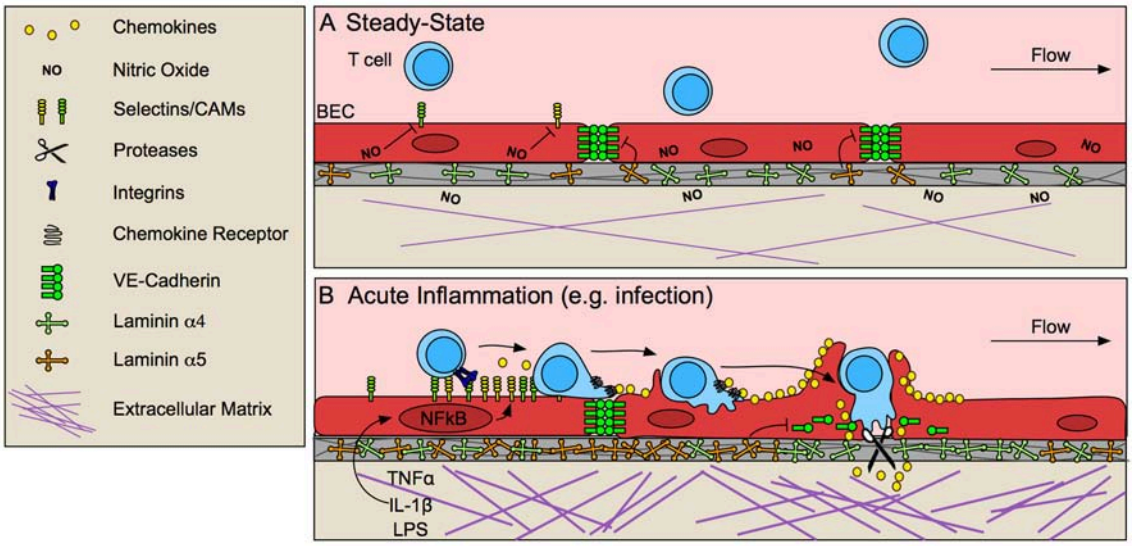

FIGURE 1 | Blood endothelial cells control T cell entry into inflamed tissue. **(A)** The vascular endothelium limits T cell infiltration at steady-state by low expression of selectins and cell adhesion molecules (CAMs), and stabilized endothelial cell-cell junctions, due in part to tonic nitric oxide (NO) signaling and laminin α5-mediated VE-cadherin stabilization. **(B)** In response to pathological challenge and inflammatory stimulus (e.g., TNFα, IL-1β, LPS), blood endothelial cells (BECs) become activated and increase expression of selectins, CAMs and chemokines, which promote lymphocyte adhesion and migration to sites permissible for transmigration. In some cases, BECs form a transmigratory cup that provides a perpendicular scaffold to direct T cell transmigration. Inflammatory remodeling of the basement membrane contributes to lymphocyte access through destabilization of VE-cadherin at endothelial junctions and by generating low-density sites permissive to lymphocyte migration.

(24), concentrating adhesion molecules into a ring structure (25). ECs often extend microvilli symmetrically around T cells to form a "transmigratory cup" (26) which further strengthens adhesion and provides a perpendicular scaffold to promote transmigration (24). Ultimately, T cells pass through the endothelium in one of two ways, either between ECs at intercellular junctions (paracellular route), or directly through individual ECs (transcellular route). Transcellular migration seems to be initiated by invadosome-like protrusions on lymphocytes (27). Paracellular migration, on the other hand, requires EC-mediated destabilization of vascular endothelial cadherin (VE-cadherin) at endothelial cell-cell junctions (28) and is further mediated by integrins, CAMs, and other adhesion molecules such as PECAM-1, JAM-1, and CD99 (18).

Destabilization of VE-cadherin at EC cell-cell junctions seems to be necessary for lymphocyte transmigration (29). ECs expressing a mutant form of VE-cadherin that is not endocytosed and therefore retained at cell-cell junctions, prevents lymphocyte recruitment to inflamed skin (28). Blockade of VE-cadherin stabilizing integrins, β1 and β3 (29) or dephosphorylation of tyrosine 731 by SHP-2 targets VE-cadherin for endocytosis and subsequently increases neutrophil transmigration *in vitro* (30). Interestingly, lymphocyte binding to ECs induces SHP-2-mediated VE-cadherin destabilization (30), indicating that lymphocyte adhesion may prime ECs to be permissive of transmigration. VE-cadherin is also cleaved by a disintegrin and metalloproteinase 10 (ADAM10) and tetrospanin 5 and 17, expressed by inflamed ECs, and EC-specific loss of ADAM-10 delays T cell, but not neutrophil or B cell, transmigration *in vitro* (31). Interestingly, proteolytically active leukocytes, such as neutrophils, may mediate cleavage necessary for lymphocyte transmigration in the absence of EC proteolysis (31). Thus, even if not intrinsically proteolytic, leukocyte protease activity may positively promote lymphocyte transmigration across inflamed endothelium *in vivo*.

Antigen-Dependence of T Cell Recruitment and Extravasation

Peripheral effector (12) and memory T cells (11) are recruited to inflamed tissue in an antigen-independent manner, indicating that local presentation of cognate antigen is not necessary for tissue infiltration. The antigen-independence of T cell recruitment is exemplified by recent studies that demonstrate abundant bystander, pathogen-specific T cells, in solid tumors (32). Interestingly, however, homing of insulin-specific $CD8^+$ T cells to pancreatic islets, but not other tissues, is reduced with loss of major histocompatibility complex class I (MHC-I) *in vivo* (33) and antigen-loaded MHC-I presented on luminal surfaces of the blood-brain barrier was functionally required for antigen-specific T cell trafficking to the brain (34). These observations have led to the hypothesis that antigen presentation by ECs may amplify antigen-specific T cell recruitment in certain tissues and disease states. ECs dynamically express MHC-I and MHC-II during inflammatory processes and possess antigen-processing machinery necessary for cross-presentation of exogenous antigens (35). Human ECs scavenge and cross-present the type I diabetes islet autoantigen GAD65 on MHC-II and this enhances the transmigration of antigen-specific T cells *in vitro* (36). Further *in vitro* evidence supports both inhibitory (37) and promotional (38, 39) roles for EC antigen presentation

in lymphocyte trafficking, indicating that antigen-presented by ECs may provide context-dependent "go" or "stop" signals that tune T cell infiltration.

Interestingly, ECs express a variety of T cell costimulatory and coinhibitory molecules (24), and as such, may represent semi-professional APCs strategically placed to interact with activated effector and memory T cell populations. In addition to tuning transmigration, data from various tissues indicate that ECs may employ their repertoire of immune checkpoints and APC-like function to mediate peripheral tolerance and modify T cell behavior as they transmigrate or arrest at the vascular interface. For example, liver sinusoidal endothelial cells scavenge and cross-present food-borne antigens and induce tolerance through T cell adhesion and sequestration in the liver (40, 41), and tumor-associated LECs cross-present exogenous antigens (42, 43) and maintain peripheral tolerance to self-antigens in LNs (44, 45) dependent on constitutive expression of programmed death-ligand 1 (PD-L1) (45). The relative significance of EC antigen presentation *in vivo*, however, is likely both tissue and disease specific. Further testing is needed and specifically, EC-specific knockdown strategies, to determine the functional relevance of EC antigen-processing and presentation *in vivo*.

Overcoming the Basement Membrane

The final and rate-limiting step in lymphocyte extravasation is crossing the basement membrane (46). The basement membrane is a 20–200 nm thick dense proteinaceous substrate composed of laminins, collagen type IV, and sulfated proteoglycans (47), that separates the vascular endothelium from extracellular matrix (ECM) in the tissue parenchyma. Laminins and collagen IV produced by ECs self-assemble into a dense sheet that is crosslinked by perlecans and nidogen and contains 2–5 μm-in-diameter pore-like regions of low protein density (48), presumed sites of lymphocyte passage. Basement membrane composition differs between developmental stage, vessel type, and activation state of the endothelium (47), with particular variability of laminin isoforms. Laminins are composed of alpha, beta, and gamma chains (e.g., laminin α4, β1, γ1 is denoted as laminin 411), and presence in basement membrane is context and location dependent. In the central nervous system (CNS), laminin α4 is ubiquitous (49), while laminin α5 expression is patchy and irregular (50), but both are increased upon inflammation (47, 51). In murine experimental autoimmune encephalomyelitis extravasation occurs predominantly at sites of low laminin α5 density (29, 51) and laminin α5 is sufficient to inhibit T cell transmigration in a dose-dependent manner *in vitro* (50). Additionally, laminin α4-deficient mice increase expression of laminin α5 in the CNS leading to decreased T cell migration across the blood brain barrier in EAE (50), suggesting that the composition of laminins in the basement membrane may selectively regulate T cell transmigration.

The mechanisms by which different laminin isoforms regulate T cell transmigration are unclear. Laminin α5 binds to integrin β1 and β3 on ECs and stabilizes VE-cadherin at EC junctions (29). Activated lymphocytes also express integrin β1 (18), however, and it is possible that laminin α5 may signal directly to infiltrating lymphocytes and instruct transmigration, although this has not been investigated. Regardless of how T cells get across the EC layer, the basement membrane is a dense, proteinaceous barrier that they must penetrate to complete diapedesis. Neutrophils express elastase to remodel regions of low basement membrane density allowing for their tissue infiltration (52), however, the specific mechanisms of lymphocyte migration through the basement membrane is unclear. The small size and pliability of lymphocytes and their nuclei may permit movement through the 2–5 μm pore-like regions of the basement membrane. However, T cell intrinsic loss of granzyme B (GrzB), which degrades both collagenous and non-collagenous ECMs (53, 54), reduces extravasation *in vivo* (54), indicating proteolysis may be required for basement membrane penetration. Further studies are needed to evaluate the contribution of the basement membrane to selective lymphocyte extravasation in acute and chronically inflamed tissue.

MOVING AROUND: T CELL INTERSTITIAL MIGRATION IN HOMEOSTATIC AND INFLAMED PERIPHERAL TISSUES

Following extravasation from the vasculature, effector and memory T cells encounter the complex heterogenous interstitial matrix through which they must traverse and locate target cells (**Figure 2**). The ECM defines the 3D structure of tissues and exhibits heterogeneity across tissue types and disease states. The topography of the interstitial matrix is determined by the combination of structural and non-structural glycoproteins such as fibrillar collagen, elastin, fibronectin, laminin, and tenascin, decorated by associating proteoglycans (e.g., decorin and versican), which contain glycosaminoglycans (GAGs; e.g., heparan sulfate and chondroitin sulfate). The physical spacing and orientation of fibrillar proteins, as well as net charge of decorating GAGs, determines matrix porosity, rigidity, and bioactive molecule presentation. Both the physical and molecular properties of the ECM determine necessary modes of leukocyte migration and thus the efficiency with which leukocytes survey tissue. Compared to innate immune cells (e.g., neutrophil and DC), the mechanisms that govern the interstitial motility and homing behavior of T cells are poorly defined, yet adaptation to and utilization of chemical and physical signals in heterogenous tissues is necessary to rapidly identify rare APCs and mediate their local effector function. Tissue biophysics, matrix rigidity and interstitial fluid flux, may be an important control point for tissue T cell dysfunction. Whether T cell motility is simply a function of the existing microenvironment, or if rather T cells may exert force within interstitial tissues to direct their movement is critical to understanding diseases where T cell infiltration is impaired, such as cancer.

Distinct Mechanisms of T Cell Interstitial Migration in Naïve and Inflamed Tissue

Molecular mechanisms of T cell interstitial migration (55, 56) have largely been determined in the context of 2D and 3D *in vitro* experimental systems that allow for control of physical and chemical cues to determine specific effects on

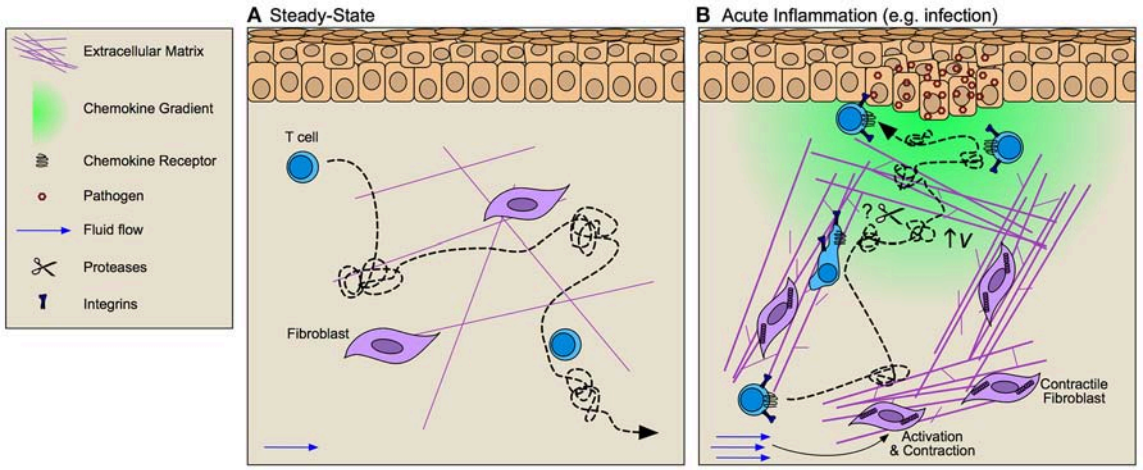

FIGURE 2 | Interstitial matrices control T cell migration through inflamed tissue. **(A)** At steady-state T cells exhibit integrin-independent amoeboid-like Lévy walk behavior (dotted line) that facilitates their surveillance of peripheral tissue. **(B)** During inflammation fibroblast activation alters tissue tension through increased deposition, bundling, and cross-linking of extracellular matrix (ECM) components thereby altering the scaffold across which T cells must migrate. Increased interstitial fluid flows that result from vascular permeability further activate fibroblasts and promote directional fiber alignment. These inflammation-induced ECM changes activate integrin-dependent lymphocyte migration along collagen bundles. Whether T cell migration is dependent upon ECM organization or if rather lymphocytes may activate proteolytic activity to promote tissue invasion remains largely unclear. Facilitating T cell position at the site of challenge are chemokine gradients (e.g., CXCL9/10) that increase lymphocyte velocity (v) thereby improving their Lévy walk search efficiency and permitting accumulation at and around target cells.

T cell motility. These studies indicate that in naïve matrices lymphocytes primarily utilize amoeboid modes of movement independent of focalized adhesions and pericellular proteolysis (57, 58). Nuclear squeezing and deformation permits lymphocyte movement along preformed structures at velocities 10–40 fold higher than adhesion-dependent, mesenchymal migration (59). These studies have importantly established guiding principles for understanding T cell behavior in 3D, however, fail to capture the full complexity of inflamed interstitial matrices, which exhibit altered collagen fiber density, orientation, and composition relative to naïve in a tissue-specific and challenge-specific manner. Highlighting the discrepancies found between naïve matrices *in vitro* and inflamed tissue *in vivo*, intravital imaging in inflamed skin indicate that Th1 T cell motility is integrin-dependent, where specifically αv integrins (paired either with β1 or β3) facilitate T cell motility along matrix fibers (60). Matrix remodeling, therefore, necessitates T cell adaptation and activation of adhesion-dependent modes of migration. Importantly, effector and memory T cells do express an array of matrix-binding integrins that increase in expression upon activation and provide ligand specificity for various matrix components (61). Furthermore, T cells extracted from gut are more adhesive to ECM *in vitro* than circulating lymphocytes (62) and $CD4^+$ effector T cells display distinct integrin repertoires when extracted from skin or lung, consistent with the differences of ECM composition in each tissue (60). Whether T cells tune their integrin repertoire in the context of their resident ECM, are imprinted at priming to prefer certain matrices, or a combination of both, remains an open question.

Interestingly, intravital imaging largely demonstrates that T cells preferentially follow pre-formed networks of fibrillar structures (55, 60, 63). 3D confinement studies *in vitro* indicate that limiting the space through which T cells can migrate significantly alters migratory speeds (64) and thus within a heterogenous interstitial matrix, it has been proposed that T cells may simply follow the path of least resistance (65). While in healthy tissue, pre-formed ECM tracks may facilitate the directional migration of T cells toward their target, under pathological conditions such as fibrosis and cancer, increased ECM density and rigidity (66) may act as a barrier to T cell infiltration and motility (discussed in more detail later). Consistent with this hypothesis, dynamic imaging reveals that matrix fiber density and orientation at tumor borders directs peripheral but not intratumoral T cell motility (63), and liver fibrosis inhibits T cell-mediated killing of infected hepatocytes (67), suggesting that T cells are incapable of proteolytically invading dense matrix structures.

Peripheral T cells, however, do express a limited array of proteases upon antigen stimulation and cytokine exposure. Matrix metalloproteinase 2 (MMP-2) is upregulated following VCAM-dependent adhesion to ECs (68), likely facilitating T lymphocyte invasion across the basement membrane. Inflammatory mediators [e.g., CCL5, prostaglandin E2, leukotriene B4 (LTB4), TNFα, transforming growth factor β (TGFβ), and IL-2] induce MMP-9 expression while type I and II interferons (IFN) are suppressive (68). Granzyme B, secreted by activated T cells, has protease activity (53, 54) and urokinase degrades laminins and fibronectin and activates some latent form of MMPs (69). Importantly, evidence for protease-dependent tissue invasion of T cells was identified in rheumatoid arthritis patients. T cells isolated from peripheral blood of rheumatoid arthritis patients display increased invasive capacity *in vitro*

and *in vivo* dependent upon elevated expression Tks5, a scaffold protein required for the formation of matrix-degrading invadopodia (70). Thus, at least in some diseased states, T cells may activate intrinsic proteolysis that supports their pathologic activity.

While the ECM provides an instructional scaffold upon which leukocytes migrate through the interstitium, diffuse matrix-bound chemokine gradients are proposed to determine positioning and directionality. Intravital imaging, however, fails to identify persistent and directional T cell homing *in vivo* and T cells rather exhibit a Lévy walk motility pattern characterized by straight runs at fixed velocity interspersed with pauses (71). Interestingly, blockade of the CXCR3 ligand, CXCL10, in a model of *Toxoplasma* infection of CNS resulted in reduced $CD8^+$ T cell velocity but did not alter Lévy walk patterns, indicating that CXCL10 may function to improve protective immunity by increasing T cell speed rather than through directional migration (71). Thus, reduced velocity impairs searching efficiency and reduces lymphocyte recruitment to and positioning at infectious foci. Consistently, CXCR3 expression on T cells was dispensable for vascular extravasation but required for localization to infected foci in cutaneous vaccinia infection (72). Continued understanding of the ways in which chemokines affect T lymphocyte behavior in tissues requires further intravital imaging studies to directly assay the dynamic behavior of T cells in the presence or absence of specific chemoattractant and chemorepellant molecules.

Organization of the Interstitial Matrix by Fluid Flows

Constitutive, interstitial fluid flows are kept in constant motion by hydrostatic and osmotic pressure differences between blood vessels, the interstitium, and lymphatic vessels (73). Interstitial fluid flow is slow, ranging from 0.1–2 μm/s at steady state and increased during inflammation and in tumors (73). Lymphatic vessels dynamically regulate fluid transport and consequently influence levels of interstitial fluid flow, which may in turn affect interstitial immunity. Cutaneous lymphatic vessels rapidly shut down fluid transport in a type I IFN-dependent manner preventing passive viral dissemination to LNs (5). Furthermore, mosquito saliva is sufficient to induce local edema and an inflammatory influx of neutrophils that when delivered in combination with virus, promotes viral retention at the bite site and enhances infection (74). Thus, modulation of fluid transport phenomena (either through enhanced vascular leakiness or altered lymphatic transport) may be a critical feature of tissue infection, which remains to be explored more carefully.

Dynamic regulation of fluid flux through tissue impacts ECM density, stiffness, and alignment and thus the scaffold within which cell motility is directed. Increased interstitial fluid pressure activates fibroblasts through integrin signaling and TGF-β upregulation of α-smooth muscle actin (αSMA) thereby increasing fibroblast contractility and subsequent alignment and bundling of collagen fibers (75). Flow additionally influences the expression and distribution of soluble and matrix bound factors along this scaffold to inform interstitial cell motility. Pericellular gradients can be established via multiple mechanisms including proteolytic release from ECM, degradation, metabolism, or removal by decoy receptors of a local source of attractants (76). Additionally, interstitial fluid flow introduces directional bias in chemokine distribution when expressed by migrating cells setting up functional gradients in the direction of flow. Termed autologous chemotaxis, this mechanism may support directional migration of tumor cells (77) and DCs to draining peripheral lymphatic vessels. How interstitial fluid flows influence T cell motility either directly, or indirectly remains to be experimentally tested.

GETTING OUT: T CELL EGRESS VIA LYMPHATIC VESSELS

Lymphocytes Exit Peripheral, Non-Lymphoid Tissues

Following entry into and surveillance of tissue at least a subset of T cells continue on and egress out through lymphatic vessels (**Figure 3**). Parabiosis experiments demonstrate that most endogenous memory T cells in peripheral tissue reach equilibrium with migratory blood-borne donor T cells indicating rapid turnover in peripheral tissue (78) [with the notable exception of resident memory lymphocytes (79)]. In sheep, where lymph can be readily sampled, afferent lymph contains 10^6 cells/ml (80). Furthermore, the number of leukocytes in lymph are increased by sometimes as much as 100 fold during acute and chronic inflammatory signals (80), indicating tissue egress is influenced by context. Whether the cellular component of afferent lymph is simply a reflection of the tissue it drains (e.g., passive, random transport) or rather represents a subset of tissue lymphocytes (e.g., active, selective transport) remains largely unknown.

At least two candidate molecules have been proposed as necessary signals for lymphatic directed egress, CCL21/CCR7 and S1P/S1PR1. In models of acute lung and skin infection, T cells egress from inflamed peripheral tissue in a CCR7-dependent manner (81, 82), such that $CD4^+$ T cells accumulate in epithelial tissue of $CCR7^{-/-}$ mice in an age-dependent manner (83). Additionally, forced overexpression of the spingosine-1-phosphate receptor (S1PR1) prevents the establishment of tissue resident memory $CD8^+$ T cells, suggesting that the inability to respond to S1P gradients maintained by lymphatic vessels is necessary for local retention (84). Treatment with FTY720 (S1PR1 agonist) only partially inhibits egress (85), but does improve CD69-deficient $CD8^+$ T cell persistence in skin after HSV infection (86). Thus, this together with low levels of CCR7 and expression of the E-cadherin binding CD103 and β1 integrin may promote retention (12, 87, 88). Conversely, egress from chronically inflamed tissue is pertussis toxin sensitive but CCR7-independent (85), indicating a role for alternative G-protein coupled receptors. CXCR4 might represent an alternative mechanism of lymphatic vessel directed egress. DC trafficking is reduced but not completely eliminated in $CCR7^{-/-}$ mice, and inhibition of CXCR4 further reduced DC trafficking to draining LNs in a model of contact hypersensitivity (89), but evidence

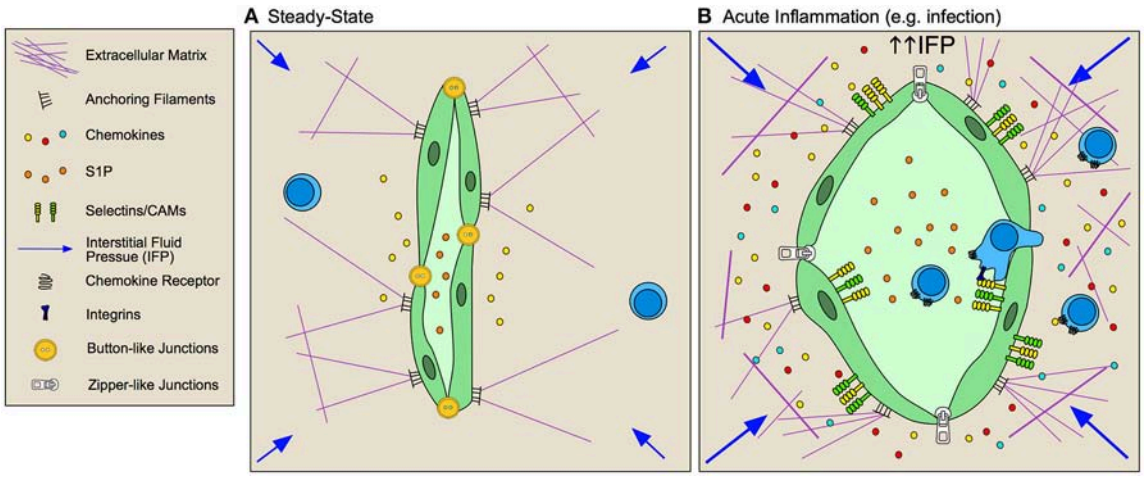

FIGURE 3 | Inflamed lymphatic vessels promote lymphocyte exit from tissue. **(A)** Steady state lymphatic endothelial cells (LEC) form loose button-like junctions in lymphatic capillaries, and constitutively transport interstitial fluid from peripheral non-lymphoid tissue to draining lymph nodes. Production of homoestatic chemokines, such as CCL21, supports immune surveillance by directing leukocyte homing toward lymphatic vessels and tissue egress. **(B)** During inflammation, lymphatic vessels respond to both biochemical and biophysical stimuli to adapt their function within the tissue. Lymphatic vessels are activated by inflammatory cytokines and elevated interstitial fluid flow resulting in increased expression of selectins, cell adhesion molecules (CAMs) and context-dependent chemokine secretion that together promotes lymphocyte egress from inflamed tissues. LECs remodel their junctions, going from loose, button-like junctions to tight, zipper-like junctions, which is associated with decreased fluid transport from inflamed tissues and subsequent increased interstitial fluid pressures.

for CXCR4-dependent T cell egress is lacking (90). Studies using quantitative models that track endogenous populations of tissue-resident lymphocytes, either through intravital imaging or photoconvertible mice, remain necessary to provide clarity regarding the molecular mechanisms that determine the context-dependence and specificity of leukocyte egress from inflamed tissues.

Lymphatic Vessels Regulate Tissue Exit

To facilitate tissue exit, lymphatic vessels express an array of chemokines in a context-dependent manner. LECs constitutively express CCL21 (91, 92) and further increase expression during chronic lung inflammation (90) and acute inflammation in skin, but not treatment with complete Freund's adjuvent (CFA) (93). TNFα stimulation of LECs causes release of CCL21 stores (94) and *de novo* production of CCL21 (94) as well as a host of other chemokines including CCL20, CXCL5, CCL5, CXCL2, CX_3CL1, and CCL2 (95). Additionally, *in vitro* analysis indicates that lipoteichoic acid, a component of gram-positive bacterial cell walls, induces TLR2-dependent expression of CXCL1, CXCL3, CXCL6, and CXCL8 (96). *In vivo* analysis of mRNA from LECs in inflamed skin confirms these *in vitro* results, and also identified several other chemokines expressed by LECs, including the $CD8^+$ T cell-homing chemokines CXCL9 and CXCL10 (93), all together indicating that the chemokine repertoire produced by LECs in peripheral tissue is context dependent. Consequently, how this diverse repertoire of chemokines produced by inflamed LECs functionally regulates lymphocyte egress from tissue remains a largely open question.

LECs, like BECs, increase expression of the T cell adhesion molecules in response to local inflammation and interstitial fluid flows. ICAM, VCAM, and E-selectin are expressed on the LEC surface rapidly following peripheral challenge *in vivo* (5, 93, 95) and following stimulation *in vitro* (97). LFA-1 is necessary for naïve T cell egress from inflamed skin (98) and inhibition of vascular endothelial receptor-1 (CLEVER-1) and macrophage mannose receptor prevented T cell migration through afferent lymphatic vessels to draining LNs (99, 100). The requirement for integrins in LEC transendothelial migration in inflamed tissue may mirror the differential integrin requirement for DCs. While DCs in skin squeeze through overlapping, button-like junctions in naïve lymphatic capillaries (101), transmigration across inflamed vessels requires integrin-mediated adhesion (97). Interestingly, cutaneous viral infection (5) and tracheal bacterial infection (102) induces lymphatic capillary remodeling of naïve button-like junctions to tight, zipper-junctions, typically found in deeper collecting vessels. These reversible changes may generate a less permeable endothelium and thus determine the integrin dependence of cellular transport. The functional relevance of lymphocyte egress at both steady-state and during inflammation remains to be determined, and in particular whether lymphocytes exit tissue to mediate immune resolution or rather enter LNs for re-stimulation by professional APCs remains an open and interesting question.

ADAPTATION IN TUMOR MICROENVIRONMENTS

Though tumors were previously thought to be poorly immunogenic and not capable of activating an immune response, we now know that somatic mutations (generated by DNA instability and environmental challenge) generate neo-antigens that are sufficiently distinct from self, such that

T cells are capable of expanding and directing tumor-specific killing. Thus, the accumulation of neo-antigens in tumors is likely a prerequisite to anti-tumor immunity across tumor types, and consistently, those tumors that exhibit highest somatic mutational burden, e.g., melanoma, exhibit good overall response to immune checkpoint blockade (103). Even in the presence of potent neo-antigens, however, some tumors still fail to respond to therapy and somatic mutational burden is not sufficient to predict T cell infiltration within and across tumor types (104). Thus, multiple overlapping mechanisms of immune suppression create a more complex immune landscape, such that, as discussed above, processes of T cell recruitment, retention, survival, and exit may underscore intratumoral T cell presence and thus influence response to therapy. Current efforts to define biomarkers that are predictive of response to immune checkpoint blockade reveal an array of factors from myeloid cells to the microbiome, that affect patient response. Here we will focus on the localization of T cells within and around the tumor parenchyma as one indicator of responsiveness and discuss how tumor-induced stromal remodeling may contribute to T cell distribution (**Figure 4**).

Multiple studies across tumor types now indicate that the presence of T cells within tumor nests is predictive of response to therapy (105). As a consequence, non-responding tumors typically exhibit T cell infiltrates that are described by three main patterns: (1) non-functional immune responses, possessing an intratumoral but seemingly ineffective infiltrate; (2) tissue excluded T cell infiltrates, possessing a T cell infiltrate that is restricted to the tumor periphery; and (3) immunological deserts, completely lacking T cell infiltrate both in the tumor nests and in adjacent stromal (106). The underlying biology that regulates these patterns of T cell infiltration is clearly multifactorial—some of the contributing factors from the perspective of the non-hematopoietic tumor stroma are discussed below.

Non-functional T Cell Infiltrates

Non-functional immune infiltrates (106), refers to tumors containing intratumoral lymphocytes in both pre- and post-therapy biopsies that do not contribute to significant clinical response. Importantly, methods to evaluate intratumoral T cell populations largely quantify changes in bulk T cell populations (CD4 or CD8), and even when enriched for markers of previous antigen exposure (CD45RO) or effector function (GrzB) likely still quantify a heterogenous pool of effector, effector memory, and central memory T cells that represent a range of antigen specificities both relevant and irrelevant to the tumor. Rapid recruitment of effector and memory T cells is antigen-independent (11, 12), and bystander, viral-specific T cells (e.g., HCMV or EBV-specific) are abundant in human tumor tissue (32). Thus, efforts to specifically quantify tumor-reactive T cell clones may be more predictive than bulk T cell populations. Consistent with this hypothesis, CD39 was recently identified as a marker to distinguish tumor antigen-specific $CD8^+$ T cells from bystander T cells across multiple tumor types (32, 107) and stratification of patients based on frequency of $CD39^+CD103^+$ double positive $CD8^+$ T cells associated with increased overall survival in head and neck cancer patients (107). Thus, because of the promiscuity of T cell infiltration across the vascular endothelium, the presence of bulk T cells in tumor microenvironments may be insufficient to indicate response. Even when tumors are well infiltrated with antigen-specific T cells, however, multiple additional mechanisms suppress their local effector function mediated by tumor, hematopoietic (108), and non-hematopoietic stromal cells.

Aberrant tumor angiogenesis and disrupted fluid flows in tumor microenvironments generate hypoxia and increased interstitial fluid pressures in solid tumors (73, 109) that influence T cell function. Hypoxia induces Warburg effect by cancer cells, leading to increased acidification and lactate production, both of which inhibit cytotoxic activity of lymphocytes *in vivo* (110, 111). Furthermore, increased interstitial fluid flow in the tumor microenvironment activates fibroblasts leading to TGFβ production (73) and ECM contraction. ECM contraction together with shear stress activates stromal stores of latent TGFβ (112), which attenuates $CD8^+$ T cell cytotoxicity (113) making them non-responsive to TCR signaling (114). Thus, the disrupted fluid mechanics within tumor tissue may itself participate in the regulation of local T cell function.

Furthermore, non-hematopoietic cells likely exert direct effects on T cells within tumor microenvironments. In the LN, LECs, and FRCs display specific immunological properties that function to maintain peripheral tolerance at steady state, and while we have drawn parallels between the structural role of LN stromal cells and non-hematopoietic cells in peripheral, non-lymphoid tissues, it remains less clear whether peripheral non-hematopoietic cells also acquire immunomodulatory properties characteristic of LN stroma. LN LECs express peripheral tissue antigens in an Aire-independent manner (44, 115); can scavenge and cross present exogenous antigens leading to dysfunctional $CD8^+$ T cell activation (42, 43); and can receive peptide-MHC-II expressing exosomes from DCs and induce $CD4^+$ T cell hyporesponsiveness (116). In tumors LECs are also capable of scavenging tumor-associated antigens and cross-presenting them on MHC-I (42), however, whether LEC antigen presentation functionally contributes to peripheral T cell responses remains unknown. LECs further are capable of inhibiting DC maturation and function (117) and T cell proliferation through the production of nitric oxide (118), demonstrating that LN-resident LECs inhibit T cell activation and proliferation both directly and indirectly. LN LECs also constitutively express PD-L1 and delete naïve, self-reactive $CD8^+$ T cells (45) and peripheral BECs and LECs express PD-L1 in tumors (119–121) and infected tissue (121, 122). Loss of non-hematopoietic PD-L1 and inhibition of IFNγ signaling on peripheral LECs, thus preventing PD-L1 upregulation, improved the persistence of anti-tumor $CD8^+$ T cell-mediated tumor killing and overall survival in melanoma-bearing mice (121). Importantly, loss of IFNγ signaling in LECs also promoted the accumulation of anti-viral T cells in infected skin and exacerbated tissue pathology (121). Thus, tumors may coopt normal non-hematopoietic-based mechanisms of tissue protection for immune escape.

Similarly, FRCs exhibit immunomodulatory function in LNs. FRCs express and present peripheral tissue antigens to T cells in LNs (115), receive peptide-MHC-II loaded exosomes from DCs

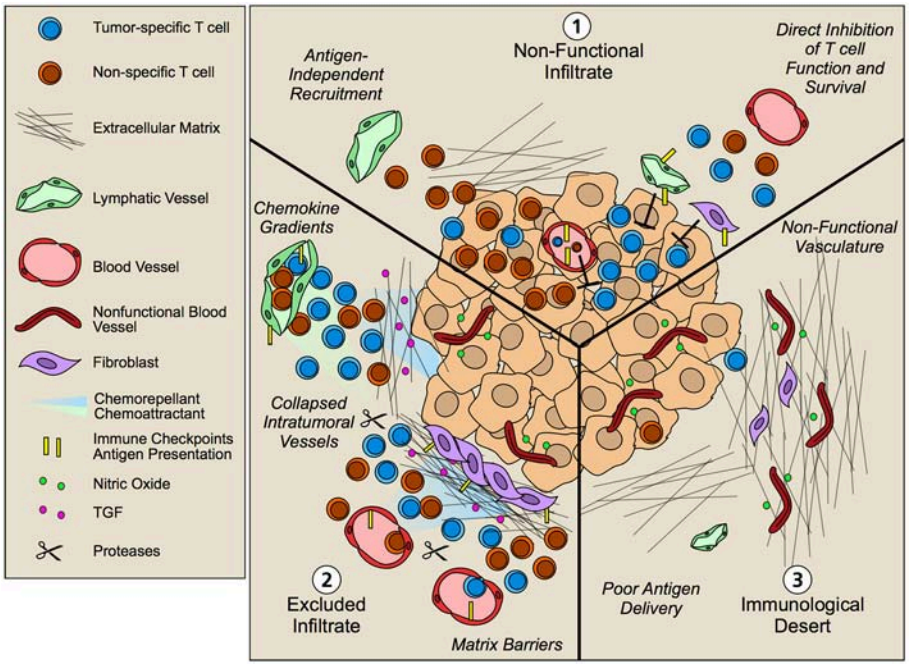

FIGURE 4 | Non-hematopoietic cell contribution to tumor immune landscapes. The geographic distribution of T cells within intratumoral and peritumoral regions is both predictive of overall survival and response to immunotherapy. Patients that fail to respond to immunotherapy often exhibit three patterns of T cell infiltrate that are governed by an array of mechanisms including contributions from tumor cells and infiltrating hematopoietic cells. Non-hematopoetic cells, however, additionally contribute to the infiltration, retention, and function of T lymphocytes in tumor microenvironments. **(1)** Non-functional infiltrate: possessing an intratumoral but seemingly ineffective infiltrate. Antigen-independent recruitment of both effector and memory T cells subsets by vascular endothelium generates a diverse repertoire of T cells both relevant and irrelevant for tumor killing. Upon tissue entry, non-hematopoietic cells further exert multiple mechanisms of immune suppression, including expression of immune checkpoints such as PD-L1 and FasL that limit local T cell function. **(2)** Excluded infiltrate: possessing a T cell infiltrate that is restricted to the tumor periphery. Establishment of matrix barriers, collapsed intratumoral vessels, poor expression of adhesion molecules, and collaborating chemoattractant and chemorepellant gradients likely all contribute to the exclusion of T cells at the periphery of tumor nests such that inhibition of these features may improve infiltration. **(3)** Immunological desert: Completely lacking a T cell infiltrate in both tumor nests and stroma. Impaired lymphatic transport may result in poor antigen delivery to lymph nodes and thus poor priming. However, even in the presence of an activated systemic T cell pool, non-functional vessels driven by the angiogenic and desmoplastic tumor microenvironment may prevent local infiltration leading to lesion-specific differences in immune infiltrates.

and induce $CD4^+$ T cell hyporesponsiveness (116), and dampen T cell proliferation through the production of nitric oxide (118, 123, 124). Interestingly, some cancer-associated fibroblasts acquire markers consistent with FRCs, namely expression of podoplanin and ER-TR7 (125). Whether these fibroblasts represent a unique subset that exhibits immune suppressive function remains unclear, though a recent study demonstrates that tumor-associated fibroblasts in melanoma models cross-present tumor antigens and inhibit T cells in a FasL and PD-L2 dependent manner (126). In LNs, migrating DCs inhibit FRC contraction through CLEC-2/PDPN interactions leading to scaffold relaxation and more space for accumulating T cells (7). Whether leukocytes directly modulate fibroblast contraction in peripheral tissue remains to be tested but might have important implications for T cell invasion into dense, desmoplastic stroma. Further work is necessary to determine the functional relevance of stromal relative to hematopoietic or tumor-mediated immune suppression.

Excluded Infiltrates

T cell exclusion, in which T cells are absent from tumor nests and rather retained in adjacent, surrounding stroma (106, 127) is a significant barrier to response to therapy. One leading hypothesis is that tissue desmoplasia, the aberrant synthesis, alignment, and crosslinking of ECM proteins by fibroblasts in tumor microenvironments (128, 129), creates a physical barrier that prevents T cell invasion. Pancreatic ductal adenocarcinoma (PDAC) is particularly fibrotic and breast carcinomas exhibit stiff collagen fibers in parallel alignment tangential to tumor borders that correlate with poor prognosis (66, 130). Furthermore, dynamic intravital imaging reveals T cell migration along collagen fibers and vessels in tumors (63, 131), consistent with their preferred amoeboid-like mode of migration described in non-malignant matrices. Thus it has been proposed that the orientation and density of matrix fibers prevents T cell infiltration into tumor parenchyma (63).

However, strategies to reduce fibrosis in mouse PDAC models have had mixed results. Though cancer-associated fibroblasts are attributed an array of tumor-promoting activities, including immune suppression, their bulk depletion in the context of PDAC did not improve tumor control but instead drove more aggressive tumor invasion and metastasis (132, 133). In contrast, strategies that rather target the composition of the ECM using enzymes that degrade specific components (e.g.,

hyaluronan) thus reducing interstitial fluid pressures (134), or reduce ECM deposition and alignment (135) improved immune infiltration and therapeutic response. Interestingly, CAR T cells have had limited utility in solid tumors at least in part due to physical tissue barriers that prevent their infiltration. Consistent with this hypothesis, CAR T cells expressing heparanase exhibit enhanced tumor infiltration and antitumor function (136). Finally, a TGF-β signature was specifically identified in a subset of PD-L1 inhibitor resistant patients exhibiting an excluded infiltrate (not dysfunctional or desert) where T cells were restricted to the fibroblast- and collagen-rich peritumoral region characteristic of metastatic urothelial carcinoma (137). Therapeutic co-administration of antibodies targeting TGF-β and PD-L1 facilitated T cell penetration into the tumor center and provoked a vigorous adaptive immune response leading to tumor regression in a large Phase II clinical trial testing atezolizumab in patients with metastatic urothelial carcinoma (137). Thus, while the ECM facilitates T cell migration in naïve or acutely inflamed tissue, tumor-associated matrix remodeling may suppress T cell motility or place new requirements for interstitial proteolysis to mediate intratumoral penetration.

Contradicting the hypothesis that a fibrotic ECM is a sufficient physical barrier to prevent T cell infiltration are desmoplastic melanomas. Desmoplastic melanomas are densely fibrotic but also exceptional responders to immunotherapy, with 70% objective response rates and 32% complete responses (138). While these tumors demonstrate a significantly higher mutational burden than PDAC, and pre-existing adaptive immune responses that correlate with PD-L1 expression, this data clearly indicates that T cells are capable of infiltrating a dense, desmoplastic fibrous stroma and that its presence is not sufficient for T cell exclusion. Furthermore, even without engineered protease expression, adoptively transferred T cells can surmount physical barriers to treat experimental PDAC models (139). While ECM composition and rigidity differs between tissue and tumor types, the specific rate-limiting factors for T cell infiltration into fibrotic tissue remain to be determined.

In addition to the effects of desmoplasia on T cell exclusion, angiogenic growth factors such as vascular endothelial growth factor A (VEGF-A), angiopoietin, basic fibroblast growth factor (bFGF), and endothelin-1 (140, 141) attenuate inflammatory-mediated endothelial activation and thus intratumoral vessels exhibit reduced expression of adhesion molecules that would mediate lymphocyte extravasation, such as ICAM, VCAM, and E-selectin (142, 143). For example, bFGF inhibits TNFα/IL-1α-mediated expression of ICAM, VCAM, and E-selectin *in vitro* (144), and VEGF-A disrupts their clustering, therefore decreasing T cell adhesion to ECs (13). Endothelin signaling on ECs increases NO production and subsequent downregulation of adhesion molecules, thus blockade of the receptor increases T cell adhesion and infiltration into tumors (142). Angiogenic signaling from tumor cells also induces FasL expression on tumor associated ECs that limits tumor infiltrating $CD8^+$ T cells, presumably through direct killing as demonstrated *in vitro* (145, 146). Consequently, factors that drive the angiogenic switch in tumors simultaneously establishes immunological barriers to limit immune surveillance and facilitates immune escape.

While anti-angiogenic therapy focused on destruction of tumor-associated vessels largely failed in most solid tumor types, adaptation of these strategies utilizing lower, normalizing doses to restore perfusion and adhesion molecule expression has proved more productive (147). Dual angiopoietin and VEGF-A blockade leads to increased T cell accumulation and function in several tumor models and synergizes with anti-PD-1 therapy (120). Furthermore, combination of anti-VEGFR2 and anti-PD-L1 antibodies induced lymphotoxin-dependent emergence of high endothelial venule-like vessels (148), which were necessary for response and are associated with better overall outcome in patients (149, 150). Interestingly, in mouse models, responders to immune checkpoint blockade exhibited rapid reperfusion of intratumoral vessels indicating that intratumoral vascular function may be required for T cell effector function and additionally that checkpoint blockade may directly affect endothelial cells (151). Thus, normalizing the angiogenic tumor vasculature may improve local T cell recruitment generating microenvironments primed to be responsive to immunotherapy. Interestingly, poorly adhesive, angiogenic vessels appear to be largely restricted to intratumoral regions, where they exhibit reduced adhesive properties and elevated expression of immune checkpoints (142). While this geographic vessel heterogeneity may limit infiltration directly into the tumor proper, it still allows infiltration into adjacent stroma perhaps contributing to the dense rings of $CD8^+$ T cells observed around tumor nests.

Additionally, antitumor effector and memory T cells restricted to peritumoral stroma may be unable to locate target tumor cells due disrupted chemokine signals. High expression of the T cell attracting chemokines CXCL9, CXCL10, CXCL12, and CCL5 correlates positively with $CD8^+$ T cell infiltration across several tumor types (152–154), indicating that if the tumors express the proper chemokines, T cells can get there. Chemokines, however, can be post-translationally modified by proteolytic cleavage, glycosylation, nitration, or deamination which results in dramatically altered activity (127). When CCL2 is nitrated, by reactive nitrogen species in the TME, for example, T cell infiltration into tumors is hindered and rather remain excluded from the tumor mass (155). In addition to the absence of chemoattractants, secretion of chemokines that serve as chemorepellants may protect tumor nests from T cell infiltration. In a mouse model of PDAC, fibroblast activating protein-expressing CAFs produce CXCL12 that coats tumor cells and prevents $CXCR4^+$ $CD8^+$ T cells from infiltrating tumor nests and controlling the tumor (156). Administration of AMD3100 (CXCR4 inhibitor) increased T cell infiltration into tumor nests, and synergized with anti-PD-L1 therapy to reduce tumor growth (156). Thus, competing chemokine gradients, initiated and maintained by multiple cell types within the tumor microenvironment, determine lymphocyte positioning and subsequent function.

Immunological Deserts

Finally, immunological deserts are defined as those tumor microenvironments completely lacking T cell infiltrates within

tumor nests and in adjacent stroma. Low somatic mutational burden and tumor immunogenicity is likely a significant driver of failed T cell responses in these tumors. However, even in the presence of immunogenic epitopes lymphatic transport and poor DC migration may limit anti-tumor T cell priming in lymphoid organs and thus prevent systemic T cell expansion. In fact, tumors induced or implanted in mice lacking dermal lymphatic vessels fail to activate and accumulate anti-tumor T cell responses (157, 158) and lymphatic vessel density correlates with T cell infiltration in colorectal cancer and melanoma patients (159, 160). Conversely, overexpression of lymphangiogenic growth factors enhances intratumoral inflammation and response to various immunotherapies (42, 161), indicating that lymphatic transport plays an important role in both adaptive immune priming and setting up an inflammatory tumor microenvironment. Thus, the non-hematopoietic stroma may dictate the systemic expansion of anti-tumor immunity and thereby restrict the pool of T cells available for tumor recruitment. Still, downstream of T cell priming, analysis of T cells in synchronous metastases reveals heterogeneous distribution of the existing systemic repertoire (162) indicating additional mechanisms of control. Furthermore, even in the absence of *de novo*, tumor-specific T cell priming, recruitment of pre-existing memory populations should lead to intratumoral accumulation of T cells. Thus, additional factors must limit extravasation and tumor residence of bulk T cell populations. Tissue-specific vascular heterogeneity or dysfunction (stromal and intratumoral) may limit T cell infiltration in a lesion-specific manner and thus contribute to immunological deserts in some and not all metastatic lesions.

FUTURE DIRECTIONS

Non-hematopoietic cells provide context to *in situ* peripheral tissue immune responses and thus may be critical local signals that determine the switch between protective immunity and immune suppression. Dynamic imaging in animal models continues to reveal the spatiotemporal control tissues exert over infiltrating T cell responses in naïve and inflamed tissues (55). Extension of mechanisms elucidated during normal tissue responses to tumors will provide critical insight into the heterogeneity of T cell recruitment and retention in synchronous metastases in patients (162); may provide novel strategies to improve the efficacy of CAR T cell therapy in solid tumors (163); and provide insight into the multiple immune barriers across solid tumor types. Importantly, careful analysis of tissue-specific differences in immune infiltrate (164, 165), if coupled with tissue-specific vasculature and matrix components through multiplexed imaging technologies, may reveal important environmental context to inform dynamics of intratumoral inflammation and thus response to therapy. Similarly, single cell sequencing, while a powerful tool for extracting novel transcriptional states in tumor, hematopoietic, and stromal cells (143, 166, 167) loses structural information that informs interpretation and thus should be coupled with validation and further discovery in matching tissue sections. The added context may not only improve the prognostic value of extracted biomarker signatures, but will also generate hypotheses for rigorous mechanistic testing in experimental models leading to new strategies for immune modulation and tumor control. Importantly, non-hematopoietic stromal interactions provide inherently local mechanisms of immune control that if targeted, may serve to unleash effector T cell responses and thus revive tumor control.

AUTHOR CONTRIBUTIONS

RSL and AWL researched the literature, created content, and wrote the manuscript.

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MINI REVIEW
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Defining Memory CD8 T Cell

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CD8 T cells comprising the memory pool display considerable heterogeneity, with individual cells differing in phenotype and function. This review will focus on our current understanding of heterogeneity within the antigen-specific memory CD8 T cell compartment and classifications of memory CD8 T cell subsets with defined and discrete functionalities. Recent data suggest that phenotype and/or function of numerically stable circulatory memory CD8 T cells are defined by the age of memory CD8 T cell (or time after initial antigen-encounter). In addition, history of antigen stimulations has a profound effect on memory CD8 T cell populations, suggesting that repeated infections (or vaccination) have the capacity to further shape the memory CD8 T cell pool. Finally, genetic background of hosts and history of exposure to diverse microorganisms likely contribute to the observed heterogeneity in the memory CD8 T cell compartment. Extending our tool box and exploring alternative mouse models (i.e., "dirty" and/or outbred mice) to encompass and better model diversity observed in humans will remain an important goal for the near future that will likely shed new light into the mechanisms that govern biology of memory CD8 T cells.

Keywords: CD8 T cell, memory, subsets, heterogeneity, protection, outbred mice, age of memory, history of Ag enounters

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INTRODUCTION

At the most basic level, a memory CD8 T cell can be defined as a CD8 T cell that has responded to cognate antigen (Ag) and persists long-term. Such a simple definition does little to account for immune-mediated protection, however, and hosts possessing memory CD8 T cells are often better protected against solid tumors and infection with intracellular bacteria, viruses, and protozoan parasites than their naïve counterparts (1–8). To encompass protective capabilities, our definition would need to expand to include quantitative and qualitative aspects of CD8 T cell memory and how these differ from naïve and effector CD8 T cells. Compared to naïve cells of the same antigen-specificity, memory CD8 T cells persist in greater numbers (9); can populate peripheral organs (10); are poised to immediately proliferate, execute cytotoxic functions, and secrete effector cytokines upon Ag re-encounter (11–16); and exist in different metabolic, transcriptional, and epigenetic states (17–20). Despite some similarities between effector and memory CD8 T cells at the molecular, epigenetic, metabolic, and functional levels (17–23), memory cells persist long-term while effector cells undergo robust contraction (18, 24), and unlike effector cells they are capable of vigorous proliferation following Ag re-encounter (25).

Expanding our definition to account for functional differences between naïve, effector, and memory cells helps to clarify why immune hosts are better protected than naïve hosts, but it does not begin to explain why some memory CD8 T cell responses are more protective than others. While a correlation between the numbers of memory CD8 T cells and the level of protection is

firmly established (26, 27), quality (or functional ability) of memory CD8 T cells also determines the degree of memory CD8 T cell-mediated protection. Characteristics of memory CD8 T cell responses best-suited to provide protection against infection vary depending upon the nature of the pathogen, and over the past 20 years it has become clear that the memory CD8 T cell pool consists of a heterogeneous population of cells that differ in phenotype, function, and protective capacity (28–34). A complete definition of CD8 T cell memory, then, should account for this diversity, and immunologists have categorized cells of distinct functional abilities into subsets to better understand memory CD8 T cell heterogeneity. Heterogeneity within memory CD8 T cell subsets uncovered by recent subsetting strategies discussed in this review also highlights the limitations of ascribing discrete functions to memory CD8 T cells expressing one or two phenotypic markers. However, despite these limitations, current subset classifications do provide valuable predictive information on the likelihood that cells of a given phenotype will be able to perform a defined function in response to a particular pathogen.

CD8 T cells of memory phenotype and function can arise in response to self-peptide and/or in a lymphopenic environment in response to cytokines that trigger homeostatic proliferation ("virtual" and "innate" memory) (35, 36). However, this review will focus solely on memory CD8 T cells generated in response to infection. Specifically, we will discuss (1) current subset classifications of memory CD8 T cells, (2) how subset composition is shaped following time after infection and upon additional Ag encounters, (3) how memory CD8 T cell subsets in humans compare to those in mice, and (4) how mouse studies that better model human biology inform our knowledge of memory CD8 T cell biology.

MEMORY CD8 T CELL SUBSETS

Effector (Tem) and Central (Tcm) Memory

Although the number of memory CD8 T cell subsets has expanded (**Table 1**), the first characterization of heterogeneity within a memory CD8 T cell pool of undefined origin in humans described two subsets—$CD62L^{lo}/CCR7^{lo}$ effector memory (Tem) and $CD62L^{hi}/CCR7^{hi}$ central memory (Tcm) cells (37). Expression of CCR7 and CD62L on Tcm cells facilitates homing to secondary lymphoid organs, while Tem cells are more cytolytic and express integrins and chemokine receptors necessary for localization to inflamed tissues (37). This description led to the paradigm that the memory CD8 T cell population consists of specialized cells that uniquely participate in the immune response to confer host protection. Mechanistic studies in mice showed that Tem cells were more prevalent in tissues, while Tcm cells were more prevalent in lymph nodes and better equipped to persist following infection and to produce IL-2 and proliferate in response to Ag (30). Transcription factors promote the development and function of Tem and Tcm cells, and T-bet, Blimp1, ID2, and STAT4 expression is associated with Tem cells, while Eomes, TCF1, BCL-6, ID3, and STAT3 expression is associated with Tcm cells (38–42, 45–49). Tcm cells provide enhanced protection against chronic infection with LCMV-clone 13 (30), while Tem cells provide superior protection against infection with vaccinia virus, and in some instances *Listeria monocytogenes* (31, 63). These studies led to the hypothesis that Tcm cells are specialized to handle systemic infections due to their centralized location within secondary lymphoid organs and superior proliferative abilities, and that Tem are specialized to handle infections arising within peripheral organs due to their cytotoxicity and ability to localize to tissues.

With identification of memory subsets came questions of when CD8 T cells of discrete function form during a response and how effector cells survive to populate the heterogeneous memory CD8 T cell pool. Interleukin 7 is required for the survival of naïve cells and promotes the survival of memory CD8 T cells (64), and initial reports suggested that the expression of CD127, the alpha chain of the IL-7 receptor, could be used to identify memory precursor effector cells (MPECs) that display increased ability to form long-lived memory cells and short-lived effector cells (SLECs) that are poor at giving rise to long-lived memory cells (65). Additional studies suggested that expression of costimulatory molecule CD27, could identify effector cells that were more likely to survive contraction (66). Later, expression of KLRG1 in addition to CD127 was used to identify SLECs ($CD127^{-}/KLRG1^{+}$) and MPECs ($CD127^{+}/KLRG1^{-}$) (38). However, despite promoting survival of effector CD8 T cells to memory, CD127 expression and IL-7 signaling are not sufficient to drive formation of memory CD8 T cells, as forced expression of CD127 expression did not rescue survival of $KLRG1^{hi}$ cells into memory (67). In addition, priming of naïve CD8 T cells in low inflammatory environment (ex. peptide-DC immunization) will generate CD127 expressing effector CD8 T cells prone to vigorous contraction (25, 68). Of note, displaying the expression pattern of markers used to identify SLECs ($CD127^{-}/KLRG1^{+}$) does not absolutely preclude long-term memory formation, as a small percentage of $CD127^{-}/KLRG1^{+}$ cells can be found months after infection (69, 70). Thus, the expression pattern of CD127 and CD27 on effector CD8 T cells mark cells with differential probability to survive contraction, but also highlights the notion that those markers cannot be used with certainty to predict effector cells that will become long-lived CD8 T cell memory.

Tissue Resident Memory (Trm)

Tissue surveillance was a function first ascribed to circulating Tem cells (71). However, elegant parabiosis experiments have made it clear that some cells within tissues are not circulating, but are permanent residents (50, 51, 72). Efforts to identify tissue resident memory T cells (Trm) have shown that, unlike circulating cells, Trm cells are not labeled by intravenous injection of antibodies (73), with the noted exception of liver Trm cells, which are exposed to the circulation (74). In addition to tissue residence, Trm cells often are identified based on expression of integrins CD103 and CD49a, which aid in tissue entry (52, 53), and CD69, which promotes tissue retention (54). However, expression of these proteins can vary depending on tissue of residence. Trm cells are also described as expressing CXCR3 and lacking expression of KLRG1, CCR7, and CD62L, and having intermediate or low expression of Cx3Cr1 (33, 43, 52).

TABLE 1 | Memory CD8 T cell subsets.

Subset	Phenotype	Function	Location/Trafficking	Transcription factors	References
Tem	CCR7^{lo}/CD62L^{lo} Cx3Cr1^{hi}/CD27^{lo} CD127^{hi} CD27^{-}/CD45RA^{-} (humans)	++ Cytotoxicity +- Proliferation	Circulation	Tbet^{hi} Blimp1^{hi}/ID2^{hi}/STAT4^{hi}	(30, 37–44)
Tcm	CCR7^{hi}/CD62L^{hi} Cx3Cr1^{lo}/CD27^{hi} CD127^{hi} CD27^{+}/CD45RA^{-} (humans)	+− Cytotoxicity ++ Proliferation	Circulation Lymph nodes	Tbet^{lo} Eomes^{hi}/TCF1^{hi}/Bcl6^{hi}/ STAT3^{hi}/ID3^{hi}	(30, 37–39, 42–49)
Temra (humans)	CCR7^{-}/CD27^{-}/CD45RA^{+} CD127^{lo}	++ Cytotoxicity +− Proliferation	Circulation		(44)
Trm	CD69^{hi}/CD103^{hi}/CD49a^{hi} (depending on tissue) CXCR3^{hi}/KLRG1^{lo}/CCR7^{lo}/ CD62L^{lo}, CD127^{hi} Cx3Cr1$^{lo/int}$	Sensing and alarm + proliferation	Tissue resident	KLF2$^{-/lo}$/Eomes$^{-/lo}$ Tbet^{lo}/TCF1^{lo} Hobit^{hi}/Blimp1^{hi}	(33, 43, 50–60)
Tpm	CCR7$^{+/-}$/CD62L$^{+/-}$/CD127^{hi} Cx3Cr1^{int}/CD27^{hi}	+ Cytotoxicity + Proliferation	Circulation Tissue trafficking Lymph nodes	Tbet$^{+/-}$	(43, 61, 62)
Others	CD27^{lo}/CD43^{lo} KLRG1^{hi}, CD127^{lo}	++ Cytotoxicity +− Proliferation	Tissue trafficking	Tbet^{hi}/Eomes^{lo}	(32, 62)

However, it was recently reported that cells that previously expressed KLRG1 can form Trm cells, and such ex-KLRG1 cells may delineate heterogeneity within the Trm population, as they express higher levels of granzymeB than Trm cells that never expressed KLRG1 (75). Responsiveness to TGF-β in most cases is necessary for Trm development (55, 76), and expression of transcription factors play an important role in promoting TGF-β responsiveness and retention of Trm cells within tissues. Transcriptionally, Trm cells are noted for reduced expression of KLF2 and Eomes (55, 56), low expression of T-bet and TCF1 (55, 57), and elevated expression of Hobit and Blimp1 (57). Trm-mediated protection in peripheral tissues is primarily mediated through sensing and alarm functions. This requires Ag recognition and IFN-γ production by Trm cells, results in global modification of gene expression within inflamed tissues and increased expression of chemokine ligands, and promotes recruitment and effector functions of cells of the innate and adaptive immune system (58, 77–79). Trm cells provide protection against diverse microorganisms in an array of tissues including the lungs (33), salivary glands (80, 81), female reproductive tract (58, 78), skin (28), and liver (74). Because of this, attempts to generate Trm cells with site-directed vaccinations are being pursued.

Tcm, Tem, and Peripheral Memory (Tpm) Subsets Based Upon Cx3Cr1 Expression

Recently, characterization of Tem and Tcm subsets was further refined, and an additional memory subset was described following the identification of Cx3Cr1^{int} peripheral memory (Tpm) T cells (43). Staining for CD27 or CXCR3 and Cx3Cr1 (fractalkine receptor) permits identification of Cx3Cr1^{-}, Cx3Cr1^{int}, and Cx3Cr1^{hi} populations at a memory time point. Cx3Cr1^{hi} cells do not migrate toward CCR7 ligand CCL19, do not re-express CD62L, are absent in lymph nodes but abundant in the circulation and tissues, proliferate and produce IL-2 poorly in response to Ag, and are efficient killers of target cells. These characteristics overlap with Tem cells and imply that expression of Cx3Cr1 may identify a homogeneous Tem population. Conversely, Cx3Cr1^{-} and Cx3Cr1^{int} populations are found in the lymph nodes and migrate in response to CCL19, suggesting that expression of Cx3Cr1 can be used to distinguish two populations among cells that would be defined as Tcm cells. Cx3Cr1^{-} cells display characteristics of classically defined Tcm cells in that they are more prevalent in lymph nodes, re-acquire CD62L faster and to a greater extent, and are better producers of IL-2 but less cytotoxic than Cx3Cr1^{int} cells. Therefore, Cx3Cr1 may allow identification of a more homogeneous population of Tcm cells. While the majority of Cx3Cr1^{-} cells express CD62L 1 year after infection, approximately half of Cx3Cr1^{int} cells express CD62L, and formation of Cx3Cr1^{int} cells is reduced but not eliminated in T-bet deficient mice, suggesting further heterogeneity within the Tpm population. This distinction may be important, as a large percentage of inflationary memory CD8 T cells in mice and humans generated in response to adenovirus-vectored vaccines or natural cytomegalovirus (CMV) infection are Cx3Cr1^{int} (82), and it was suggested that a CD62L^{hi}/Cx3Cr1^{+} population within the lymph nodes is important in providing protection against chronic infection (61). Importantly, Tem, Tcm, and Tpm populations identified based on Cx3Cr1 expression display different migratory patterns (43). Contrary to previous descriptions as tissue surveyors, Tem cells were excluded from tissues, but were highly represented in the circulation. Instead, the tissue surveyor role was ascribed to Tpm cells, which could traffic to the tissues and return to lymph nodes via afferent lymphatics. These data call for a refinement to the hypothesis of the role of memory CD8 T cell subsets in providing host protection and suggest that immuno-surveillance

is mediated by discrete actions of Tem cells, which are cytotoxic and present in the circulation and can be easily recruited to sites of inflammation; Tcm cells, which are centrally localized within lymph nodes and are highly proliferative following Ag re-encounter; Trm cells, which respond to infections arising in peripheral tissues and proliferate and recruit other immune cells following infection; and Tpm cells, which survey peripheral tissues and may be important for mediating protection against chronic infections.

Additional Memory Cell Subset Classifications

Classifications of memory CD8 T cells into, Tem, Tcm, Trm, and Tpm subsets informs our understanding of immuno-surveillance provided by CD8 T cells of discrete functionality, but it does not capture the complete diversity within the memory CD8 T cell pool. Additional subsets have been described based upon expression of CD27 and CD43, a glycosylated form of sialic acid (32, 62). $CD27^{lo}/CD43^{lo}$ memory cells are $KLRG1^{hi}$, $CD127^{lo}$, $T\text{-}bet^{hi}$, and $Eomes^{lo}$ (32), an expression pattern that overlaps with, but is not identical to either Tem or Trm cells. Importantly, $CD27^{lo}/CD43^{lo}$ memory cells provide superior protection against Sendai virus and *Listeria monocytogenes* infection, perhaps due to an ability to localize to tissues. Thus, Tem, Tcm, Trm, and Tpm classification does not completely capture memory CD8 T cell diversity. Examination of additional markers may improve resolution of existing subsets and expand the number of identifiable subsets in the future, and lead to an improved understanding of memory CD8 T cell-mediated immuno-surveillance.

EFFECTS OF TIME AND AG-ENCOUNTERS ON MEMORY CD8 T CELL POOL COMPOSITION

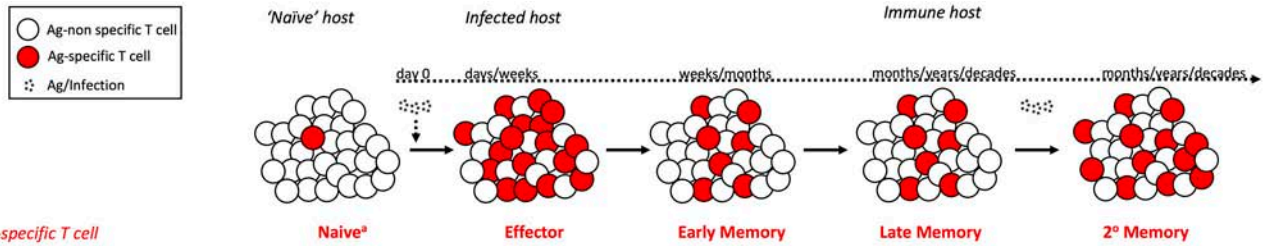
Time

Long-lived hosts can re-encounter pathogens at any time, and studies have indicated that the phenotype, function, and protective abilities of Ag-specific memory CD8 T cells change with time following infection. The percentage of circulating pathogen-specific memory CD8 T cells expressing CD27 and CD62L increases with time after infection, (30, 83–85), and the percentage expressing Cx3Cr1 decreases (43, 75), indicating that representation of Tcm cells among pathogen-specific memory CD8 T cells increases with time after infection. As would be expected of Tcm cells, aged or late memory cells proliferate and produce IL-2 to a greater extent than early memory cells in response to Ag (69, 70, 86, 87), and provide enhanced protection against chronic viral infection (69, 70). Changes observed in late memory cells extended beyond phenotype and functions normally attributed to Tcm cells, including increased ability to up-regulate expression of FasL and CD40L and to produce XCL1; decreased expression of many cytokine and chemokine receptors including IL-10R, components of IL-12R and IL-18R, CCR2, and CCR5; and decreased ability to produce IFN-g in response to inflammatory cues in the absence of cognate antigen recognition (bystander activation) (70, 88). Strikingly, phenotypic heterogeneity of Tcm cells was diminished with time after infection, and progressive changes in transcriptomic, phenotypic, and metabolic profiles of Tcm cells indicated an improved proliferative capacity of Tcm cells with time after infection, leading to an increased ability to provide protection against LCMV-clone 13 infection (69). In contrast, the percentage of $CD62L^{lo}$ cells decreases with time after infection (69, 70, 83, 84), indicating decreased representation of Tem cells. Of note, the CD62Llo subset is comprised of not only functional, IFN-g producing Tem but also of recently identified T death intermediate memory (Tdim) cells (89). Tdim arise from the process of memory CD8 T cell homeostatic proliferation, are non-functional, and are destined to die, (89) and their representation increases among CD62Llo Tem subset with time after infection (69).

Like Tem cells, numbers of Tpm cells decrease initially after infection, but following an initial period of decline, they are maintained at stable numbers (43). However, the percentage of $CD62L^{hi}$ Tpm cells increases with time after infection. Few studies have examined the properties of long-term Trm cells, and it is unclear how the functions of Trm cells are affected by time. Trm cells in the skin persist for >300 days after infection and are long-lived (28). However, influenza-specific Trm cells in the lungs are shorter-lived (90) and require replenishment by circulating CD62Llo memory cells (91). Together, these studies indicate that with time after infection, the circulating Ag-specific memory CD8 T cell population is comprised of a more homogeneous population of Tcm cells with enhanced proliferative capacity, which impacts host CD8 T cell-mediated protection against infection (**Figure 1**).

Ag-encounters

Hosts are often exposed to the same pathogens throughout life, and prime-boost vaccination protocols intended to increase memory cell numbers result in memory CD8 T cells that have encountered Ag multiple times. Initial experiments utilizing adoptive transfer of purified TCR transgenic or tetramer positive memory cells of known Ag-encounter history showed that additional Ag-encounters result in decreased representation of circulating memory CD8 T cells expressing CD62L, CD27, and CD127, and increased representation of cells expressing KLRG1 and GranzymeB (83, 84) (**Figure 1**). Phenotype of secondary memory CD8 T cells is also greatly impacted by systemic inflammation elicited during the infection/vaccination (92). Successive Ag-encounters also result in stepwise changes in transcriptomic signature of memory CD8 T cells, but not in progressive enrichment in Tem associated genes (93), suggesting that additional Ag-encounters result in generation of a more transcriptionally diverse Tem population. Differences in memory CD8 T cell composition and function with additional Ag-encounters translate to differential ability to provide protection against re-infection, with memory CD8 T cells that have encountered Ag multiple times being more protective against re-infection with *Listeria monocytogenes*, LCMV-Armstrong, and Vaccinia Virus, and less protective against re-infection with MHV and LCMV-clone 13 (29). However, recurrent homologous



Ag-specific T cell	Naive[a]	Effector	Early Memory	Late Memory	2° Memory
Numbers	+	++++	++	++	+++
Subset representation	-	SLEC>MPEC	Tem>Tpm>Tcm	Tcm>Tpm>Tem	Tem>Tpm>Tcm
Phenotypic and/or transcriptomic diversity	+/-	++	+++	++	++++
Persistence/survival	+++	+	+++	+++	++
Proliferation capacity	++++	+/-	++	+++	+
Lymph node homing	++++	+/-	++	+++	+
Ag-driven IFN-γ/TNF-α production	+/-	++++	++++	++++	++++
Ag-driven IL-2 and/or XCL1 production	+/-	+/-	++	+++	+
Cytotoxicity	-	++++	++	+	+++
IL-12 and IL-18 cytokine receptor expression	-	++++	++	+	+++
Bystander IFN-γ production	-	++++	++	+	+++
Ability to repopulate lung Trm	-	++++	++	+	+++
Protection against acute, systemic infections	+/-	+++	+	+	++
Protection against chronic infections	+/-	+/-	++	+++	+

FIGURE 1 | Phenotypic and functional changes within the circulating Ag-specific CD8 T cell pool with time after infection and with additional Ag encounters. Following infection or vaccination, rare naïve CD8 T cells that recognize their cognate Ag robustly proliferate and give rise to an effector CD8 T cell population. Following contraction of the effector pool, memory CD8 T cells are stably maintained at numbers greater than the naïve pool. Upon re-infection or booster vaccination, primary memory CD8 T cells proliferate and generate a secondary effector and memory CD8 T cell pool that is larger in size than the primary memory pool. Properties of cells comprising the Ag-specific CD8 T cell pool, including expression of phenotypic markers and subset representation, ability to traffic to and localize within tissues, ability to execute effector functions, and ability to provide protection against infection with diverse pathogens differ between naïve, effector, and memory CD8 T cells, and among memory CD8 T cells of different age relative to initial Ag-encounter and of different number of Ag-encounters. – symbols indicate reduced quantity or ability, while + symbols indicate increased quantity or ability. [a]Virtual and/or innate memory cells within the naïve CD8 T cell pool are not considered here.

boosting preserves numerical stability and increases phenotypic and functional complexity of the memory CD8 T cell pool (94), and sequential heterologous infection results in a pool of Ag-specific memory CD8 T cells with a phenotypic profile reflective of Tcm cells that are metabolically fit, proliferate robustly following re-infection, and provide protection against LCMV-clone 13 (34). Although homologous and heterologous infection strategies likely result in mixed memory populations with cells that have encountered Ag a different number of times, and thus are not reflective of pure memory populations of known number of Ag-encounters, they may more accurately reflect sequential infections in humans.

Recent examinations of Trm cells that re-encounter Ag have shown that Trm cells proliferate within tissues and contribute to formation of secondary Trm cells (95, 96), and can migrate to, and form Trm populations within tissue-draining lymph nodes (97). Importantly, although Ag-exposure history defines CD8 T cell dynamics and protection during localized pulmonary infections (98) lung Trm derived from repeatedly stimulated influenza-specific circulatory memory CD8 T cells exhibit extended durability and protective heterosubtypic immunity relative to primary lung Trm (99). Parabiosis studies reveal that repeated antigen encounters resulted in generation of long-lasting circulating effector memory (Tem) cells that maintained their ability to be recruited to the lung parenchyma and converted to Trm (99). Thus, successive Ag-encounters also results in diversification of the Trm subset, which impacts their ability to provide protection against subsequent infections arising at peripheral locations.

MEMORY CD8 T CELL HETEROGENEITY AND SUBSETS IN HUMANS

Humans are exposed to an array of infections throughout life and often re-encounter the same infection. Additionally, it is often difficult to determine precisely when infection was encountered, and due to obvious difficulties in acquiring human tissue samples, the majority of human studies rely on analysis of CD8 T cells in peripheral blood. These considerations have presented difficulties for examining memory CD8 T cells of known age relative to Ag-encounter and of known number of Ag-encounters in humans, but recent studies have provided insight into subset composition and heterogeneity present within the memory CD8 T cell population of humans. Most human studies rely on analysis of bulk CD8 T cell populations, and similar to mice, Tcm and Tem subsets can be identified in humans, along with a terminally differentiated subset that expresses CD45RA (Temra). Initial characterization of these subsets was based on expression of CD45RA and CD27 (44), while later studies distinguished Tem and Tcm subsets based upon expression of CCR7 (37), and staining for CD45RA and either CD27 or CCR7 identifies naïve ($CD45RA^+/CD27^+/CCR7^+$), Tem ($CD45RA^-/CD27^-/CCR7^-$), Tcm ($CD45RA^-/CD27^+/CCR7^+$), and Temra ($CD45RA^+/CD27^-/CCR7^-$) CD8 T cells. Memory CD8 T cells of distinct phenotypes accumulate with age, and accumulation of Temra cells in humans is influenced by chronic infections, such as CMV (100, 101).

Recent studies with organ donors of diverse ages have provided some clarity on the compartmentalization of human

memory subsets, describing large populations of Trm cells, and regional surveillance by Tem, Tcm, and Temra cells that varied depending on the tissue and were not reflective of subset representation within the blood (59, 60). As in mice, protection against infection is likely mediated by cells of discrete phenotype and function that cannot be fully described based upon classification of Tem, Tcm, Temra, and Trm subsets. Recently, human memory subsets were described based on Cx3Cr1 expression, and a highly cytotoxic Cx3Cr1$^+$/CD62L$^+$ subset that resides within the lymph node was suggested to be important for mediating protection against chronic infections including CMV (61).

Due to the endemic nature of most pathogens that humans are vaccinated against, it is difficult to examine Ag-specific memory CD8 cells of known age relative to initial activation and number of Ag-encounters in humans. However, experiments with vaccines for small pox and yellow fever virus (YFV), which are not endemic within the United States, have allowed for examination of primary memory CD8 T cells of known age relative to initial Ag-encounter. Expression of CD45RA, CD127, and CCR7 on Ag-specific memory CD8 T cells increased, while expression of perforin and granzymeB decreased with time after infection, suggesting that similar to mice, representation of Tcm cells within the Ag-specific human memory CD8 T cell population increases with time after infection (22, 102). However, while cytotoxic functions of memory CD8 T cells appeared to decrease with time after infection based upon expression of perforin and granzymeB at steady state, memory cells retained open chromatin configurations at locations relevant for cytotoxicity and cytokine production, suggesting that genes encoding for effector functions are readily open for transcription following Ag re-encounter (19, 22). Recent reports in mice have also shown dynamic epigenetic regulation of genes driving CD8 T cell localization and function during differing differentiation states (23). DNA methylation patterns of *Sell* (the gene encoding CD62L) were restrictive in effector cells, but demethylated in naïve and memory cells. Conversely, *GzmB* (the gene encoding granzymeB) displayed restrictive methylation patterns in naïve cells, but were demethylated in effector cells and memory precursor cells (23). These recent studies have indicated that, as in mice, the memory CD8 T cell pool in humans is composed of subsets with discrete functionalities, and subset composition likely impacts host immuno-surveillance in response to diverse pathogens.

CD8 T CELL RESPONSES IN ALTERNATIVE MOUSE MODELS

Human studies have pointed to many similarities between CD8 T cell responses in mice and humans. However, differences that exist between mice and humans may limit translational value of mouse research. Recent efforts to extend mouse models outside of traditional inbred mice housed in specific pathogen free (SPF) facilities have provided valuable insight into CD8 T cell biology. In contrast to the CD8 T cell compartment of SPF laboratory mice, which consists primarily of naïve T cells and is similar to that of a neonatal human, sequential infections with common pathogens or co-housing laboratory mice with wild/pet store ("dirty") mice generates a CD8 T cell compartment that is similar to adult humans and is comprised of a large number of Ag-experienced CD8 T cells with increased representation of cells in peripheral tissues (103). Additionally, a greater percentage of memory phenotype CD8 T cells of "dirty mice" displayed phenotypic markers expressed by Tem cells and were more cytolytic than memory phenotype cells of SPF laboratory mice (103). *De novo* immune responses in "dirty mice" resulted in reduced Ab production compared to SPF mice, and displayed transcriptional similarities to adult human blood in contrast to SPF mice, which displayed transcriptional similarities to neonatal humans (103, 104). Furthermore, memory CD8 T cells of "dirty mice" that developed following infection with *Listeria monocytogenes* were more skewed toward a SLEC phenotype compared to SPF mice, and "dirty mice" were better protected against infection with *Listeria* and *Plasmodium berghei* (103). These studies suggest that "dirty mice" may more closely model the immune system of adult humans, and that history of pathogen exposure shapes the immune system and impacts phenotype of memory CD8 T cells generated and protection provided following *de novo* infection. Future studies should more closely examine the innate and adaptive immune factors that are shaped following sequential infection with unrelated pathogens, and how these interact to generate a qualitatively different CD8 T cell response following *de novo* infection.

Additional insight has been gained from studies utilizing outbred mice to model genetic diversity present in the human population. Ag-driven changes in expression of CD8α and CD11a have been used as "surrogate activation markers" approach to track pathogen-specific CD8 T cell responses to infection in outbred mice without a priori knowledge of MHC class I restriction and/or specific epitopes (105). Data revealed that compared to uniformity in size of the effector and memory responses generated in inbred mice, magnitude of effector and memory CD8 T cell responses are highly variable in individual outbred mice (105, 106). Furthermore, while memory CD8 T cells in inbred mice progressed linearly from a Tem to Tcm phenotype with time, percentages of memory cells expressing Tcm markers (CD62L^{hi}, CD27^{hi}, CD127^{hi}, KLRG-1^{lo}) did not increase or increased very slowly with time after infection in some outbred mice (106). Importantly, differences in CD8 T cell responses generated to a primary infection in outbred mice led to differences in CD8 T cell-mediated protection provided against a secondary infection, and degree of protection did not always correlate with size of the memory CD8 T cell pool prior to secondary infection (105, 106). These studies suggest that vaccine strategies that generate a memory CD8 T cell pool of sufficient size and quality to provide protection against re-infection in inbred mice may not generate a protective memory CD8 T cell response in all outbred mice, a finding that has direct relevance to the outbred human population.

Differences in memory CD8 T cell response size and phenotype following infection in individual outbred mice could have been caused by a number of immunologic factors including differences in cells of the innate compartment or differences in

Th bias of the CD4 T cell compartment. However, underlying causes for divergent CD8 T cell-mediated immune outcomes were unable to be fully explored in the studies discussed due to a lack of tools available for study in outbred mice. Collaborative cross mice, a recombinant inbred panel of mice that displays vast genetic diversity due to unique inheritance from eight founder strains (107, 108), and diversity outbred mice, which are generated by outcrossing collaborative cross strains at various stages of the inbreeding process (109), may prove to be useful models for deciphering the answers to this question. Studies with collaborative cross mice have revealed a range of immune cell composition, phenotype, and function among strains prior to infection that is more representative of the human population (110), and post-infection outcomes relevant to the human population that are not observed in traditional inbred mice (111). Genetic tools uniquely suited for collaborative cross mice, including quantitative trait locus mapping (QTL) (112), may provide additional insight into factors underlying divergent memory CD8 T cell outcomes in genetically diverse organisms, and how memory CD8 T cells of diverse phenotype and function arise and participate in immune-mediated protection against re-infection.

CONCLUSION

Protection against diverse pathogens that have evolved for unique interactions with hosts, different points of host entry, and colonization and replication within particular host cells requires a diverse and adaptable immune system. Heterogeneous memory CD8 T cells that can persist in and localize to different areas within the host, and that are functionally adapted to respond in discrete ways within their host niche, contribute to the diversity and adaptability needed to efficiently provide host immuno-surveillance. The effects of time following infection and additional Ag encounters further shape diversity of the memory CD8 T cell pool, which impacts efficacy of CD8 T cell-mediated protection against re-infection. Efforts to subset memory CD8 T cells have informed our knowledge of how CD8 T cells with discrete functionalities contribute to host immuno-surveillance against diverse microbial pathogens, and improved animal models that more accurately reflect the human immune system may improve our understanding of the origins and functions of memory CD8 T cells of diverse phenotype and improve translational value of current animal studies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Tissue Adaptations of Memory and Tissue-Resident Gamma Delta T Cells

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Epithelial and mucosal barriers are critical interfaces physically separating the body from the outside environment and are the tissues most exposed to microorganisms and potential inflammatory agents. The integrity of these tissues requires fine tuning of the local immune system to enable the efficient elimination of invasive pathogens while simultaneously preserving a beneficial relationship with commensal organisms and preventing autoimmunity. Although they only represent a small fraction of circulating and lymphoid T cells, γδ T cells form a substantial population at barrier sites and even outnumber conventional αβ T cells in some tissues. After their egress from the thymus, several γδ T cell subsets naturally establish residency in predetermined mucosal and epithelial locations, as exemplified by the restricted location of murine Vγ5$^+$ and Vγ3Vδ1$^+$ T cell subsets to the intestinal epithelium and epidermis, respectively. Because of their preferential location in barrier sites, γδ T cells are often directly or indirectly influenced by the microbiota or the pathogens that invade these sites. More recently, a growing body of studies have shown that γδ T cells form long-lived memory populations upon local inflammation or bacterial infection, some of which permanently populate the affected tissues after pathogen clearance or resolution of inflammation. Natural and induced resident γδ T cells have been implicated in many beneficial processes such as tissue homeostasis and pathogen control, but their presence may also exacerbate local inflammation under certain circumstances. Further understanding of the biology and role of these unconventional resident T cells in homeostasis and disease may shed light on potentially novel vaccines and therapies.

Keywords: memory γδ T cells, resident γδ T cells, innate γδ T cells, adaptive γδ T cells, barrier infections

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INTRODUCTION

Epithelial and mucosal tissues form physical barriers separating the body from the outside world. They are constantly exposed to a wide range of stressors such as infectious agents and their toxins capable of damaging barrier tissues. Barrier surface interactions with microorganisms extend far beyond encounters with pathogenic microbes; indeed, these tissues are typically mutualistic ecosystems that maintain beneficial relationships for resident commensal organisms while providing support to the tissue (1). Because of the complexity of these interfaces, the immune system is tightly regulated in order to eliminate invading pathogens while maintaining a robust commensal environment. It is now well established that the microbiota plays a significant role in

educating immune cells and promoting protective anti-infectious responses (2–4). However, the microbiota may also play an important role in aberrant inflammation (5, 6). In addition, pathogenic agents also leave their imprint on the immune system and generate long-lasting memory responses. Protective immunity has mainly been the purview of conventional effector memory (T_{EM}) or central memory (T_{CM}) T cells and B cells. More recently, the discovery of conventional resident memory T cells (T_{RM}) (7, 8), innate immune memory also known as trained immunity (9, 10), and other unconventional memory responses (11, 12) has focused attention on tissue-specific immunity at barrier locations.

γδ T cells are an unconventional T cell population that display immunologic features common to both the innate and adaptive immune systems (13). This dual nature of γδ T cell biology is typified by their non-MHC-restricted antigenic specificity while mounting rapid immune responses to a wide range of tissue stressors (14), generally referred to as "lymphoid-stress surveillance" (15). γδ T cells are the first T cells generated during embryonic development and quickly seed peripheral tissues where specialized subsets are maintained for life in residence. These unconventional T cells are only found at low frequencies in lymphoid tissues and the blood in adult humans and rodents; however, they are enriched in epithelial and mucosal tissues (16–19). Generally, distinct barrier tissues harbor mostly non-overlapping γδ T cell subsets with non-redundant functions (17). Some tissues contain unique and highly specialized γδ T cell subsets that are not found elsewhere in the body. For example, $V\gamma3V\delta1^+$ skin dendritic epidermal T cells (DETC) reside exclusively in the skin epidermis while $V\gamma5^+$ T cells reside exclusively in the intestinal epithelium [the Garman nomenclature (20) is used throughout this review for murine γδ T cells] (21). The development and selection processes that regulate the differentiation of these cells are unique and result in the generation of highly adapted cells that actively survey neighboring cells, sense and respond to stresses of various nature and participate in many tissue processes. Thus, these natural tissue-resident γδ T cells are programmed sentinels that are also shaped by and highly adapted to their tissue environment.

Because of their preferential location in barrier sites, γδ T cells are often directly or indirectly influenced by the microbiota or the pathogens that invade these sites. The steady-state microbiota may influence the generation, effector functions, or maintenance of γδ T cells (22–24). These commensal-induced γδ T cells adapt to their tissue of residence where they add another level of immune surveillance and may be mobilized in many pathological contexts including inflammation (25–27) and cancer (28, 29). These tissue-resident γδ T cells are also mobilized during infection to promote anti-pathogen immunity (30) and represent innate first responders during infection. Alternatively, pathogen-induced adaptive γδ T cells appear to follow a more conventional T cell maturation pathway, resulting in delayed activation and expansion while favoring the establishment of long-lasting memory and heightened protective potential upon pathogen re-exposure. Throughout this review, the term "adaptive" will be utilized to describe γδ T cells having features consistent with conventional αβ T cells. This review will focus on the tissue adaptation of tissue-resident natural γδ T cells and adaptive γδ T_{RM} cells in barrier tissues while highlighting their development, maintenance and role in health and disease.

γδ T CELLS OF HUMANS AND MICE

Murine γδ T cells are often segregated into different subsets based on their Vγ T cell receptor (TCR) chain, as it is generally associated with tissue tropism and a bias in effector function (31, 32) (**Table 1**). It is well established that γδ T cell ontogeny is temporally controlled and manifested by "waves" of development (76). The factors regulating γδ T cell development have been recently reviewed (77, 78). Most barrier tissue γδ T cells develop early during fetal development in the fetal/neonatal thymus with the first thymic wave of γδ T cells starting at embryonic day 13 and giving rise to DETC characterized by surface expression of an invariant Vγ3Vδ1 TCR (16). $V\gamma3V\delta1^+$ DETC migrate to the skin epidermis (18, 76, 79) and produce IFNγ (80) and other cytokines (81, 82), and growth factors (83, 84). From embryonic day 14 to the perinatal period, the fetal/neonatal thymus generates other innate-like [also called "natural" (85)] γδ T cells, including the IL-17A biased quasi-invariant $V\gamma4V\delta1^+$ T cells which preferentially migrate to the genital tract, the tongue and the lungs (16, 76, 86). Fetal-derived γδ T cells are typically considered innate-like due to their reduced TCR sensitivity (87) and rapid functional response to innate stimuli like cytokines and pathogen- or danger-associated molecular patterns (72, 88, 89). IL-17A-producing γδ T cells (referred to as γδ17 T cells in this review) are characterized by the expression of the transcription factor RORγt (90), chemokine receptor CCR6 (86, 90, 91), scavenger receptor SCART2 (92), CD25 (93), but lack CD27 (86, 90, 94). In contrast, IFNγ-producing γδ T cells express the transcription factor T-bet and surface receptors NK1.1 and CD27. Consistent with other IFNγ producing lymphocytes, they also express high levels of the IL-2/IL-15 receptor β chain CD122 (93, 95). It was initially thought that γδ17 T cells acquired their peripheral effector fate due to a lack of antigenic selection in the thymus; antigen-experienced cells were programmed to make IFNγ in the periphery while antigen-inexperienced cells were programmed to make IL-17A (80, 87, 95, 96). However, recent evidence suggests that signaling through the TCR is required for γδ17 T cells development and that the strength of the signal is the critical factor determining their functional lineage. A strong TCR signal promotes an IFNγ-dominant lineage whereas a weak TCR signal promotes an IL-17A-dominant lineage (97–99). An additional level of regulation comes from the thymic cytokine milieu: while signaling through IL-15Rα restrains γδ17 T cell development *in cis* (100), IL-7 promotes their expansion (101). An interesting feature of γδ17 T cells is their functional plasticity, which allows them to co-produce IL-17A and IFNγ under certain circumstances (61, 102). Although $CD27^-$ γδ T cells have a permissive chromatin state at the *Il17a* and *Ifng* loci, only a handful of situations have been associated with IL-17A and IFNγ co-production *in vivo*, including oral *Listeria monocytogenes* (*L. monocytogenes*) infection (61, 62) and peritoneal tumor (102). Post-transcriptional repression of IFNγ production has

TABLE 1 | Memory and tissue resident γδ T cells in infection and disease.

Tissue	Subset(s)	Role	Response	Cytokines	Other features	Context	References
Systemic	Vδ2$^-$	Protective	Memory?	IFNγ	Stress surveillance against CMV and cancer	CMV	(33–37)
	ND	Protective	Memory?	IFNγ	Antigen specific expansion	Vaccinia	(38)
	Vγ9Vδ2	Protective	Memory	IFNγ	Cross-reactive to HMBPP	Monkeypox	(39)
	Vγ9Vδ2, Vδ1	Protective	Memory?	IFNγ	Late expansion after initial exposure	*P. falciparum*	(40–43)
	Vγ1.1Vδ6.3	Protective	Memory?	M-CSF, CCL5, CCL3	Oligoclonal expansion	*P. chabaudi*	(44)
	Vγ9Vδ2	Protective	Memory	IFNγ	Cross-react with *M. tuberculosis*	BCG	(45, 46)
Lungs	Vγ9Vδ2	Protective	Memory	Granzyme B	Activated by HMBPP	*M. tuberculosis*	(45–47)
	Vγ1.1$^-$, Vγ2$^-$	Protective	Innate	IL-17A	High expression of IL-1R1, IL-18R, and IL-23R	*B. pertussis*	(48)
	Vγ2	Protective	RM	IL-17A	*B. pertussis*-specific	*B. pertussis*	(48)
Peritoneum	Vγ4	Protective	RM	IL-17A	CD27-CD44+ Effector memory phenotype	*S. aureus*	(49)
	Vγ1.1, Vγ2	Protective	Innate	ND	Polyclonal response	*S. aureus*	(49)
Skin	Vγ4Vδ1	Protective	RM	IFNγ, TNFα	TLR2/IL-1β dependent response	*S. aureus*	(50)
	Vγ2Vδ4	Pathogenic	RM	IL-17A/F	CCR2-dependent recruitment to tissue	Psoriasis	(25, 26)
	Vγ2	Pathogenic	RM	IL-17A	Constitutive expression of CCR6, RORγt, and IL-23R	Dermatitis	(51)
	Vγ9Vδ2, Vδ1	Variable	Memory	IL-17A, IFNγ, TNFα	Pathogenic IL-17A; Protective IFNγ	SCC/Melanoma	(52, 53)
	Vγ3Vδ1	Protective	Innate	IFNγ, KGF-1/2	Immotile; semi-activated	Wound, dermatitis, *S. aureus*, cancer	(54–58)
Intestine	Vγ9Vδ2	Protective	Memory	IL-17A, IFNγ, IL-4, TNFα	Multifunctional cytokine production	*L. monocytogenes*	(59, 60)
	Vγ4Vδ1	Protective	RM	IL-17A, IFNγ	Multifunctional cytokine production	*L. monocytogenes*	(61, 62)
	Vδ1	Pathogenic	Infiltrating	IFNγ	Interacts with colonic fibroblasts	IBD	(63, 64)
	Vγ9Vδ2, Vδ1	Pathogenic	RM	GM-CSF, IL-17A	Pathogenicity dependent on MDSC regulation	CRC	(65)
	Vγ5, others	Protective	Innate	IFNγ, Granzymes	Highly motile; semi-activated	*S. enterica, T. gondii*	(66–69)
Breast	Vγ2	Pathogenic	RM	G-CSF, IL-17A	Pathogenicity dependent on MDSC regulation	Breast Cancer	(70)
Brain	Vδ2, Vδ1	Protective	Memory	IFNγ, TNFα, Granzyme B	Found in the context of γδ expansion methodology	Neuroblastoma	(71)
	Vγ2	Pathogenic	Innate	IL-17 cytokines, IL-21	IL-23- and IL-1β-dependent activation	EAE/MS	(72)
	ND	Pathogenic	Innate	IL-17A	Part of a microbiota-gut-brain axis	Ischemic stoke	(27)
Joints	Vγ1.1, Vγ1.2	Pathogenic	Innate	IL-17A	IL-23- and IL-1β-dependent activation	CIA	(73)
	ND	Pathogenic	Innate	IL-17A	IL-23-dependent activation	Ankylosing spondylitis	(74)
Eyes	Vγ1.1, Vγ2	Pathogenic	Innate?	IL-17A	Enhanced uveitogenic αβ T cell development	Uveitis/EAU	(75)
	Vγ2	Protective	Resident	IL-17A	Induced by *C. mastidis* colonization	Ocular *P. aeruginosa*/	(24)
					CD1d- and IL-1β-dependent	*Candida albicans*	
Primate γδ T cells		Rodent γδ T cells					

ND, not determined; RM, resident memory; CMV, cytomegalovirus; BCG, M. bovis BCG strain; SCC, squamous cell carcinoma; IBD, inflammatory bowel disease; CRC, colorectal cancer; EAE/MS, experimental autoimmune encephalomyelitis/multiple sclerosis; CIA, collagen-induced arthritis; EAU, experimental autoimmune uveitis.

recently been reported in γδ17 T cells (61); however, whether co-production of IL-17A and IFNγ is regulated by derepression has not been evaluated.

Although most γδ17 T cells fall into the innate-like category, adaptive-like differentiation of naïve γδ T cell precursors into mature γδ17 T cells in peripheral lymphoid organs has also recently been reported in multiple models. After the identification of phycoerythrin (PE) as a γδTCR antigen, PE-specific γδ T cells were shown to transition from a naïve $CD44^{lo}$ $CD62L^{hi}$ to an activated $CD44^{hi}$ $CD62L^{lo}$ phenotype after immunization with PE (103). These γδ T cells expressed RORγt and inflammatory cytokine receptors IL-1R1 and IL-23R which drove production of IL-17A without extensive proliferation (103). Similarly, imiquimod (IMQ)-induced skin inflammation and MOG-induced experimental autoimmune encephalomyelitis (EAE) induced the *de novo* generation of γδ17 T cells in draining lymph nodes (104, 105). These unrelated models demonstrate that the differentiation of some γδ17 T cell subsets is optimal with a TCR signal and in the presence of IL-23, reminiscent of the multistep development of naïve $CD4^+$ T cells. In contrast to natural γδ17 T cells, these *de novo* generated cells are often referred to as inducible γδ17 T cells (14).

γδ T cell subsets in human and non-human primates are generally divided into two major populations based on the Vδ TCR chain: $Vδ2^+$ and $Vδ2^-$ γδ T cells. $Vδ2^+$ T cells appear to develop almost exclusively in the fetal liver and fetal thymus (106, 107) and form the predominant γδ T cell population in the peripheral blood of adult humans (108, 109). Most fetal, cord blood and adult $Vδ2^+$ T cells express the semi-invariant Vγ9Vδ2 TCR with a public germline encoded CDR3γ sequence and a more diverse CDR3δ sequence (110). Despite their preferential localization in the blood, $Vγ9Vδ2^+$ T cells can also be recruited to inflamed tissues where they can participate in pathogen clearance or promote inflammation (39, 45, 47) (**Table 1**). The TCR combination allows the majority of $Vγ9Vδ2^+$ T cells to recognize prenyl pyrophosphate metabolites (111), broadly referred to as phosphoantigens (PAgs), presented in the context of butyrophilin (BTN)3A1 and BTN3A2 (112–115). PAgs are metabolic intermediates produced by the eukaryotic mevalonate pathway and the microbial 2-C-methyl-D-erythriol 4-phosphate (MEP) pathway, which generates one of the most potent $Vγ9Vδ2^+$ T cell activator (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) (111). Fetal $Vγ9Vδ2^+$ T cells express genes found in adult cells and can expand and produce IFNγ in response to HMBPP stimulation (110). By 1 year of age, almost all $Vγ9Vδ2^+$ T cells have acquired a memory phenotype and can rapidly produce IFNγ and cytotoxic molecules (108, 116), similar to circulating adult cells (108, 116, 117). These data suggest that human $Vγ9Vδ2^+$ T cells are preprogrammed fetal-derived effectors with a restricted TCR specificity. Thus, $Vγ9Vδ2^+$ T cells seem to belong to the natural, innate-like population of lymphocytes.

In contrast to $Vγ9Vδ2^+$ T cells, the $Vδ2^-$ γδ T cell subset is heterogenous (106) and preferentially resides in epithelial tissues such as the skin (118) and intestines (119) and appears to form resident populations in the liver (120) (**Table 1**). $Vδ2^-$ γδ T cells mainly consist of $Vδ1^+$ T cells, with fewer $Vδ3^+$ and $Vδ5^+$ T cells. While most antigens recognized by $Vδ2^-$ γδ T cells remain unknown, the antigens identified to date suggest a broad reactivity to MHC-like molecules like endothelial protein C receptor (EPCR) (33) and CD1 molecules (33, 121, 122), stress-induced ligands (123) and algal phycoerythrin (103). $Vδ2^-$ γδ T cell TCR are highly diverse in cord blood but their TCR repertoire becomes more restricted into adulthood (124). Furthermore, they clonally expand in response to cytomegalovirus (CMV) infection and differentiate into $CD45RA^+$ effector memory T (T_{EMRA}) cells (34, 35, 125–127). Thus, the $Vδ2^-$ γδ T cell repertoire appears to be shaped by TCR-dependent selection events mediated by microbial encounters throughout life. As $Vδ2^-$ γδ T cells can recognize stress antigens, non-infectious events that trigger a response, such as cancer development, may also shape their repertoire (36, 128).

γδ T cells can provide different physiologic roles depending on the nature and context of the insult, the tissue involved and the γδ T cell populations mobilized. At steady state, γδ T cells are involved in many biological processes aiming at maintaining barrier integrity (e.g., by promoting epithelial cell survival and homeostasis) (82–84, 129) and regulating thermogenesis (130). Because of their rapid sensing of stress and recruitment to inflamed sites, γδ T cells are often involved in shaping early immunologic events. They can promote the activation, maturation, and recruitment of dendritic cells (DC), neutrophils, B cells, and conventional T cells [for a detailed review see (131)]. γδ T cells are also a direct and potent source of critical inflammatory cytokines like IFNγ, TNFα and IL-17A in many pathological contexts, including infection (59, 111, 132–134), autoimmune disease (25, 26, 72, 135) and cancer (29, 136–138). As such, they are also an integral part of the effector response. At later phases, γδ T cells can promote the resolution of the inflammation through the production of anti-inflammatory molecules like TGFβ (139, 140). Finally, they sustain tissue repair and remodeling after infection or injury (54, 83, 132, 141). Thus, γδ T cells are critically involved in regulating health during homeostasis and disease.

THE FIRST TISSUE-RESIDENT T CELLS: INTESTINAL AND EPIDERMAL γδ T CELLS

Many γδ T cell subsets are constrained to specific tissue locations. DETC and intestinal intraepithelial lymphocytes (IEL) with a γδTCR (γδ IEL) populate the two largest interfaces of the body, the skin and the intestines, respectively. DETC and γδ IEL are shaped within their respective tissues where they provide adapted support to maintain tissue homeostasis and respond to stresses or invading pathogens. These populations have recently been the focus of an in-depth review (21). Thus, only features relevant to this review will be discussed here.

Dendritic Epidermal T Cells– DETC

DETC are the first T cells to develop during embryogenesis and by far the most abundant T cell subset present in the mouse skin epidermis (142). Their name stems from the unique DC-like morphology observed during homeostasis. DETC form a

highly uniform population characterized by the expression of a canonical Vγ3Vδ1 TCR with no junctional diversity. The mouse fetal thymus supports the generation of the entire DETC precursor pool between embryonic day 13 and 18, after which mature DETC are maintained life-long in the skin epidermis by self-renewal (18, 76, 79, 143). The narrow developmental window of DETC progenitors may result from the temporally restricted expression of a Btn-like protein, Skint-1, by embryonic medullary thymic epithelial cells (144–146). Expression of Skint-1 is required at various stages of DETC thymic development to regulate their biology. First, Skint-1 promotes the thymic maturation of Vγ3Vδ1$^+$ T cell progenitors, without which the skin epidermis would be devoid of mature DETC (144–146). Second, Skint-1 educates DETC precursors by promoting IFNγ production over IL-17A (80), instructing skin-homing (147), and attenuating TCR responsiveness by increasing its activation threshold (87). Similarly, TCR signaling seems required for the maturation of DETC precursors (148–150) and the establishment of a mature population with innate-like properties in the skin epidermis (87, 148–151). It is also indirectly involved in the thymic egress and subsequent migration to the skin of positively selected progenitor cells. Indeed, TCR signaling induced the expression of sphingosine-1-phosphate receptor 1 (S1P1) and the skin-homing chemokine receptor CCR10, which mediates T cell exit from the thymus and migration toward keratinocyte-derived CCL27, respectively (152, 153). Additional molecules like E- and P-selectin ligands and CCR4 may also play a role in the establishment or maintenance of the DETC population in the skin (154).

During homeostasis, mature DETC are maintained in a semi-activated state and constantly survey the epidermis through the extension of motile basal dendrites and by projecting dendrites toward the apical epidermis. These dendrites establish stable synapses at the squamous keratinocyte junctions that allows DETC to survey several surrounding cells simultaneously (155). Each apical dendrite ends with phosphorylated tyrosine–rich aggregates in synapse-like structures enriched with TCR and phosphorylated TCR signaling intermediates. Therefore, mature DETC might receive continuous TCR-mediated signals from neighboring cells residing in the epidermis, which are necessary for their long-term maintenance in the tissue (156). Although healthy skin does not appear to express DETC TCR ligand detectable by soluble Vγ3Vδ1 TCR tetramers (157), exposure of the skin to low grade stresses might sustain basal expression of ligands sufficient for their survival but below the sensitivity of this detection method. Indeed, DETC express basal levels of the type-2 cytokine IL-13 in resting skin, consistent with some level of activation at steady state (82). Absence of DETC-derived IL-13 induces an epithelial cell stress response that disrupts barrier integrity. As such, DETC play a key role in preserving skin homeostasis at steady state.

The skin is constantly exposed to a variety of pathological conditions and stresses. Superficial damage to the epithelium induces a stress response associated with upregulation of the NKG2D ligand Rae-1 and leads to the further activation of DETC (82, 158). Enhanced production of DETC-derived IL-13 induces keratinocyte maturation, which promotes efficient epithelial cell renewal, restoring tissue integrity (82). Shortly after deep wounding, damaged keratinocytes in close proximity to the lesion quickly and transiently upregulate a yet unidentified stress antigen (156, 157, 159). DETC rapidly become activated in a TCR dependent-manner and their activation is associated with retraction of their dendrites and cellular rounding (54, 155, 159). Full activation of DETC in this context requires engagement of the TCR and costimulation provided by the junctional adhesion molecule JAML (81), CD100 (semaphorin-4D) (160) or NKG2D (161, 162), whose ligands are all upregulated in damaged skin. Activated DETC provide anti-apoptotic signals to keratinocytes and promote their survival through the production of insulin-like growth factor-1 (84). DETC also produce many additional growth factors, including keratinocyte growth factor (KGF)-1 and KGF-2 (54, 83), inflammatory cytokines like IFNγ and TNFα (81, 163) and chemokines (164) that favor epithelial regeneration and wound closure. The important and non-redundant contribution of DETC to wound repair was demonstrated in *Tcrd*$^{-/-}$ mice or animals deficient in DETC costimulatory signals. Lack of DETC or their impaired activation led to a substantially delayed wound healing (54, 81, 160–162). Additional roles of DETC include regulation of aberrant inflammation in a model of contact dermatitis (55) and protection against UV-mediated DNA damage (165), cutaneous infection (56) and development of malignancies (57, 58, 166). Interestingly, DETC may mediate their anti-cancer effect by direct cytolytic activity in a TCR- and NKG2D-dependent manner *in vitro* (57). Additionally, IL-13 production by DETC favors the production of IgE (158), that promotes protective anti-cancer immunity through a yet undetermined mechanism involving tumor infiltrating FcεRI$^+$ cells (166).

Mucosal and epithelial sites are not only patrolled by natural resident cells like DETC, they are also kept under the surveillance of pathogen-induced CD8$^+$ and CD4$^+$ αβ T_{RM} cells which provide local long-lived protection against reinfection (7, 8). Natural and induced resident T cells occupy a similar space. Cutaneous infection by herpes simplex virus (HSV) generates CD8$^+$ T_{RM} that remain in the basal epidermis around the lesion site (167, 168). Surprisingly, the increased CD8$^+$ T_{RM} density at the site of infection inversely correlated with DETC numbers even several months after pathogen clearance. Conversely, distant DETC-rich areas had a reduced CD8$^+$ T_{RM} population. One potential explanation for the redistribution of resident T cell subsets is that infection may lead to selective loss of DETC, creating a niche for CD8$^+$ T_{RM} cell seeding. Indeed, DETC are rapidly infected by HSV after cutaneous exposure (169). HSV infection of non-neuronal cells is typically lytic and may induce their death. However, alternative mechanisms may also lead to loss of DETC as their redistribution was also observed after intradermal injection of effector CD8$^+$ T cells in the absence of infection (168). DETC can also be temporarily displaced by infiltrating NKT cells following acute stress (58), demonstrating that conventional and unconventional αβ T cells can colonize the skin and create a niche at the expense of DETC. It has been proposed that these cells may compete for maintenance signals like IL-15 or aryl hydrocarbon receptor (AhR) ligands (170), which are necessary for mature DETC

survival in the skin (171–174). Such competition should also occur between αβ T_{RM} generated by different, non-overlapping infections as both populations would be expected to have similar homeostatic requirements. However, it was recently reported that the generation of new αβ T_{RM} cells does not result in the replacement of previously established T_{RM} cells (175), suggesting that limited resources like IL-15 may not be responsible for redistribution of DETC and αβ T_{RM} cells. Identifying the factors involved in the maintenance of natural and induced T cell populations is necessary to better understand their apparent competition and would be beneficial for the design of targeted local therapies.

Intestinal Intraepithelial Lymphocytes–γδ IEL

The intestinal epithelium is actively patrolled by IEL, a large fraction of which are unconventional γδ T cells expressing a CD8αα homodimer in mice (19, 176). The intestine is colonized by γδ IEL during the perinatal period. In contrast to the essential role of the thymus in the generation other γδ T cell subsets, its contribution to intestinal γδ IEL development is more limited. Intestinal γδ IEL can develop extrathymically in athymic mice but at lower numbers than in euthymic animals (177–180). IL-7 production has been shown to be fundamental for γδ IEL thymic and extrathymic intestinal development (181, 182). A large fraction of γδ IEL express the Vγ5 TCR (79, 183). The preferential expression of Vγ5 is controlled at the chromatin level by IL-15-STAT5 signals, which regulate the accessibility of the Vγ5 gene and favor its expression in thymocytes and immature IEL (184). Despite the overrepresentation of the Vγ5 TCR among γδ IEL, the overall γδTCR repertoire in the intestinal epithelium is diverse. Indeed, several mechanisms contribute to the diversity of intestinal γδ IEL including various Vδ and Vγ chain pairings, usage of the Jδ1 or Jδ2 segment and addition of non-germline encoded nucleotides (79, 183). Because of their TCR heterogeneity, γδ IEL have the potential to recognize a wide array of potential antigens or ligands that include host-derived molecules such as nonclassical and nonpolymorphic MHC class Ib molecules T10 and T22 (185). Despite the similarity to MHC class I molecules, T10 and T22 do not present peptide antigens. T10/T22 reactivity is conferred by a specific W-(S)EGYEL CDR3δ motif, which allows some $V\gamma5^+$, $V\gamma1.1^+$ and $V\gamma2^+$ γδ IEL to bind T10/T22 (185). To date, the antigenic specificity of the non-T10/T22 reactive γδ IEL remains obscure.

γδ IEL precursors do not require S1P1 for their emigration from the thymus (186). However, γδ thymocytes and unconventional ($CD8\alpha\alpha^+$) recent thymic emigrants express high levels of the gut homing receptors CCR9 (187, 188) and $\alpha_4\beta_7$ integrin (187–189). Interestingly, CCR9 is preferentially expressed by antigen-inexperienced $CD122^{lo}$ or $CD62L^{hi}$ $CD44^{int/lo}$ thymocytes (189, 190), suggesting they have more potential to home to the gut and that some γδ IEL did not encounter their antigen prior to their migration into intestinal tissues. This assumption was confirmed by the presence of similar numbers of T10/T22 reactive γδ T cells in the intestinal epithelium of $B2m^{-/-}$ mice, which lack surface expression of T10/T22 (190). Intestinal γδ IEL might be selected based on their TCR affinity more than their specificity, as suggested by the inverse correlation between TCR affinity and CCR9 expression (190). This unusual "non-selection" of a diversified γδ T cells likely reflects the need to maintain a heterogeneous broadly reactive population that can respond appropriately to the wide variety of stresses and antigens encountered in the intestine.

Within the first few weeks of life, $V\gamma5^+$ T cells expand in the intestinal epithelium and transition from an immature to a mature phenotype (180). Despite the heavy microbial colonization of the gut, γδ IEL expansion and maturation are independent of the microbiota (66, 178). Instead, expansion and maturation are regulated in a TCR-dependent manner by the BTN-like (Btnl)1 and Btnl6 heterocomplex expressed on the surface of enterocytes (180), reminiscent of Skint-1-mediated selection of DETC in the thymus (144–146). Upon selection by cells co-expressing Btnl1 and Btnl6, $V\gamma5^+$ T cells upregulate CD25 and produce pro-inflammatory cytokines like IFNγ, growth factors like GM-CSF and chemokines like CCL4 (180). The Btnl-mediated selection of intestinal γδ IEL may occur in a similar fashion in humans, with $V\gamma4^+$ T cells being activated by cells co-expressing BTNL3 and 8 (180). Once established in the tissue, γδ IEL rely on the production of IL-15 by microbiota stimulated intestinal epithelial cell (IEC) (191–193) and AhR ligands (174) for their maintenance and survival. In return, γδ T cells participate in the maintenance of tissue homeostasis and barrier integrity. γδ IEL promote IEC proliferation and maturation through multiple mechanisms that may include production of KGF (83, 129, 141), regulating tight junctions (67), producing anti-microbial peptides in response to pathobiont invasion (68), limiting tissue damage, and promoting epithelial repair after injury (141).

γδ IEL from specific pathogen-free (SPF) mice constitutively express cytotoxic genes, including granzyme A and B (194), and can lyse target cells directly *ex vivo* (195), consistent with an anti-infectious role of intestinal γδ IEL. The absence of γδ T cells in $Tcrd^{-/-}$ was associated with enhanced dissemination of enteric bacteria (*Salmonella enterica* serovar Typhimurium) or parasites (*Toxoplasma gondii*), rendering mice more susceptible to systemic infection (67–69). Additionally, γδ IEL indirectly protect from murine norovirus infection by secreting type I and III interferons and increasing the resistance of IEC to viral infection (196). They are also important in controlling dissemination of commensals that may occur with loss of barrier integrity after pathogen invasion or epithelial injury (197). Thus, γδ IEL serve multiple functions in regulating immunity at the mucosal interface with the environment.

Intestinal γδ IEL were initially thought to have limited mobility within the epithelium (188). This view has recently been challenged by two compelling studies that demonstrated that intestinal γδ IEL are highly dynamic and constantly migrate within the intestinal tissue. During tissue homeostasis, individual γδ IEL survey a large surface area and contact numerous IEC within a short period of time (66, 198). γδ IEL mainly remain in the middle region of the intestinal villi, between the basement membrane and the epithelial layer, but they also appear to occasionally migrate to the intercellular space between IEC for

a short period of time (66, 198). Although commensals do not impact γδ IEL numbers, microbial colonization is required for their normal distribution within the villi and their migratory behavior in the tissue (66), and also promotes their cytotoxic and anti-microbial functions (68, 195). These patterns drastically change upon enteric infection with invasive bacteria or parasites. Shortly after pathogenic exposure, γδ IEL preferentially localized to pathogen-rich areas and decreased their normal surveillance behavior. Reduced surveillance coverage was associated with increased movement between IEC and the lateral intercellular space in a behavior termed "flossing" (66, 69) that is regulated by the tight junction protein occludin (198). These behavioral and functional changes result from the MyD88-dependent sensing of pathogenic microbes by IEC, and the specific abrogation of MyD88 signaling in IEC severely blunted γδ IEL responses (66, 68). γδ IEL at steady-state may also be activated through their TCR as injection of a TCRδ-specific antibody diminished intracellular calcium flux (199). It is therefore conceivable that the IEC-γδ IEL dialogue could also involve TCR-mediated tissue surveillance. Thus, γδ IEL continually survey epithelial integrity via cross-talk with IEC which dictates γδ IEL behavior and leads to their adaptation in the intestinal environment. While the exact function of γδ IEL flossing remains unclear, its association with pathogen hotspots and the importance of γδ T cell responses to anti-infection immunity suggests an important role of flossing in controlling intestinal infections or promoting epithelial repair.

Natural tissue-resident γδ T cells are remarkably adapted to their tissue of residence, where they provide signals necessary to maintain tissue homeostasis and barrier integrity while also providing a rapid front-line defense against infectious assaults continually encountered in epithelial tissues. Both DETC and intestinal γδ IEL are adapted to efficiently survey their respective tissues, through their placement/migration into the tissue and communication with neighboring epithelial and immune cells. Despite this, natural tissue-resident T cells may have to compete for limited space or nutrients with *de novo* generated conventional T_{RM} cells after local infections. Whether direct competition for resources and space or an undefined crosstalk between these cells regulate tissue colonization is unclear and an area of much interest.

MICROBIOTA-INDUCED γδ17 T CELLS: DIVERSIFIED EFFECTORS WITH MULTIFACETED ROLES

Almost all tissues exposed to the environment are colonized by established commensal communities, with the exception of the eye for which the presence of a resident microbiome remains a matter of debate (1). The presence of these microorganisms shapes the local immune system and promotes protective anti-infectious immunity, as exemplified by the anti-bacterial, -fungal or -parasitic type-17 and type-1 responses triggered by segmented filamentous bacteria in the intestines (2) or *Staphylococcus epidermidis* (*S. epidermidis*) and other commensals in the skin (3, 4), respectively. However, commensal-specific T cells (especially intestinal T_H17 cells) can also have detrimental effects at remote sites under certain circumstances, inducing pathological inflammatory responses that lead to the development of diseases like arthritis and autoimmune encephalomyelitis (5, 6).

As for conventional T cells, the microbiota also impacts γδ T cell responses at many body sites. Interestingly, commensal-induced γδ T cell responses appear to largely involve IL-17A-producing cells regardless of their tissue distribution among diverse sites such as the skin (4, 200), the liver (22), the oral and peritoneal cavities (23, 201), the eye (24), the lungs (28) and the intestines (29, 197). The generation and activation requirements of microbiota-induced γδ T cells appear uniquely adapted to the tissue location. First of all, the presence of a microbiota is a prerequisite for the development of some, but not all, tissue tropic γδ T cells. Indeed, antibiotic-treated SPF or germ-free (GF) mice harbor fewer activated liver-resident (22), pulmonary (28), peritoneal, and small intestinal lamina propria (siLP) γδ17 T cells (23). In contrast, γδ IEL numbers are independent of a microbiota (66, 178, 197). Second, few identified microorganisms have been specifically associated to particular γδ T cell populations: *Corynebacterium mastidis* (*C. mastidis*) colonization with ocular $V\gamma2^+$ γδ17 T cells (24), *Corynebacterium accolens* (*C. accolens*) and other bacteria from the *Corynebacterium* genus producing mycolic acid with skin $V\gamma2^+$ γδ17 T cells, and *S. epidermidis* with skin $V\gamma2^-$ γδ17 T cells (200). The expansion of $V\gamma2^+$ and $V\gamma2^-$ γδ T cell subsets by *C. accolens* and *S. epidermidis* association, respectively, demonstrates that the γδ T cell responses can adapt within the same niche. In contrast, other γδ T cell subsets only require the presence of a microbiota without any distinction between bacterial species (22, 28). Lastly, many different signals control the activation and/or expansion of commensal-induced γδ17 T cells, including lipid presentation by the non-classical molecule CD1d (22), DC-mediated expansion (24, 201) and activation/polarization (27, 29, 200) or MyD88 signaling pathways (23, 197). Cytokines like IL-1β (23, 24), IL-23 (200) and IL-6 (28), either alone or in combination with other activation signals, also participate in the induction or propagation of IL-17A from microbiota-induced γδ T cells.

IL-17 family cytokines, including IL-17A, are key regulators of mucosal and epithelial immunity. Over the past decade, a multitude of roles, from the induction of protective anti-infectious responses to the promotion of pathological inflammatory processes, have been attributed to IL-17A (202). Accordingly, the induction of γδ17 T cells by microbial colonization has also been associated with seemingly contrasting effects. Commensal-induced γδ T cells can mediate local protection against penetrating commensals (197), pathogenic bacteria or even yeast, as exemplified by the resistance displayed by *C. mastidis* colonized animals to ocular *Candida albicans* infection (24). In this model, induced γδ T cells were driving the production of antimicrobial peptides such as S100A8 and S100A9 and the recruitment of neutrophils through the production of IL-17A. As IL-17A can elicit these responses in virtually all mucosal and epithelial surfaces, similar broad-spectrum anti-infectious immunity might occur in other γδ T cell rich tissues. In contrast to their protective effect against infection, microbiota-elicited

γδ17 T cells may be beneficial (28) or harmful (29) in cancer. Other local detrimental effects attributed to microbiota-induced γδ17 T cells include the acceleration of nonalcoholic fatty liver disease by liver-resident γδ17 T cells (22) and the exacerbation of imiquimod-induced skin inflammation following *C. accolens* association (200).

Microbiota-elicited γδ T cells can also impact distal immune function. They express a plethora of homing receptors that allows them to navigate to distant tissues and impact health or disease. For example, γδ T cells are recruited to the ischemic penumbra after ischemic stroke in a CCR6-dependent manner (203). There, they contribute to exacerbate brain injury through the production of IL-17A and subsequent recruitment of neutrophils (203–205). In a recent study using a transient middle cerebral artery occlusion mouse model, the γδ17 T cells recruited to the ischemic brain originated from the small intestine and were dependent on specific commensal species for their maintenance (27). Alteration of the gut microbiota by antibiotic treatment led to a reduction in intestinal γδ17 T cells and diminished γδ T cell infiltration to the meninges, limiting injury. Thus, commensal-induced γδ T cells may have local and distal effects on pathological or physiological tissue processes.

It is now well established that the microbiota is a critical component of human health and disease. In addition to providing many enzymatic and metabolic pathways and colonization resistance to invading pathogens, commensals also participate in the development of and shaping of the immune system (206). Dysbiosis can be sensed by the immune system and has been associated with the development or exacerbation of many diseases in many organ systems. Given their preferential association with epithelial and mucosal tissues, it is not surprising that some γδ T cell populations are also influenced by the microbiota.

INFLAMMATORY DISEASE AND MEMORY-LIKE γδ17 T CELL RESPONSE

In addition to γδ T cell responses to the microbiota or after infection, γδ T cells have also been implicated in innate responses in inflammatory disease. Inflammatory diseases with γδ T cell contributions include multiple sclerosis or EAE (72), psoriasis (135), collagen induced arthritis (73), ankylosing spondylitis (74), inflammatory bowel disease (63, 64), and uveitis (75). One factor of inflammatory disease progression attributed to γδ T cells is IL-17A production, a feature often associated with changes in the microbiota (72, 73, 135). Inflammation-induced tissue damage may allow bacteria to bypass the epithelium leading to a positive feedback inflammatory loop. Interestingly, memory-like γδ T cell formation has been seen in inflammation of the skin (25, 26, 51, 207). IL-17A-producing Vγ2Vδ4$^+$ T cells initially derive from the neonatal thymus where they are instructed with tissue tropism. IMQ-induced psoriasis-like skin inflammation triggers a potent long-lived Vγ2Vδ4$^+$ T cell response (**Figure 1**) (25, 26). These Vγ2Vδ4$^+$ T cells were phenotypically memory-like with a CD44^{hi} CD62L^{lo} CD27$^-$ expression pattern. Vγ2Vδ4$^+$ T cells expanded after primary challenge and migrated from the draining lymph nodes to both the inflamed and uninflamed skin in a S1P1-dependent manner where they persisted. Migration of Vγ2Vδ4$^+$ T cells from the circulation to the skin may also be influenced by signals including cutaneous lymphocyte antigen (CLA) binding to P- and E-selectins, CD103 interactions with E-cadherin, and C-C chemokine receptor type 2 (CCR2), and CCR6. CCR2 appeared essential for γδ17 T cell recruitment to inflamed tissues in B16 melanomas and EAE while CCR6 appeared necessary for dermal γδ17 T cell residence (208). Subsequent IMQ administration on previously untreated skin induced an accelerated and robust re-expansion of skin resident Vγ2Vδ4$^+$ T cells that produced IL-17A/F and exacerbated disease (25, 26). IL-17 production and subsequent neutrophil recruitment for skin disease appeared be partially dependent on an NFκB-inducing kinase (207). Enhanced inflammation with subsequent exposure was also associated with the Vγ2Vδ4$^+$ T cell recall response but independent of αβ T cells (26). These findings were also noted in an acute contact dermatitis model using dinitrofluorobenzene where a similar memory Vγ2$^+$ γδ17 T cell population appeared predominately tissue-resident in classical parabiosis experiments (51). Together, these studies suggest that γδ T cells can modulate inflammatory diseases of the skin by forming long-lived tissue resident memory populations that exacerbate disease through the production of IL-17 family cytokines. While these studies suggest the establishment of long-lived memory T cells, whether this response is driven by a specific antigenic responsiveness or is broadly reactive is unclear.

INFECTION-INDUCED ADAPTIVE γδ T CELLS: LONG-TERM PLAYERS IN MUCOSAL IMMUNITY

Anamnestic immunity was thought to be mediated solely by conventional αβ T cells and B cells. The recent identification of several innate and unconventional memory responses challenged this belief and has reshaped our view of immunological memory. γδ T cells bridge innate and adaptive immunity in many contexts by rapidly responding to stresses such as infections and promoting conventional adaptive immunity. For that reason, most mouse studies focused on γδ T cell responses in the first few hours to days after pathogen exposure or inflammatory insult. However, mounting evidence in humans, non-human primates and mice demonstrated that γδ T cells can mount adaptive-like responses. One of the most studied pathogens in that context is CMV. Indeed, the involvement of γδ T cells in the protective response to CMV infection was first suggested in kidney transplant patients whose γδ T cells underwent a massive and long-lasting expansion in the blood (34, 209, 210). γδ T cell expansion to CMV was also observed in the context of immunosuppression or immunodeficiency (35, 36, 126, 211–215), neonatal infection (216) and in otherwise healthy individuals (35, 125). Analysis of the repertoire of CMV-selected γδ T cells revealed an oligoclonal and in some individuals even monoclonal population (34, 35, 125), which, surprisingly, did not involve circulating Vγ9Vδ2$^+$ T cells but tissue tropic Vδ2$^-$ γδ T cells. Expanded cells displayed a T_{EMRA}

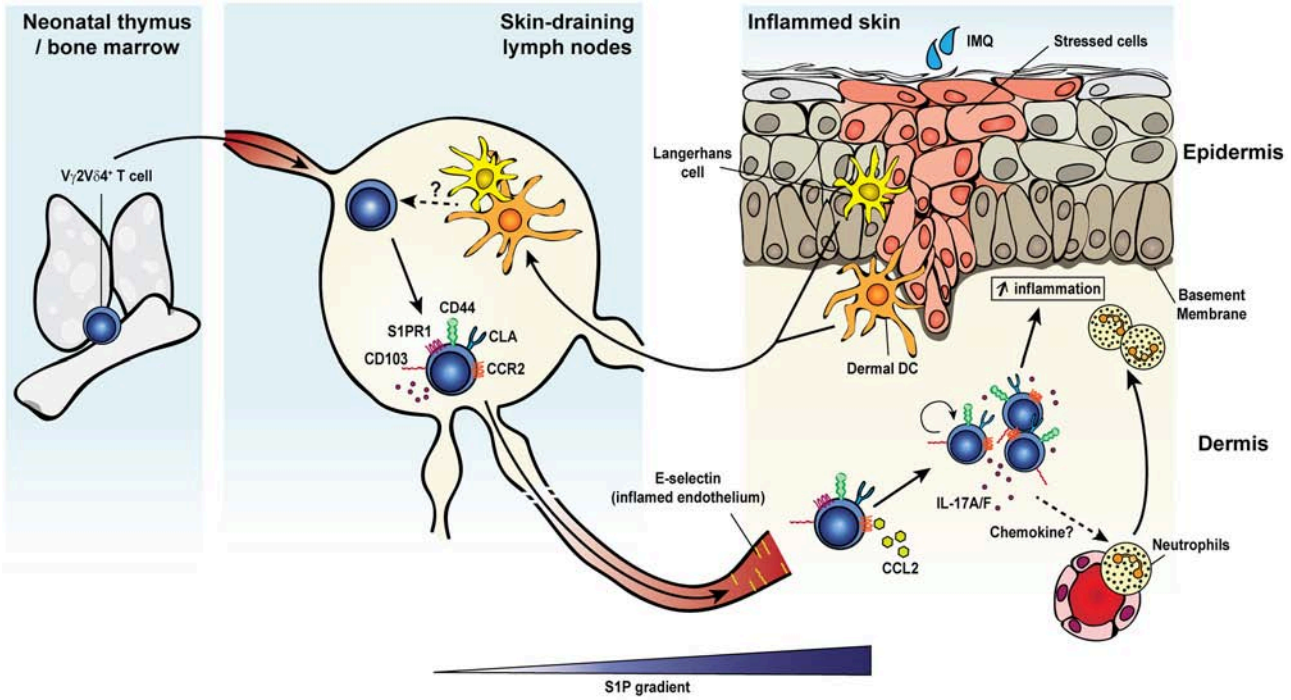

FIGURE 1 | Inflammation-induced dermal memory γδ T cells sensitize mice to imiquimod-induced psoriasis. Topical skin exposure of naïve mice to the TLR7/8 ligand imiquimod (IMQ) activates regional dendritic cells (Langerhans cells or dermal DCs) which migrate to the draining lymph nodes and present antigens to Vγ2Vδ4$^+$ T cells. Activated γδ T cells proliferate, acquire an effector/memory CD44^{hi} CD27$^-$ CD62L$^-$ phenotype and upregulate several migration molecules favoring their egress from the lymph nodes (S1P1) and homing to the inflamed and resting skin dermis (CCR2 and cutaneous lymphocyte-associated antigen or CLA), where the cells establish memory. Secondary IMQ skin application at the same or a distant site leads to the local proliferation and activation of dermal memory Vγ2Vδ4$^+$ T cells, which produce large amount of IL-17A/F and promote the recruitment of neutrophils and thereby exacerbate skin inflammation.

phenotype, similar to CMV-specific CD8$^+$ T cells (127), and only responded to CMV infection (34, 128). Importantly, the expansion of Vδ2$^-$ γδ T cells correlated with the resolution of the acute infection in humans (210) and adoptive transfer of murine CMV-expanded γδ T cells conferred full protection to susceptible immunodeficient mice (217, 218). Thus, CMV-elicited γδ T cells display many features classically attributed to conventional memory T cells. Another long-lived γδ T cell response to virus has been reported in the context of vaccinia virus immunization in humans (38) and rhesus macaques (39). Interestingly, vaccinia virus immunized macaques were protected against monkeypox virus challenge infection and this was associated with the expansion of circulating and pulmonary Vγ9Vδ2$^+$ T cells. Long-lasting adaptive-like γδ T responses were also reported in the circulation of individuals infected with the protozoan *Plasmodium falciparum* (*P. falciparum*) (40–43) and the circulation and peripheral tissues of animals infected with *Plasmodium chabaudi* (44). Interestingly, γδ T cell distribution to parasite-targeted tissues raises the possibility that these cells might provide unique functions to control parasite replication during the blood and liver stages. Collectively, these studies provide compelling evidence of adaptive γδ T cell responses triggered by unrelated pathogens in humans, non-human primates and rodents. However, the chronic or latent nature of the infections and their associated antigen and inflammation in conjunction with some inherent challenges associated with human studies has hindered conclusive demonstrations of the memory potential and long-term tissue residency of these populations.

Infection-Induced *bona fide* Memory γδ T cell Responses

Adaptive γδ T cells survey exposed mucosal and epithelial barriers where they may participate in pathogen clearance or control and have tissue-adapted functions. γδ T cells are one of the first immune responders in many bacterial infections, where they act concurrently with cells of the innate immune system. However, this innate γδ T cell response does not preclude the establishment of a subsequent localized memory γδ T cell response. A mouse model of peritonitis induced by repeated intraperitoneal exposure to *Staphylococcus aureus* (*S. aureus*), induced a rapid Vγ1.1$^+$ and Vγ2$^+$ γδ17 T cell response in the peritoneum and the draining mediastinal lymph nodes a few hours after exposure (49). After this early polyclonal innate response, a long-lived predominantly IL-17A-producing Vγ4$^+$ T cell population emerged in both tissues. Surprisingly, secondary challenge with *S. aureus* of previously exposed but pathogen-free mice induced a conventional memory response of Vγ4$^+$ T cells. Recalled Vγ4$^+$ T cells underwent secondary expansion, displayed an activated CD44^{hi} CD27$^-$ phenotype,

and produced elevated levels of IL-17A. Adoptive transfer of purified *S. aureus*-elicited Vγ4$^+$ T cells was sufficient to protect naïve recipients against peritonitis and bacterial dissemination to the liver and kidneys (49). In contrast to the fundamental role of IL-1β in the induction of IL-17A production by naive γδ T cells during primary *S. aureus* exposure, memory Vγ4$^+$ T cells were IL-1β-independent suggesting that memory γδ T cells have an altered ability to respond to unique environmental cues to provide effector functions. Localized *S. aureus* infection of the skin in *Il1b*$^{-/-}$ mice resulted in poor bacterial control during primary infection but protection against reinfection, revealing the potential presence of an additional memory γδ T cell subset. Indeed, intradermal infection induced the selective expansion of skin resident Vγ4Vδ1$^+$ and Vγ3Vδ1$^+$ T cell clones with conserved CDR3δ and CDR3γ motifs that were maintained during the convalescent phase and present after secondary infection of WT and *Il1b*$^{-/-}$ mice (50). Protection during secondary infection was conferred by IFNγ- and TNFα-producing γδ T cells. Adoptive transfer of purified *S. aureus*-elicited γδ T cells, but not CD4$^+$ T cells, neutrophils or serum from convalescent mice, was associated with bacterial clearance. Thus, different memory γδ T cell responses can be induced by the same pathogen and local memory γδ T cell populations may be tissue adapted to provide distinct protective mechanisms.

In addition to the memory responses involving Vγ4$^+$ T cells, a long-lasting protective response of Vγ2$^+$ T cells was observed after pulmonary *Bordetella pertussis* (*B. pertussis*) infection (**Figure 2**) (48). After an early innate response dominated by IL-17A-producing Vγ1.1$^-$ Vγ2$^-$ γδ T cells, effector memory CD44$^+$ CD27$^-$ Vγ2$^+$ T cells started accumulating from day 14 and were maintained long-term in the lungs. The later emergence of Vγ2$^+$ T cells coincided with the expansion of T_{RM} precursors and T_{EM}-like CD4$^+$ T cells in the lungs (219). Expanded pulmonary Vγ2$^+$ T cells share several features with *B. pertussis*-specific memory CD4 T cells: (i) they reside in the lungs for a prolonged period of time after bacterial clearance and rapidly and locally proliferated in response to secondary pulmonary challenge, (ii) a considerable fraction expresses the T_{RM} marker CD69 and some also co-express CD103, (iii) they have a strict reactivity to *B. pertussis*, (iv) they are biased toward IL-17A production, and (v) they contribute to enhanced bacterial clearance after challenge (48, 219). Thus, *B. pertussis*-elicited memory γδ T cells closely resemble conventional T_{RM} cells. In contrast to the reported displacement of skin DETC by virus-specific CD8$^+$ T_{RM} (168), CD4$^+$ T_{RM} and memory γδ T cells were able to coexist in the lungs of infected mice and both subsets expanded after infection and participated in conferring protection, suggesting that they may reside in distinct niches within the tissue or do not compete for space or survival factors.

Microorganisms producing PAgs are potent activators of human and non-human primate Vγ9Vδ2$^+$ T cells. Mycobacteria, including *Mycobacterium bovis* BCG strain and *Mycobacterium tuberculosis* (*M. tuberculosis*), produce HMBPP (220–222), the most potent Vγ9Vδ2$^+$ T cell activator. Correspondingly, intravenous (i.v.) BCG vaccination of macaques triggered a drastic expansion of these circulating cells in the blood, but also in the lungs and the intestines (45). Pulmonary *M. tuberculosis* infection led to a similar expansion of mucosal but not circulating Vγ9Vδ2$^+$ T cells (47), demonstrating tissue-adapted responses by adaptive γδ T cells that may be

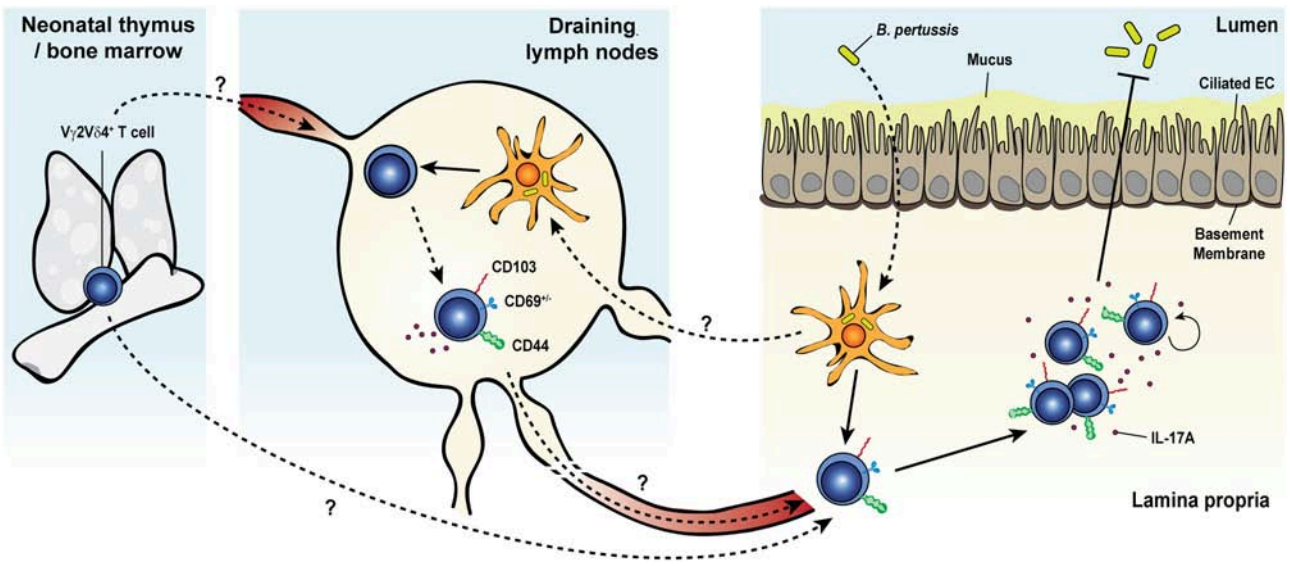

FIGURE 2 | Memory γδ T cell response to pulmonary *Bordetella pertussis* infection. Upon primary intranasal infection with *Bordetella pertussis* (*B. pertussis*), Vγ2Vδ4$^+$ T cells are activated by *B. pertussis* antigen-presenting dendritic cells either in the draining lymph nodes or directly in the lung tissue. Activated γδ T cells expand, display a CD44$^+$ and CD103$^+$CD69$^{+/-}$ activated resident memory phenotype and remain at an elevated number in the lungs after bacterial clearance. Secondary exposure to *B. pertussis* induces a recall expansion of memory Vγ2Vδ4$^+$ T cells in the lung tissue and a protective and robust IL-17A response leading to an enhanced pathogen clearance.

predicated on immunization route. BCG challenge of vaccinated monkeys induced a more rapid and robust clonal expansion of Vγ9Vδ2$^+$ T cells but no other γδ T cell subsets. Thus, Vγ9Vδ2$^+$ T cells are capable of forming long-lived clonally-expanded memory responses (45). Interestingly, direct contact with antigen presenting cells was required for the recall-like expansion of Vγ9Vδ2$^+$ T cells (46). The recall response of Vγ9Vδ2$^+$ T cells in BCG immunized macaques was associated with enhanced clearance of challenge infection and protection against fatal tuberculosis (45). In line with these findings, Vγ9Vδ2$^+$ T cells induced in BCG vaccinated volunteers that were previously unexposed to any *mycobacteria* showed an enhanced responsiveness to *M. tuberculosis ex vivo* (223), suggesting that BCG vaccination also primes γδ T cells to respond to *M. tuberculosis* in humans. Although human and monkey Vγ9Vδ2$^+$ T cells share many features, including a memory-like response to *mycobacteria*, it remains to be established whether human γδ T cells, like their non-human primate counterparts, are maintained in peripheral tissues following BCG immunization to confer some protection against *M. tuberculosis* infection.

Multifunctional Memory γδ T Cells to *L. monocytogenes*

L. monocytogenes is known to be a potent inducer of γδ T cell responses. In humans, expansion of Vγ9Vδ2$^+$ T cells has been detected in the blood of pregnant women, newborns, infants and the elderly early after *L. monocytogenes* exposure (224, 225). These γδ T cells displayed an activated (HLA-DR$^+$) and memory (CD45RO$^+$) phenotype. Consistent with a predetermined innate response, stimulation of PBMC from healthy donors with heat-killed *L. monocytogenes* (225), listeria lysate or culture supernatant (226) led to rapid proliferation of Vγ9Vδ2$^+$ T cells.

A similar mobilization of circulating γδ T cells during *L. monocytogenes* infection has also been reported in rhesus macaques. In a model of disseminated *L. monocytogenes* infection, Vγ9Vδ2$^+$ T cells increased in the blood of rhesus macaques infected with an attenuated *L. monocytogenes* strain through an intramuscular, and to a lesser extent i.v. route (59). These cells were also elevated in bronchoalveolar lavages and rectal biopsies suggesting that they actively traffic to and seed mucosal tissues during infection. More interestingly, *L. monocytogenes* challenge of immunized animals led to a rapid and robust re-expansion of Vγ9Vδ2$^+$ T cells that correlated with the resolution of infection (59). One peculiar feature of *L. monocytogenes* is its ability to use both the classical mevalonate and the alternative MEP pathways for isoprenoid synthesis (227). Both primary and recall-like responses of Vγ9Vδ2$^+$ T cells have been shown to rely on the bacteria's ability to co-produce mevalonate-derived isopentenyl pyrophosphate and MEP-derived HMBPP, the latter being much more efficient at inducing primary and secondary expansion of primate Vγ9Vδ2$^+$ T cells and promoting their differentiation into CD27$^+$ CD45RA$^-$ CD28$^-$ memory cells (60). *L. monocytogenes*-elicited γδ T cells displayed various effector functions after secondary challenge, including production of IFNγ, IL-4, IL-17A, and TNFα (59). Surprisingly, a substantial portion of these cells were multifunctional and simultaneously produced IFNγ and IL-17A, IFNγ and IL-4, or TNFα and perforin in response to HMBPP (59, 60). Expanded Vγ9Vδ2$^+$ T cells were also potent bactericidal effectors capable of efficiently lysing *L. monocytogenes*-infected DC and restraining intracellular bacterial growth in macrophages *ex vivo*. Thus, *L. monocytogenes* infection elicits a multifunctional circulating γδ T cell response in non-human primates. Because this response is accompanied by the colonization of epithelial tissues, infection-elicited mucosal γδ T cells may also have distinct effector functions that provide tissue-adapted responses.

A large body of evidence has convincingly demonstrated the involvement of γδ T cells in the early phase of the primary immune response to systemic *L. monocytogenes* infection of mice (228–244) and rats (245, 246). More recently, our group reported a *bona fide* memory γδ T cell response in mice after food-borne infection with a mouse-adapted *L. monocytogenes* capable of intestinal epithelial cell invasion (**Figure 3**) (62, 134, 247). Food-borne infection induced a long-lived Vγ4Vδ1$^+$ T cell population in the gut draining mesenteric lymph node (MLN) with a CD44^{hi} CD27$^-$ phenotype (62). By 7 days after infection, these cells were mobilized into the blood, up-regulated the gut-homing integrin $\alpha_4\beta_7$ and trafficked to the intestinal lamina propria similarly to conventional *L. monocytogenes*-specific CD8$^+$ (248) and CD4$^+$ (249) αβ T cells. Like *L. monocytogenes*-induced CD4$^+$ and CD8$^+$ αβ T_{RM} cells, *L. monocytogenes*-elicited γδ T cells established residency in MLN and intestinal lamina propria where they were maintained long term in the absence of further antigenic stimulation (62, 134). The generation of this γδ T cell subset was restricted to tissues associated with the gastrointestinal system and was induced by food-borne (62) but not i.v. infection (232, 233). *L. monocytogenes*-elicited γδ T cells demonstrated enhanced anamnestic response upon *L. monocytogenes* challenge infection and were fully competent for immunologic boosting upon tertiary exposure (62). Although *L. monocytogenes*-elicited γδ T cells appeared to share a similar anatomical niche as *L. monocytogenes*-specific CD4$^+$ and CD8$^+$ αβ T cells (248, 249), all populations expanded robustly after infection and were maintained without any apparent competition for limiting resources or anatomic space.

Memory γδ T cells cooperated with αβ T cells to confer optimal protection in the MLN and the small intestine during food-borne *L. monocytogenes* challenge infection. Indeed, only the concomitant antibody-mediated depletion of αβ T cells (both CD8$^+$ and CD4$^+$) and forced internalization of the γδ TCR resulted in the complete loss of protection afforded to immunized mice, whereas the sole removal of αβ T cells only partially impaired *L. monocytogenes* control (62). One striking feature of *L. monocytogenes*-elicited γδ T cells was their ability to produce IFNγ and IL-17A during each stage of the immune response. Moreover, subsets within the CD44^{hi} CD27$^-$ γδ T cell population co-produced both cytokines during the primary and secondary responses (62), reminiscent of the multifunctional response described in rhesus macaques after

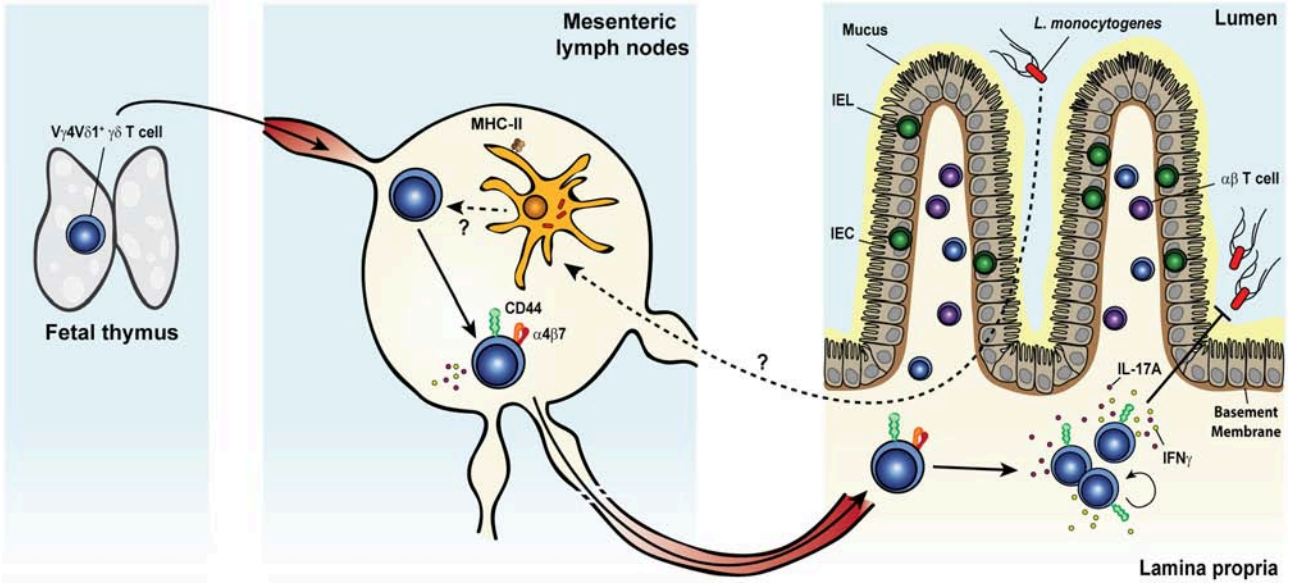

FIGURE 3 | *Listeria monocytogenes* elicits a multifunctional protective memory response from fetal γδ T cells. Shortly after oral infection, the foodborne pathogen *Listeria monocytogenes* (*L. monocytogenes*) crosses the intestinal barrier and migrates to the mesenteric lymph nodes (MLNs). Whether *L. monocytogenes* reaches the MLNs extracellularly or is carried intracellularly by migratory intestinal dendritic cells is yet unclear. Colonization of the MLNs leads to the expansion of a population of semi-invariant Vγ4Vδ1$^+$ T cells characterized by a CD44^{hi} CD27$^-$ phenotype and the rare ability to co-produce both IL-17A and IFNγ, in a process that would likely involve MHC-II$^+$ cells. Activated cells upregulate the gut-homing integrin $\alpha_4\beta_7$ and migrate through the blood circulation to the intestinal lamina propria (LP). After pathogen clearance, *L. monocytogenes*-elicited γδ T cells become resident memory cells and persist long term in both tissues. Memory γδ T cells undergo a rapid and dramatic re-expansion upon re-exposure to *L. monocytogenes* and cooperate with conventional T cells to confer heightened protection against the bacterium.

secondary challenge (59). During the recall response, the majority of IL-17A was derived from reactivated memory γδ T cells in the MLN. This production of IL-17A was a critical component of anti-listerial immunity as it mediated the formation of *L. monocytogenes*-containing immune cell clusters composed of memory γδ T cells and IL-17RA$^+$ inflammatory monocytes and neutrophils (134).

Collectively, these studies demonstrate that systemic and food-borne *L. monocytogenes* infection generates long-lived multifunctional memory γδ T cells in rhesus macaques and mice, respectively. Thus, a population of pathogen-elicited γδ T cells appears to behave very similarly between mice and primates, and this may suggest a conserved biology among mucosal γδ T cells. These studies also highlight the important influence of infection route and models that mimic natural infection on understanding the γδ T cell response. Interestingly, amongst the memory and memory-like responses described to date, *L. monocytogenes* is the only agent known to induce multifunctional γδ T cells in two distinct species. Although γδ17 T cells are known to have a permissive chromatin state for IFNγ expression (102), other memory γδ T cell populations reported in mice only produce IL-17A (25, 26, 48, 49). Conversely, only IFNγ was shown to be produced by virus-activated memory-like Vγ9Vδ2$^+$ T cells (39). miR-146a has recently been shown to negatively regulate IFNγ production by murine γδ17 T cells, including during oral *L. monocytogenes* infection (61). Elucidating the mechanisms by which *L. monocytogenes* partially breaks miR-146a-mediated inhibition of IFNγ production by γδ17 T cells and understanding why other pathogens do not would provide important clues about the fine regulation of γδ17 T cell functions and might open new avenues for the manipulation of these cells.

ANTI-TUMOR MEMORY γδ T CELLS IN CANCER

A substantial body of research has focused on the beneficial nature of γδ T cells in anti-cancer immunity and their potential as a targetable therapeutic since a landmark study demonstrated that γδ T cells in the epithelial compartment play a substantial role in prevention of cutaneous carcinogenesis (57). Indeed, the presence of an intra-tumoral γδ T cell gene signature was associated with the single most favorable prognostic indicator of patient outcome for a wide range of cancers (250). γδ T cells can have a wide range of effects ranging from reshaping the tumor microenvironment (251, 252), being integral in promoting a diverse cancer protective IgE repertoire through NKG2D stress surveillance (166), or IFNγ production (52). Substantial effort has focused on resolving the anti-tumor activity of Vγ9Vδ2$^+$ T cells, the predominant γδ T cell population in human PBMC, in multiple cancers (253–257). Tissue resident Vδ2$^-$ γδ T cells may also substantially contribute to anti-tumor immunity. Vδ2$^-$ γδ T cells typically predominate over Vδ2$^+$ T cells within tumors (52, 65) as well as in tissues from healthy individuals (120). This Vδ2$^-$ γδ T cell population is principally composed of Vδ1$^+$ T

cells but also contain a significant population of Vδ3$^+$ T cells. Due to Vδ2$^-$ γδ T cell prevalence in tumor microenvironment, it is likely that this subset also substantially contributes to anti-tumor activity.

Vγ9Vδ2$^+$ T cells were previously delineated based on expression of CD45RA and CD27 as naive (CD45RA$^+$ CD27$^+$) cells or effector and memory T_{CM} (CD45RA$^-$ CD27$^+$), T_{EM} (CD45RA$^-$ CD27$^-$), and T_{EMRA} (CD45RA$^+$ CD27$^-$) cells (117). While naive T cells and T_{CM} cells primarily reside in secondary lymphoid tissues, T_{EM} and T_{EMRA} migrate to inflammatory sites to perform effector functions. These latter populations have been investigated in multiple cancers including squamous cell carcinoma (SCC) (52), CRC (65), neuroblastoma (71), and melanoma (53) due to their proliferative capacity and tendency to migrate toward inflammatory sites. Substantial effort has also sought to leverage the anti-tumor properties of Vγ9Vδ2$^+$ T cells using approaches like *in vitro* expansion of patient-derived γδ T cells and chimeric antigen receptor T cells for potential adoptive immunotherapies (258, 259). Vγ9Vδ2$^+$ T cells can be selectively activated through PAgs or amino bisphosphonates such as zoledronic acid (zoledronate) in combination with various growth factors, cytokines, or costimulatory molecules (260). While various adoptive transfer methods have been primarily explored in a number of pre-clinical studies (261–267), to date, clinically favorable outcomes appear limited to prostate cancer (137). However, challenges remain in the rapid and robust generation of the large numbers of cells that would be necessary for successful adoptive immunotherapies (268). Zoledronate also has various indirect effects on γδ T cells by independently impacting the tumor microenvironment (251, 269, 270), which can provide a pro-tumor or anti-tumor outcome (271, 272). As such, it will be important to assess the contribution of γδ T cells and the impact of any therapies in individual tumor types.

A protective role of tissue resident γδ17 T cells has been readily described in the context of infectious disease, but they have also been implicated in exacerbating chronic inflammatory diseases like psoriasis. Chronic inflammatory disease is a risk factor and clinical precursor to a number of cancers including pancreatic cancer (273), skin cancer (274) and CRC (275). A growing body of literature has also demonstrated a γδ T cell response that promotes tumor growth. This pro-tumor outcome of some γδ T cell responses appears predominately a consequence of IL-17A production that is often associated with the up-regulation of proliferation pathways in cancerous lesions (276) (**Figure 4**). These apparent anti- and pro-tumor discrepancies are likely due to the dichotomous functional outcomes associated with type-1 or type-17 γδ T cell responses. A pro-tumor role of IL-17A-producing γδ T cells is evident in a number of cancers such as SCC (52), CRC (29), and metastatic breast cancer (70). In human SCC, tumor infiltration of IL-17A-producing Vδ1$^+$ and Vδ2$^+$ T cells was associated with a negative prognosis, in contrast to a more favorable outcome associated with tumor-infiltrating IFNγ-producing γδ T cells (52). Similar results were seen in human CRC where a predominately Vδ1$^+$ IL-17A-producing γδ T cell population positively correlated with a more advanced tumor stage. This correlation was attributed

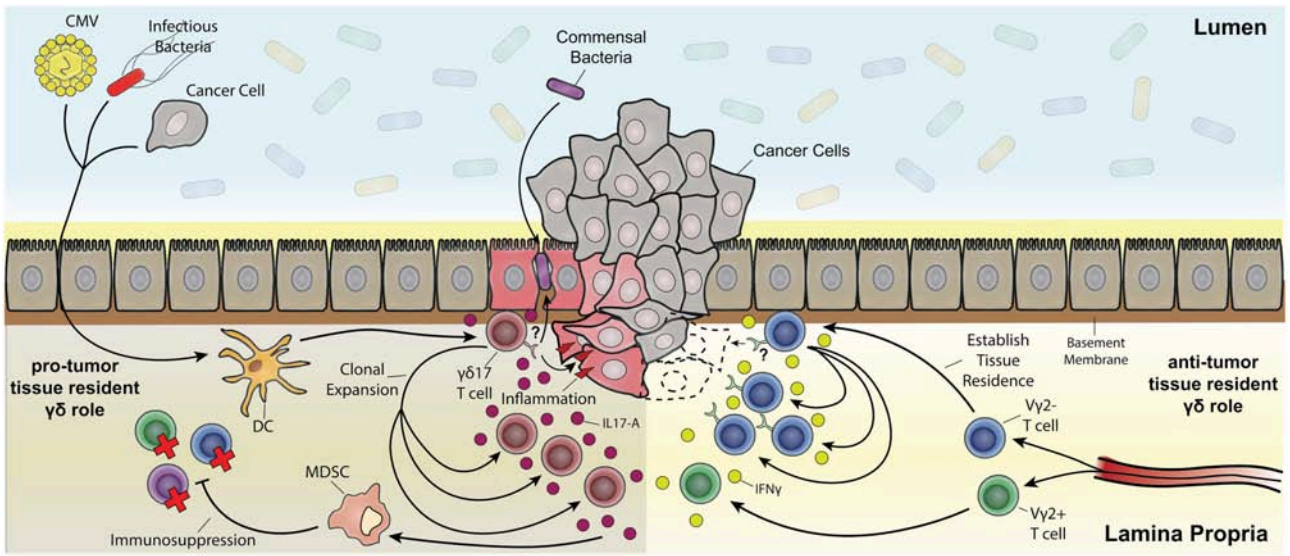

FIGURE 4 | The multifaceted role of resident memory-like γδ T cells in tumorigenesis. Depicted are Vδ2$^-$ γδ T cells establishing tissue residency upon being primed by various means (e.g., CMV, bacterial infection, and tumor associated antigens) through localization from the draining lymph nodes to the tissue's epithelial layer. Vδ2$^+$ T cells also localize to the tissue but do not establish permanent residence. Both Vδ2$^+$ and Vδ2$^-$ γδ T cell subsets can be polarized from IFNγ anti-tumor subsets toward pro-tumor IL-17A-producing subsets through inflammatory dendritic cell cytokine signaling (e.g., IL-23). One possibility is that pro-inflammatory tissue damage causes a leaky barrier to commensals and other bacteria and a positive feedback loop of inflammation resulting in expansion of IL-17A-producing γδ T cell subsets. Chronic inflammatory exacerbation opens the window for cancer upon mutagenesis due to constant tissue regeneration. IL-17A signaling also causes myeloid-derived suppressor cells (MDSC) to have an immunosuppressive effect on effector T cells. On the other hand, IFNγ-producing tissue resident Vδ2$^-$ subsets clonally expand upon recognition of antigen (in part through stress recognition but it has yet to be thoroughly elucidated) causing tumor cell death.

to an inflammatory DC - γδ17 T cell - MDSC regulatory axis (29). Interestingly, tissue resident memory $V\gamma2^+$ T cells were also seen in a metastatic mouse model of breast cancer. These $V\gamma2^+$ T cells produced IL-17A and G-CSF, which promoted the establishment of immunosuppressive intratumoral MDSC (70). Collectively, these studies implicate tissue resident $V\delta1^+$ and $V\gamma2^+$ T cells as tumor growth promoting through IL-17A-mediated MDSC recruitment and immunosuppression in cancer. More importantly, these findings segregate deleterious γδ T cell responses from those which may have a beneficial outcome.

On the other hand, $V\delta2^-$ γδ T cells are not limited to pro-tumor effects and effort has been invested into their therapeutic benefits. Intrahepatic $V\delta1^+$ and $V\delta3^+$ T cells express a $CD45RA^+$ $CD27^-$ and $CD45RA^-$ $CD27^-$ phenotype that is nearly absent from the blood. Intrahepatic $CD45RA^-$ $CD27^-$ $V\delta1^+$ and $V\delta3^+$ T cells were competent producers of IFNγ and TNFα and also expressed receptors for early activation and tissue retention, such as CD69, that have also been noted in both liver resident NK and $CD8^+$ αβ T cell populations (120, 277). CMV infection has notably been one of the drivers of hepatic $V\delta2^-$ γδ T cell expansion and memory formation, and these factors appear to have a protective effect against tumor formation. CMV-seropositive patients (infected pre- or post-transplantation) have a reduced risk of skin cancer development and leukemia relapse after kidney or bone marrow transplant, respectively (36, 37). $V\delta2^-$ γδ T cells from CMV-infected kidney transplant patients were capable of killing HT29 colon cancer cells *in vitro* (128) and CMV-induced $V\delta2^-$ γδ T cells had anti-tumor activity against primary and metastatic tumors in a HT29 xenograft mouse model (278, 279). The characterization of the antigenic specificity of one highly expanded γδ T cell clone from a CMV-seropositive transplant patient revealed that its recognition of stressed (infected or transformed) cells was mediated by the direct binding of the TCR to EPCR, independently of its cargo (33). Similarly, Annexin A2 is upregulated at the surface of stressed cells and can activate another $V\delta2^-$ γδ T cell clone (123). However, regardless of which epitope is being recognized, TCR sequencing of intrahepatic $V\delta2^-$ γδ T cell populations has revealed that CMV infection can induce expansion, memory phenotypes, and tumor reactivity in a clonally expansive manner (120). Overall, these studies suggest that $V\delta2^-$ γδ T cells form T_{RM} cell populations that can clonally expand and cross-react with tumor epitopes to provide anti-tumor immunity.

Knowledge of resident γδ T cell biology is integral for future cancer therapies. Despite intra-tumoral γδ T cell gene signatures being regarded as a favorable prognostic, there is a delicate balance between becoming pro-tumor and anti-tumor γδ T cells (**Figure 4**). Pro-tumor populations are characterized by γδ17 T cells and their indirect immunosuppressive activity through MDSC (29). On the other hand, anti-tumor populations are characterized by IFNγ producing γδ T cells (52). Notably, IgE response mediated by DETC stress surveillance can have anti-tumor effects (166) as well as potential autoimmune effects (280). A better understanding of how signals in tumor microenvironment shape and potentially polarize γδ T cell cytokine production and signal to other cells would be of great benefit.

CONCLUDING REMARKS

The roles of γδ T cells in response to pathogens and commensals and in inflammatory disease and cancer have been an area of expanding interest over the last decade generating significant advances in knowledge. However, our basic understanding of γδ T cell biology is still largely incomplete and lags far behind our understanding of their αβ T cell counterparts, particularly in the area of anamnestic responses. γδ T cells are adapted to their tissue environment which in turn shapes the immune landscape of that environment. Like most cells of the immune system, γδ T cells can appear duplicitous under certain circumstances. On one hand, they can provide beneficial outcomes to the host by conferring anti-pathogen and anti-tumor immunity. On the other hand, they can lead to negative outcomes or exacerbated disease in some inflammatory disorders and cancers. Regardless of their impact, it is now clear that γδ T cell responses encompass both innate inflammatory responses and more traditional adaptive memory responses that provide substantial opportunities for therapeutic targeting. Memory γδ T cell responses may advance a new arm of rationale vaccine design that has broad implications for boosting anti-pathogen or anti-tumor immunity. Vaccines that elicit broadly reactive long-lived circulating or tissue-resident memory γδ T cells may provide protection against a wide range of cancers and infections. Similarly, innate inflammatory or adaptive effector responses may be targeted to enhanced therapeutic modalities with far ranging implications. In the context of a detrimental impact on human health, γδ T cell responses may be blunted or, in the context of cancer, diverted to a lineage that promotes tumor eradication. Thus, memory and tissue-resident γδ T cells represent a lineage of the adaptive immune system that necessitate greater understanding to facilitate the generation of novel therapeutics to promote human health and reduce disease.

AUTHOR CONTRIBUTIONS

CK wrote the first draft of the manuscript. THC wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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REVIEW
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Tissue Resident CD8 Memory T Cell Responses in Cancer and Autoimmunity

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Resident memory (T_{RM}) cells are a distinct tissue-localized T cell lineage that is crucial for protective immunity in peripheral tissues. While a great deal of effort has focused on defining their role in immunity to infections, studies now reveal T_{RM} cells as a vital component of the host immune response to cancer. Characterized by cell-surface molecules including CD103, CD69, and CD49a, T_{RM}-like tumor-infiltrating lymphocytes (TILs) can be found in a wide range of human cancers, where they portend improved prognosis. Recent studies in mouse tumor models have shown that T_{RM} cells are induced by cancer vaccines delivered in peripheral tissue sites, or by the depletion of regulatory T cells. Such tumor-specific T_{RM} cells are recognized as both necessary and sufficient for long-lived protection against tumors in peripheral tissue locations. T_{RM} responses against tumor/self-antigens can concurrently result in the development of pathogenic T_{RM} responses to self, with a growing number of autoimmune diseases and inflammatory pathologies being attributed to T_{RM} responses. This review will recount the path to discovering the importance of resident memory CD8 T cells as they pertain to cancer immunity. In addition to highlighting key studies that directly implicate T_{RM} cells in anti-tumor immunity, we will highlight earlier work that implicitly suggested their importance. Informed by studies in infectious disease models, and instructed by a clear role for T_{RM} cells in autoimmunity, we will discuss strategies for therapeutically promoting T_{RM} responses in settings where they don't naturally occur.

Keywords: T_{RM}, TIL, CD103, T_{CM}, melanoma, vitiligo, immunotherapy, biomarker

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INTRODUCTION

Cancer can be considered a disease of immune dysfunction, with a failure of immune recognition leading to the outgrowth of malignant cells as tumors (1). Tumor development has been said to occur in three distinct steps: inefficient elimination of early transformed cells, development of a state of equilibrium between tumor cells and immune cells, and tumor escape from immune pressure (2). While innate immune cells are important for early tumor immune surveillance, T cells are fundamentally recognized for their crucial role in the antigen-specific recognition and elimination of malignantly transformed cells (2). Indeed a wealth of studies from humans and mouse models establishes a particularly potent role for CD8 T cells in controlling the outgrowth of malignancies (3).

The recent success of T cell immune checkpoint inhibitor (ICI) therapies for cancer has revealed CD8 T cells as potent mediators of immunity against advanced cancers (4, 5). Following effective priming in lymph nodes, T cells traffic to tumors and other peripheral tissues. In a growing number of cases, CD8 T cells have been shown to mediate the regression of large bulky tumors, resulting in durable long-term disease remissions (5). The persistence of such responses is fundamentally thought to be based on the ability of T cells to act as potent effectors and, subsequently, generate long-lived memory (6). T cell memory is antigen-specific, and can provide durable host-wide protection. As the field of cancer immunotherapy advances rapidly, it is now crucial to understand how the dissemination and maintenance of tumor-specific T cells can be optimally achieved.

Studies in infectious disease models have provided a wealth of information regarding memory CD8 T cell generation and localization. Classical definitions of memory T cells derive from the belief that T cells localize and recirculate predominantly throughout the blood and secondary lymphoid organs (7, 8). Such memory T cells were traditionally defined as being comprised of both central memory (T_{CM}) and effector memory (T_{EM}) subsets (9). T_{CM} cells were shown to persist and recirculate through the blood, bone marrow, lymph nodes, and spleen; whereas T_{EM} cells were shown to recirculate predominantly through blood, and peripheral tissues (10). In the early 2000's, large and persistent populations of antigen (Ag)-specific CD8 T cells in peripheral tissues were initially classified as T_{EM} cells in recirculation from the blood (8, 11).

These early classifications of T cell memory were quickly brought to bear on the question of what T cell subset provides the best immunity against cancer. A series of mouse adoptive T cell therapy studies published a decade ago showed that *in vitro* activated melanoma Ag (gp100)-specific T_{CM}-like CD8 cells have a greater ability to control established melanomas in comparison with clonally-identical T_{eff}/T_{EM}-like cells (12, 13). Subsequent work in humans identified a third major subset of memory T cells known as stem cell-like memory (T_{SCM}) cells (14). This less-differentiated T cell subset was capable of generating both T_{CM} and T_{EM} cells, and was shown in adoptive immunotherapy studies to have even greater anti-melanoma potency as compared with T_{CM} cells (15, 16). However, these early studies relied on definitions of memory that had been generated from a myopic focus on blood and lymphoid tissues. The concept that tumor-specific T cells could persist in peripheral tissues and tumors, without recirculation from the blood, was not yet being seriously considered.

Studies in viral models have now revealed a distinct lineage of memory T cells that resides in peripheral tissues and can provide orders of magnitude stronger protection than their T_{CM} cell counterparts (17). It is now recognized that peripheral host cells are surveyed overwhelmingly by T_{RM} cells that vastly outnumber their recirculating counterparts in peripheral tissues (18). The role of these tissue-resident memory (T_{RM}) cells in immune responses against cancer is only beginning to be explored. However, early studies have revealed that T_{RM} cells are induced by vaccination, present in human tumors, and sustained by the same molecular mechanisms that were defined by infectious disease models. As the concepts of tumor immunity and autoimmunity remain closely linked, a better understanding of T_{RM} responses to cancer has also provided new insights regarding a role for T_{RM} cells in autoimmune disease. In turn, lessons regarding T_{RM} responses in autoimmune disease have begun to inform the field of tumor immunotherapy.

The goal of this review is to discuss new advances in our understanding of resident-memory T cells as they pertain to cancer immunity and associated autoimmunity. In addition to discussing recent studies that have directly implicated T_{RM} cells in anti-tumor immunity, we will highlight key early studies that implicitly suggested a contribution from T_{RM} cells before their existence was known. As the field has grown out of studies in infectious diseases, we will draw heavily on such models in forming the groundwork for studies in cancer. The focus of this article will be on CD8 T_{RM} cells as key mediators of the anti-tumor response, but not to imply an unimportant role for CD4 T cells. While CD4 T_{RM} cells have been described in multiple infectious disease settings (19), their role in immunity to cancer remains as yet undefined.

FEATURES OF T_{RM} CELLS IN INFECTIOUS DISEASE MODELS

CD8 T_{RM} cells are defined based on their long-term persistence in peripheral tissues without recirculation from the blood. Since the earliest discovery of extra-lymphoid memory T cells in peripheral tissues of mice infected with vesicular stomatitus virus (VSV), and listeria monocytogenes (LM) infections (11), T_{RM} responses have been documented in response to a myriad of infections including lymphocytic choriomeningitis virus (LCMV) (20, 21), herpes simplex virus (HSV) (20, 22, 23), chlamydia (24), influenza (23, 25), vaccina virus (VACV) (17), human immunodeficiency virus (HIV) (26), tuberculosis (TB) (27), mouse cytolomegalovirus (MCMV) (28), and human papilloma virus (HPV) (29, 30). Thus, the formation of T_{RM} responses upon productive host infection can be viewed as a rule rather than an exception.

Phenotypic Features of T_{RM} Cells

As a unique memory T cell lineage, CD8 T_{RM} cells can be distinguished from other T cell subsets based on their cell surface phenotype. Like all memory T cells, T_{RM} cells are differentiated from naïve T cells based on their expression of CD44; a marker of antigen experience (31). T_{RM} cells also lack expression of CD62L (L-selectin); which differentiates them from naïve T cells and T_{CM} cells that require CD62L for entry into secondary lymphoid organs (10). To distinguish T_{RM} cells from effector and T_{EM} cells, more detailed phenotypic considerations are necessary, and tissue retention markers; most notably CD103 and CD49a (VLA-1) are typically used. CD103 is a TGF-β induced molecule that promotes T_{RM} cell tissue retention by binding to e-cadherin, which is expressed on normal host epithelial cells (32). CD49a promotes tissue retention and survival through binding to collagenase type IV (33). While studies have largely focused on

CD103 and CD49a as markers of tissue residency, it is important to note that their expression is not absolute, nor required. CD103 expression has been shown to be dispensable for T_{RM} formation in the liver (34) and gut (35), and CD49a is dispensable for T_{RM} formation in the lung (27). Additional retention markers have been used to identify T_{RM} cells, such as cutaneous leukocyte antigen (CLA) in the skin (36, 37) and LFA-1 in the liver (38). Thus, while T_{RM} cells are exclusively $CD44^{hi}$ and $CD62L^{low}$ they can express an array of tissue-specific retention markers, with no single marker being sufficient to definitively identify a T_{RM} population.

Another key phenotypic feature of T_{RM} cells is the expression of CD69, a marker of T cell activation, which blocks T cell expression S1PR1 (20). CD69 thus promotes tissue retention and residency by interfering with the ability to sense the S1P gradient that is essential for tissue egress (39, 40). Similarly, T_{RM} cells typically lack the expression of CCR7, which cooperates with S1PR1 for tissue egress through lymphatic vessels. However, as with CD103 and CD49a, CD69 can be dispensable for T_{RM} formation (18) and has been shown to be non-definitive in distinguishing recirculating T cells from T_{RM} cells in the steady state (41). In humans, a role for CD69 may be more pronounced than in mice, as peripheral tissue T_{RM} cells in healthy individuals overwhelmingly expressed (42).

T_{RM} cells occupy a unique niche in their tissue of residence, and take on a dendritic morphology that is uncharacteristic of circulating memory T cells (43). T_{RM} cells continually scan the peripheral tissue where an initial insult occurred (44), exhibiting limited migratory ability, and tending to accumulate at sites of antigen persistence (45). T_{RM} cells adapt well to their surroundings by exploiting the features of tissues in which they reside. In skin, T_{RM} cells have been shown to cluster around niches formed by keratinocytes, near hair follicles, which secrete IL-15, IL-7 (46), and TGF-β (47). In anatomical regions with a high tissue turnover rate, such as the lamina propria of the gut, immune cells such as macrophages support the formation of T_{RM} aggregates (48). In other barrier tissues, T_{RM} cells occupy *de novo* niches, such as repair-associated memory depots (RAMD) in the lung, and mucosa-associated lymphoid tissue (MALT) in the female reproductive tract (FRT) (49, 50). Localization to barrier sites of mucosal tissues exerts a metabolic burden that typically limits the persistence of T cells. However, T_{RM} cells utilize fatty acid beta-oxidative phosphorylation to support their longevity (51–53). In contrast to conventional memory T cells which conduct their own fatty acid synthesis, T_{RM} cells in the skin have been shown to express high levels of fatty acid binding proteins FABP4 and 5, to facilitate the necessary uptake of fatty acids (51). These properties of T_{RM} cells enable them to function in diverse peripheral tissue niches.

Transcriptional Profiles of T_{RM} Cells

Transcriptional profiling has demonstrated that T_{RM} cells are distinct from their T_{CM} and T_{EM} counterparts, and thus represent a unique T cell lineage (20, 54). Although unique genes define T_{RM} subpopulations in different tissues (20), a core transcriptional signature has been proposed for T_{RM} cells (54, 55). This signature highlights the distinctive nature of T_{RM} cells as a hybrid between effector and memory cells, which are armed in an effector like-state even during quiescence (54, 55).

Master transcriptional regulators of T_{RM} cell differentiation across multiple tissue types include Hobit, Blimp1 (42), and Runx3 (54). In contrast, *Tbx21* (T-bet) and *Eomes*—the master regulators of effector and lymphoid memory T cell lineages—have been shown to impede the development of T_{RM} cells (56). Hobit and its homolog Blimp1 act in synergy as negative regulators of tissue egress, by directly binding to *S1pr1*, *Ccr7,* and *Tcf7* motifs in mice (55). Additionally, Blimp-1 has been shown to initiate cytotoxic function while Hobit maintains deployment-ready cytotoxicity in T_{RM} cells (57). On the other hand, Runx3 acts to promote the expression of T_{RM} tissue retention markers such as CD103 and CD69 (54). In addition to these canonical T_{RM} transcription factors, the NR4A family has also been shown to be highly upregulated in T_{RM} cells, with the absence of *Nr4a1* resulting in a reduced capacity to generate T_{RM} (58).

While most in-depth transcriptional analyses have, to date, been conducted in murine infectious disease models, transcriptional characteristics of T_{RM} cells from humans are also beginning to be reported. CD8 T_{RM} cells from human lungs showed high CD69 expression, and variable CD103 expression (59). These lung T_{RM} cells could be distinguished from their circulating counterparts by high levels of *GZMB*, *IFNG*, *TNF, and NOTCH1* transcripts, with NOTCH signaling shown to promote *IFNG* gene expression (59). Separate studies showed that $CD69^{+}$ memory T cells across multiple tissues of human cadavers exhibit a conserved transcriptional profile including *ITGA1* (CD49a), *ITGAE* (CD103), and *PDCD1* (PD-1) expression (42). In contrast to mouse T_{RM} cells however, human cells lacked expression of *ZNF683* (HOBIT*)* and *PRDM1 (*BLIMP-1*)* (42). It is important to note that T_{RM} cell transcriptional signatures have been largely generated from pooled T cell samples, thus lacking single cell resolution and missing the complexity and heterogeneity that potentially exists within a T_{RM} cell pool. Single-cell RNA-sequencing of T_{RM} cells from mice and humans may, in the future, reveal heterogeneous CD8 T_{RM} cell subsets.

Protective Function of T_{RM} Cells

T_{RM} cells have been shown to play a dominant role in protection against peripheral infections, in some cases mediating orders of magnitude stronger protection than lymphoid memory T cells (17). However, because infections generate both resident and circulating memory T cell compartments, specialized techniques have been needed to isolate the contribution of T_{RM} cells from that of their lymphoid memory counterparts (60). FTY720 is a small molecule S1PR antagonist that inhibits T cell egress from lymph nodes, and thereby prevents circulating memory T cell subsets from accessing peripheral tissues (61). In mice infected by VACV-OVA through skin scarification (s.s.), it was shown that treatment with FTY720 had no effect on protection against cutaneous viral re-challenge, indicating that skin T_{RM} cells are sufficient for long-lived protection (17). In the setting of influenza viral infection, a protective role for lung T_{RM} cells was also first established by studies involving FTY720 treatment (62). Low dose monoclonal antibody (mAb) depletion strategies can also be employed based on their ability to efficiently deplete circulating

and lymphoid T cells, while sparing tissue-resident T cell niches (49). Using this technique, HSV skin infection was shown to generate long-lived protective immunity that was unperturbed by the depletion of circulating memory T cells (20). Lastly, the surgical joining procedure known as parabiosis, has been used to isolate the contribution of T_{CM} from that of T_{RM}. Parabiosis allows the equilibration of circulating immune compartments between immune and naïve mice, thus transferring circulating memory to a naive recipient (8). Through elegant parabiosis studies in conjunction with the use of FTY720, skin $CD8^+$ T_{RM} cells were shown to be superior to T_{CM} in protecting against cutaneous VACV re-infection (17). These fundamental studies established a crucial role for T_{RM} cells in mediating long-lived protection against peripheral infections.

RESIDENT MEMORY T CELL RESPONSES TO CANCER

The above characteristics of T_{RM} cells have, more recently, been recognized for their relevance to cancer immunity. Indeed, as infections occur in peripheral tissues, so do cancers arise in the same tissues. As such, it stands to reason that populations of tumor-specific T_{RM} cells can occupy tumors themselves, and the tissues from which they arise (**Figure 1**). Despite this, the role of T_{RM} cells in mediating immunity to cancer has only recently been described.

Prior to the term "resident memory," a growing body of literature had already identified CD103 expressing CD8 T cells within human tumors, and linked these cells to improved prognosis. With CD103 now recognized as a common T_{RM} cell marker, these studies can be viewed as the earliest evidence of tumor infiltrating lymphocytes (TILs) having T_{RM}-like properties. Importantly, these findings formed the ground work for the extensive characterization of T_{RM} cell transcriptional signatures in a variety of human tumors, and mechanistic mouse work to establish a crucial role for this memory T cell subset in immunity to cancer.

Identification of $CD103^+CD8^+$ TILs

Studies examining CD103 expression on tumor infiltrating lymphocytes date back 30 years, with an initial focus on the identification of cellular localization patterns within tumors. A 1988 analysis was the first to identify CD103-positive tumor infiltrating lymphocytes (TILs) (63). These studies found that a majority of TILs in gastrointestinal tumors were positive for CD103 (at the time referred to as HML1), and that these cells were localized throughout the tumor mass (63). A decade later, studies involving pancreatic cancer patients revealed that approximately 20% of all CD8 T cells in tumors expressed CD103 as well as CD45RO, a human memory T cell marker (64). Unlike the prior study (65) these studies showed $CD103^+$ T cell aggregation in fibrous stromal tissue, and exclusion from tumor cell nests (64). Tumor-excluded T cell distribution was further supported by a 2001 analysis of bladder cancer samples in which the majority of $CD103^+CD8^+$ TILs were found on the periphery of the tumor, potentially suggesting immune failure (66). However, a 2003 study reported high levels of $CD103^+CD8^+$ T cells infiltrating intratumoral regions of microsatellite-unstable colorectal cancers (65). Later work revealed that $CD103^+$ TILs were not limited to tumor masses, and could also be found in the ascites fluid of high-grade serous ovarian cancer (67). This early collection of studies established CD103 expression on $CD8^+$ TILs in various solid cancers, but indicated their variable distribution throughout the tumor microenvironment (TME).

With CD103 substantiated as marker of tumor-infiltrating CD8 T cells, several early studies also sought to define the function of CD103 in the interaction between T cells and tumor cells. Attempts at defining these interactions focused predominantly on two molecules: TGF-β, as an inducer of CD103 expression (68, 69), and e-cadherin, as the only known binding partner for CD103 (70). An early study showed that TGF-β-induced CD103 expression significantly enhanced the lysis of e-cadherin-transduced pancreatic cancer cells by CD8 T cells *in vitro* (71). This was found to occur by increasing the adhesion of CTL's to tumor cells, a process which depended on the expression of both CD103 and e-cadherin (71). However, it remained unknown whether such interactions occur in response to tumor cell encounter, and whether Ag recognition plays a role in CD103 induction. Subsequent studies involving human HER2/NEU-specific T cell clones revealed that cognate antigen and TGF-β were both required for the *in vitro* upregulation of CD103 on T cells (72). Furthermore, a human colon cancer cell line was capable of secreting enough TGF-β to promote T cell expression of CD103 upon Ag-recognition *in vitro*, suggesting that tumor-derived TGF-β could induce CD103 expression on TILs (72). This study further showed that T cells exposed to TGF-β during priming, will re-express CD103 more readily upon recall (72). Follow-up studies in a human lung cancer xenograft mouse model showed that CD103 is induced upon T cell trafficking to tumors, and that *in vivo* neutralization of TGF-β impairs the recruitment of $CD103^+$ CD8 T cells into the TME (73). These studies together substantiated the role of tumor-derived TGF-β in the induction of CD103 on intratumoral T cells.

While CD103 upregulation on TILs was shown to be TGF-β dependent, the mechanism governing this process in cancer still remained unclear. In 2014, it was shown that TGF-β induces Smad2/3 phosphorylation which allows nuclear translocation and binding to the proximal regulatory elements of CD103 (74). Indeed pSmad2/3 nuclearization was recently used as a marker of $CD103^+$ TIL's in human cervical cancers, in which TGF-β signaling is abundant (75). The key role that TGF-β plays in $CD103^+$ TIL biology was further validated using a tumor-specific human T cell clone, by showing that binding of TGF-β to its receptor promoted the recruitment and phosphorylation of integrin-linked kinase (ILK) to the CD103 intracellular domain, inducing integrin inside-out signaling that may further promote T_{RM} cell migration and function (76, 77).

Subsequent studies have also shed light on the putative role of CD103/e-cadherin interactions in promoting tumor cell killing. *In vitro* studies used CD103 expressing tumor-antigen specific T cell lines from lung cancer patients to demonstrate that e-cadherin expression was required by tumor cells for

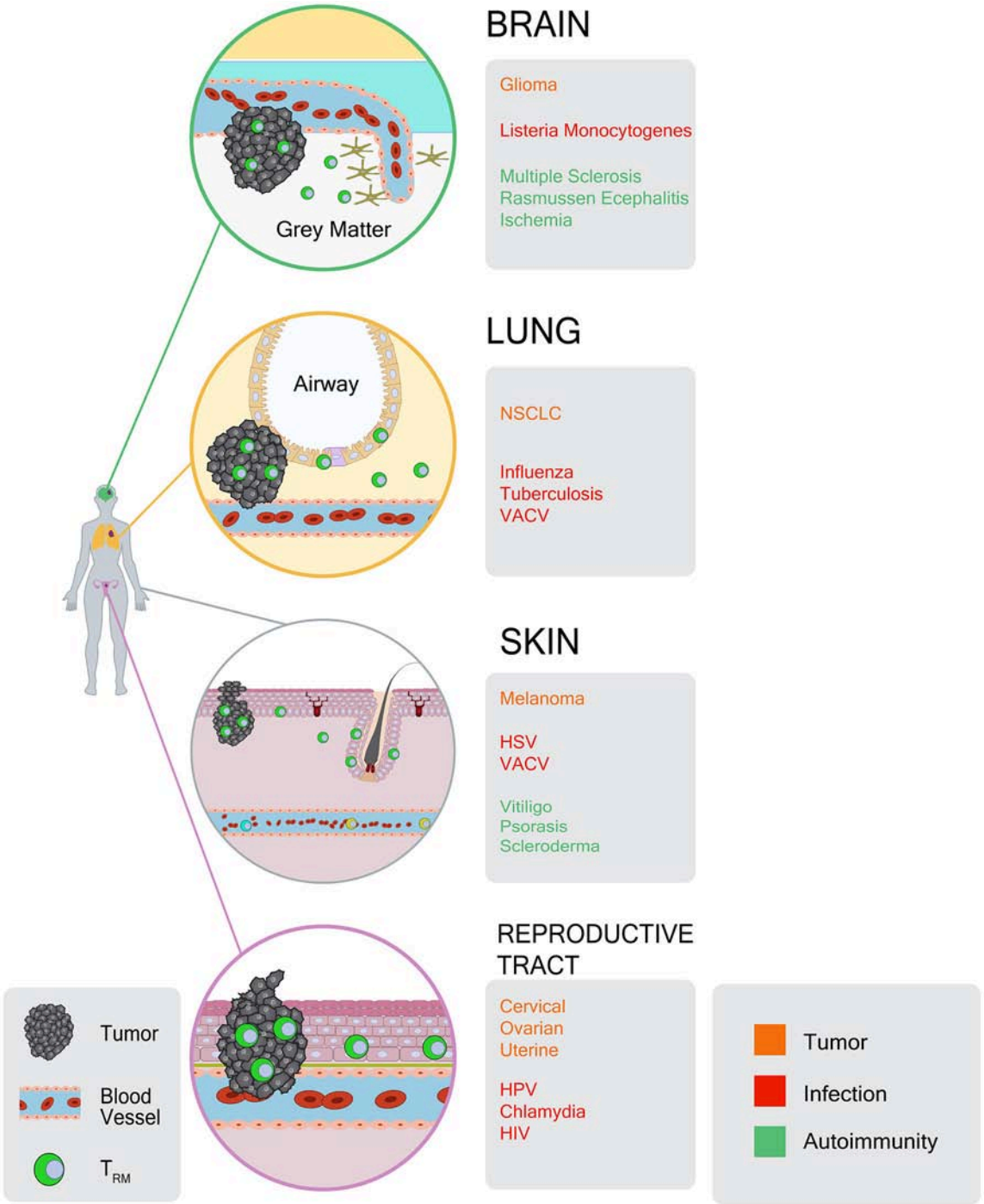

FIGURE 1 | Atlas of T_{RM} responses in peripheral tissues and tumors**.** $CD8^+$ T_{RM} cells occupy a diverse array of tissues and tumors. For each tissue location, gray boxes indicate: (1) tumor types in which T_{RM}-like TILs have been identified (orange), (2) infections that give rise to T_{RM} responses (red), and (3) autoimmune or inflammatory conditions in which T_{RM} cells have been identified (green).

effective killing via granule polarization and exocytosis (78). It was separately shown that CD103 ligand engagement together with TCR binding, enhances the strength of human TIL/tumor cell interactions (79). CD103 engagement with e-cadherin was found to shape immunological synapse morphology, which was essential for the polarization of cytokine and lytic granules containing granzyme B and IFN-γ (79). A co-stimulatory role for CD103 was subsequently established by showing that CD103

triggering promotes phosphorylation of ERK1/2 kinases and phospholipase Cγ1 (PLCγ1)—a process that was required for cytotoxicity by tumor-specific human T cells (80). CD103-downstream signaling in TILs was separately shown to require paxillin phosphorylation and binding to the CD103 cytoplasmic tail, potentiating effector function against cognate lung tumor cells (81). Thus, ligation of CD103 has been proposed to enhance tumoricidal T cell functions *in vivo*.

While the above studies employed e-cadherin-expressing tumor cell lines and/or transfectants, it should be noted that the expression of e-cadherin is often lost on epithelial cells during malignant transformation and tumorigenesis (82). This may suggest that CD103-ecadherin interactions are unlikely to play a major role in the TME. This is underscored by analyses of bladder (66), ovarian (83), lung (84), and pancreatic cancer (85) specimens showing either a lack of co-localization between CD103-positivie TILs and e-cadherin expressing tumor cells, or a lack of correlation between e-cadherin expression levels and $CD103^+$ TIL infiltration. However, some studies do report the contrary (86). Interestingly, CD103 has been shown to have unknown binding partners in peripheral tissue (87, 88), and one could speculate that such partners function in engaging CD103 on CD8 T cells in e-cadherin negative tumors, or negative regions within tumors. Alternately, following upregulation of CD103 by tumor-derived TGF-β, this integrin might serve as a marker of the T_{RM} transcriptional program, rather than a functional player that engages its ligand in the TME.

Prognostic Significance of $CD103^+CD8^+$ TILs

While early studies confirmed the existence of $CD103^+CD8^+$ TlLs in solid tumors, evidence that these cells had prognostic value for patients did not appear in the literature until 2014, when it was shown that this subset is strongly associated with survival in high-grade serous ovarian carcinoma (HGSOC) (83). In this study, $CD103^+$ TILs were clearly localized to intratumoral regions, as opposed to associated tumor stroma. Interestingly, the presence of $CD8^+CD103^-$ cells conferred no benefit when compared to tumors devoid of all CD8 T cells, suggesting that the $CD103^+CD8^+$ subset dominates the protective response in HGSOC (83).

Studies followed in several tumor types, all demonstrating correlation between $CD103^+CD8^+$ cells localized in tumor nests (also referred to as "intraepithelial" regions), and improved patient prognosis. In urothelial (bladder) cancer, high intratumoral $CD103^+CD8^+$ TIL density was inversely correlated with tumor size and could be used to predict improved overall survival (86). Similarly, a large breast cancer study demonstrated that $CD103^+$ TIL infiltration to intratumoral (but not stromal) regions of tumor masses was prognostic of survival in a basal-like subtype (89). Investigation of endometrial adenocarcinoma showed that CD103 expression clearly delineated CD8 T cells localized to intratumoral regions (as opposed to stromal regions), and was an independent predictor of improved survival, particularly for high risk disease (90). Intraepithelial CD103-positive TILs were separately showed to be a valuable biomarker for therapeutic response in cervical cancer patients undergoing chemotherapy and radiation therapy (91). In contrast to earlier studies, these studies all supported CD103 as a marker of T cells infiltrating tumor nests as opposed to stroma. Importantly, these studies highlighted the superior prognostic strength of $CD103^+CD8^+$ TILs as compared with total $CD8^+$ TILs.

Several studies have also supported this association for lung cancer. Early work showed that total $CD103^+CD8^+$ TILs have prognostic value in non-small cell lung cancer (NSCLC) patients with regards to early disease-free survival (92). Further subsetting of NSCLC patients showed that high numbers of $CD103^+$ TILs in tumor nests were an independent predictor of disease-free survival for patients with pulmonary squamous cell carcinoma (84). Interestingly, this study also reported a strong correlation between smoking status and increased density of $CD103^+$ TILs (84). A separate study of human NSCLC tumors showed that CD8 T cells in intratumoral regions were more likely to express CD103 than those in stromal regions, which was again highly predictive of survival (93). Further validation of CD103 as a biomarker was provided through stratification of NSCLC patients in The Cancer Genome Atlas (TCGA) database, which showed that patients with high tumor *ITGAE* (CD103) expression have improved overall survival (93).

For pancreatic cancer, the association between CD103 expression and prognosis is less straight-forward. Whereas high numbers of total CD8 T cells predicted improved prognosis in pancreatic ductal cell adenocarcinoma (PDAC), $CD103^+CD8^+$ TIL numbers did not predict survival, nor did $CD103^+CD8^+$ TILs in intratumoral regions (85). Interestingly though, a high ratio of $CD103^+$ TILs in intratumoral vs. stromal locations was predictive of prognosis, potentially indicating the importance of spatial relationships between T_{RM}-like cell subsets in PDAC (85). Indeed T_{RM} cell localization to intratumoral regions would appear to situate them for optimal tumor control, although $CD103^+$ TIL subsets may not be prognostic in all tumor types.

Phenotypic Characteristics of T_{RM}-Like TILs

More detailed phenotypic analyses of $CD8^+CD103^+$ TILs have revealed additional T_{RM}-like characteristics of these cells. Investigation of HGSCOC, endometrial adenocarcinoma, and ovarian cancer, all showed that $CD8^+CD103^+$ TILs express high levels of the exhaustion marker PD-1 (75, 83, 90). CD8 T cells in pediatric glial tumors exhibited a $CD45RO^+$ $CD69^+$ $CCR7^-$ T_{RM}-like phenotype, in addition to multiple inhibitory checkpoints including PD-1, PD-L1, and TIGIT (94). Detailed flow cytometric and qPCR analyses of $CD103^+CD8^+$ TILs from lung cancer patients also revealed high levels of *PDCD1* (PD-1) and *HAVCR2* (TIM3), and low levels of the tissue egress marker *S1PR1* (92). Using an immunofluorescence technique to visualize T_{RM} in NSCLC, co-expression of CD49a (VLA-1) was identified on $CD103^+CD8^+$ T cells (93). This study also showed elevated levels of PD-1 and TIM-3 on $CD103^+CD8^+$ TILs as compared to CD103-negative TILs. Accordingly Cy-TOF analysis of melanoma-infiltrating T cells showed that a $CD69^+$ subset (among which ∼50% expressed CD103), co-expressed

high levels of inhibitory checkpoint molecules CTLA-4 and PD-1 (95). Many of these studies have thus hypothesized that T_{RM}-like TILs are key targets of immune checkpoint inhibitor therapy.

It is important to note that inhibitory checkpoint molecules, while overwhelmingly expressed on T_{RM}-like TILs, have demonstrated somewhat more variable expression on T_{RM} cells in peripheral infection models. PD-1 is expressed on $CD103^+CD8^+$ brain T_{RM} cells in response to listeria monocytogenes, MCMV, and VSV infection (96, 97). Interestingly, upregulation of PD-1 on brain T_{RM} cells is independent of chronic antigen stimulation or inflammation (98). However, a study of VSV infection found PD-1 expression absent on T_{RM} cells in the brain (99). In the skin, HSV infection induces T_{RM} cells that express PD-1 (20, 100), and CTLA-4 (20). However, VACV infection produced PD-1-negative T_{RM} cells in the skin (17), and LCMV infection induced T_{RM} cells in the small intestine that lacked PD-1 (101). Thus, inhibitory checkpoint molecule expression does not appear to be a defining feature of T_{RM} cells in peripheral infection models. Moreover, it remains unclear if PD-1 expression levels differ between $CD103^+$ cells in normal peripheral tissues and those in tumors.

Considering the widespread expression of inhibitory checkpoint molecules on $CD103^+$ TILs, it has remained unclear if these cells represent true T_{RM} cells or, rather, exhausted TILs that simply express CD103 in the TGF-β rich TME. Indeed no studies as-yet have demonstrated the long-term persistence of $CD103^+$ CD8 T cells in tumors, without ongoing input from circulation—the true hallmark of a T_{RM} response. In HGSCOC tumors it was further shown that $CD103^+$ TILs have phenotypic characteristics of varying T cell differentiation states including T_{CM}, T_{EM}, and T_{RM} cells (75), which may suggest that these cells are replenished from circulation. In the next section we will highlight several studies that transcriptionally profiled $CD8^+$ TILs to generate a more comprehensive profile of their gene expression signature. These results, although varied across different tumor types, collectively support the conclusion that subsets of TILs are regulated by T_{RM} transcriptional programs.

Transcriptional Profiles of T_{RM}-Like TILs

Transcriptional profiling of TILs from NSCLC tumors has provided new insights into the characteristics of T_{RM}-like TILS that extend beyond lung cancer. NSCLC tumors with a high TIL infiltration score were shown to have more pronounced gene expression characteristics of T_{RM} cells including higher transcript levels for *ITGAE (CD103)*, *CD69*, *ITGA1 (*CD49a*)*, *CXCR6*, *PDCD1 (PD-1), HAVCR2* (TIM3), *LAG3*, and *TIGIT*, but lower expression of *KLRG1, CCR7, SELL (*CD62L), and *S1PR1* (102). Even among tumors with high CD8 T cell density, high expression of CD103 conferred patient survival advantage (102). Focusing on $CD103^{hi}$ CD8 TILs, this study also identified elevated expression of components of the NOTCH signaling pathway, as well as CD39, the cell surface ectonucleotidase that dephosphorylates ATP (102). This $CD103^+CD39^+$ TIL subset was the focus of a subsequent study that identified this populations in head and neck squamous cell carcinoma (HNSCC), melanoma, HNSCC, ovarian, lung, and rectal cancer tumors (103). In HNSCC patients, the $CD103^+CD39^+$ TIL population was found to be a better predictor of survival as compared with CD39 and/or CD103-negative CD8 T cell subsets (103). Transcriptional analysis of this sorted $CD103^+CD39^+$ subset from five tumors (HNSCC and ovarian) revealed enrichment of gene transcripts associated with exhaustion and reduced expression of T cell recirculation associated genes, suggesting that $CD103^+CD39^+$ TILs may have the most pronounced T_{RM}-like character in tumors (103).

TIL characterization through bulk RNA-sequencing provides a wealth of transcriptional data, but obstructs the detection of small heterogenous populations within the TME. On the other hand, single-cell RNA sequencing (scRNA-seq) allows for fine resolution of TIL sub-populations at a cost of failing to detect poorly expressed transcripts. Indeed scRNA-seq of over six thousand TILs isolated from two patients with triple negative breast cancer (TNBC) showed at least four differential clusters of CD8 T cells, all expressing CD69 (104). One TIL cluster, identified based on high CD103 expression, was shown to express low levels of transcripts for *KLRG1* and *SELL* (CD62L), as well as tissue egress related genes, *KLF2* and *S1PR1* (104). This TIL subset also expressed high transcript for inhibitory checkpoint genes *HAVCR2*, *PDCD1, CTLA4, TIGIT,* and *LAG3*, and cytotoxicity-related genes *GZMB* (granzyme) and *PRF1* (perforin). Coupled with bulk RNA-seq data on sorted TIL populations, this study showed that $CD8^+CD103^+$ TILs in breast cancer exhibit multiple features of T_{RM} differentiation. Importantly, this T_{RM}-like TIL gene signature was predictive of survival in TNBC patients from the METABRIC consortium, and could be used to distinguish melanoma responders to ICI therapy (104).

Further highlighting the heterogenicity of the TIL population, another study sequenced >12,000 TILs from twelve NSCLC tumors and identified seven clusters with one expressing the T_{RM} specific transcription factor, *ZNF683* (*HOBIT*) (105). In contrast to the above study in TNBC, HOBIT-expressing lung T_{RM}-like TILs expressed low levels of CD103 and showed reduced expression of PD-1 as compared to other CD8 clusters, suggesting a unique transcriptional program in NSCLC T_{RM}-like TILs (105). As in TNBC, the scRNA-seq derived gene signature was used to stratify patients in a TCGA lung adenocarcinoma (LUAD) dataset, to show that enrichment of a CD8-ZNF683 gene-signature is predictive of survival when compared to other TIL derived signatures (105).

Taken together, the above studies highlight variability in T_{RM}-like TIL gene signatures in cancer. Discrepancies likely arise due to unique tumor microenvironmental factors associated with individual patients, potentially relating to tumor tissue of origin, stage, and mutational status and/or burden. In comparing T_{RM}-like TILs to bona fide T_{RM} cells in normal peripheral tissues, it is also important to note that tumors, by their nature, lack many of the structural and molecular features of normal tissues that provide a hospitable niche for T_{RM} cells. Thus, one might expect that T_{RM}-like TILs would never perfectly match T_{RM} cells that reside in the normal tissue counterpart of a tumor. Despite this, the discovery that TILs with features of T_{RM} cells portend improved patient survival across multiple tumor types,

underscores the importance of these cells, and represents a key recent advance in the field of cancer immunology.

Role of T_{RM} Cells in Mediating Immunity to Cancer; Lessons From Mouse Models

While the above studies provide strong correlative associations between T_{RM} cells and improved patient survival, until very recently, formal evidence that T_{RM} cells can mediate immunity to cancer was lacking. In retrospect, preclinical cancer vaccine studies published 15 years ago, inferred the crucial contribution of a long-lived, tissue-localized T cell population, however the importance of resident memory had not yet been recognized. This section will illustrate the path to our discovery of a role for T_{RM} cells in mediating durable anti-tumor immunity.

Early Indications of T_{RM} Cells From Studies of Cancer Vaccination Route

One of the earliest studies to infer T_{RM} cell responses to cancer, published in 2003, revealed that melanoma-specific memory CD8 T cells distribute to distinct tissue locations, depending on the route of vaccination (106). By administering a peptide-pulsed dendritic cell (DC) vaccine via various routes, it was shown that only subcutaneous vaccination could reliably protect against a subcutaneous melanoma tumor rechallenge (106). This concept of regionally localized tumor-specific CD8 T cell memory was revolutionary at the time, although lymphoid tissues remained the focus of this early work. As such, tumor protection in these mice was attributed to memory populations in local lymph nodes, rather than in peripheral tissues (106).

Subsequent work extended the concept of tissue-localized tumor immunity, while further demonstrating that tumor-specific CD8 T cells could localize to peripheral tissue. In a 2010 study, mice infected through various routes with recombinant vaccinia virus (rVACV) expressing $OVA_{257-264}$, were challenged with an OVA-expressing B16 melanoma cell line 6 weeks later (107). Reminiscent of the 2003 study, optimal protection against dermal melanoma rechallenge was only afforded by prior infection in the skin (107). This study further identified OVA-specific T cells in the skin, and referred to them as "skin-resident T_{EM} cells" (107). While it was inferred that such T_{EM} cells could mediate tumor protection in the skin, no formal experiments were performed to isolate the effects of this tissue-resident population from those of the lymphoid memory T cell compartment, both of which were present in tumor-bearing mice.

The field again approached this concept in 2013, with studies showing that growth of orthotopic head and neck TC1 tumors (implanted in the tongue) could only be inhibited when prior vaccination had been delivered via the intranasal (i.n.) route (108). In this study, i.n. vaccination with Shiga toxin B subunit fused to the HPV16-E7 tumor/viral peptide (STxB-E7) gave rise to $E7_{39-47}$-specific $CD8^+$ T cells in mucosa-draining lymph nodes. These tumor-specific T cells expressed CD103 and CD49a however, in contrast to the 2010 study, T cell responses were not analyzed in peripheral (mucosal) tissue itself. Interestingly, mAb-mediated blockade of CD49a was shown to block CD8 T cell infiltration into TC1 tumors during acute STxB-E7 treatment, indicating CD49a as an important determinant of T cell infiltration into tumors (108). One other report in 2016 similarly showed that *in vivo* mAb-mediated blockade of CD49a or CD103 significantly impaired the control of subcutaneous B16-OVA tumors, supporting important roles for these molecules in T cell mediated anti-tumor immunity (109). While these studies revealed important new concepts regarding vaccine route and markers of tissue (or tumor) residence, the question of whether T_{RM} cells directly mediate tumor protection remained open.

A Key Role for T_{RM} Cells in Mediating Anti-tumor Immunity

Several notable studies in the past 2 years have now definitively demonstrated a role for T_{RM} cells in mediating immunity to cancer. Importantly, each of these studies employed techniques originally used in infectious disease models, to isolate the contribution of T_{RM} cells from that of the lymphoid memory compartment. In doing so, these studies illustrate a definitive role for T_{RM} cells in providing long-lived protection against multiple tumor types, and in various tissue locations.

In follow-up work to the 2014 study involving STxB-E7 vaccination, it was shown that intranasal vaccination indeed generates a large pool of Ag-specific memory T cells in lung mucosal tissue (93). This was in clear contrast to intramuscular vaccination, which generated effector-like T cells in the spleen. E7-specific CD8 T cells in the lung expressed T_{RM} markers including CD103 and CD49a, that were absent on T cells in the spleen. Further transcriptomic analysis of E7 Ag-specific CD8 T cells from the spleen showed that they expressed higher levels of lymphoid homing and tissue exit markers (i.e., *Sell* and *S1pr1*) compared with lung, while lacking adhesion and retention markers. Three key experiments were conducted to implicate T_{RM} cells in the recall response against E7-expressing TC1 head and neck tumors. First, FTY720 was used to illustrate a minimal contribution of circulating T cells to tumor protection (93). Second, *in vivo* mAb-mediated TGF-β blockade was used to demonstrate a reduction in the generation of T_{RM} populations, in conjunction with significantly decreased tumor protection. Finally, parabiosis was used to demonstrate that vaccinated mice were protected against tumor rechallenge, while no protection was afforded to parabiosed naive mice. These studies thus convincingly showed a dominant role for E7-specific T_{RM} cells in protection against orthotopic head and neck cancer.

A thorough investigation of VACV-OVA vaccination route separately implicated T_{RM} cells as important players in anti-tumor immunity (110). Mice infected with VACV-OVA by the dermal, nasal, or peritoneal routes showed distinct patterns of antigen-specific T cell memory formation in circulation and in peripheral tissues (110). Using i.p. vaccination to generate circulating memory without resident memory, or FTY720 as a means for blocking T cell access to the skin, it was shown that either circulating or resident memory are sufficient for protection against B16-OVA re-challenge in the skin (110). Moreover, parabiotic transfer of circulating memory to naïve recipient mice conferred reduced tumor protection compared with vaccinated parabiotic donor mice, demonstrating that T_{RM}

cells are significant contributors to tumor immunity induced by viral vaccination (110).

This same year, our own work illustrated that T_{RM} cells in the skin are both necessary and sufficient for long-lived protection against B16 melanoma (111). Employing therapeutic depletion of regulatory T cells to break tolerance to melanoma differentiation antigens, followed by surgery to curatively excise residual B16 primary tumors, we identified the formation of tumor/self (gp100) Ag-specific CD8 T cells in the skin with a $CD44^{hi}$ $CD62L^{low}$ $CD103^+$ $CD69^+$ CLA^+ T_{RM} phenotype (**Figure 2**) (111). These tumor-specific T cells persisted for several months even following extended FTY720 treatment or upon skin grafting onto T cell deficient mice, indicating their true T_{RM} nature. Importantly, generation of this T_{RM} population in the skin depended on CD8 T cell expression of CD103 and Fut7 (the enzymatic determinant of cellular CLA production) (111). As Treg-depleted mice also generated memory T cell responses in lymphoid tissues, a requirement for T_{RM} cells in protection against B16 dermal re-challenge was shown by two methods. First, long-lived tumor immunity was shown to be unperturbed by the continual administration of FTY720. Second, genetic knockout of CD103 in the mouse CD8 T cell compartment, while having no effect on lymphoid memory generation, was shown to completely abrogate skin tumor protection. Thus, CD103-dependent T_{RM} cells in the skin were the sole mediators of long-lived immunity against the dermal B16 melanoma (111).

This study also extended a link between tumor immunity and autoimmunity formed by our earlier work that autoimmunity against normal melanocytes (i.e., vitiligo) maintains lymphoid memory T cell responses against melanoma/melanocyte shared antigens (112, 113). Indeed we found that T_{RM} cells only developed in the skin of mice with treatment-related vitiligo (111). Vitiligo was shown to be required for the seeding of gp100-specific T_{RM} precursors throughout pigmented and depigment skin, but preferentially in melanocyte-depleted hair follicles (111) (**Figure 2**). The concept of generating T_{RM} responses against tumor/self-antigens was also illustrated in studies involving intradermal DNA vaccination against gp100 (114). This study showed that vaccination induced the development of gp100-specific T_{RM} cells in the skin, in association with autoimmune vitiligo. Importantly T_{RM} cells (but not circulating memory T cells) were refractory to low dose anti-CD8 mAb depletion, a technique used to demonstrate that T_{RM} cells mediate long-lived protection against B16 tumor rechallenge (114). Thus, in the generation of protective T_{RM} responses to tumor antigens that are shared by normal tissues, autoimmunity clearly plays an important role.

These mechanistic studies in mouse tumor models, coupled with data from patient tumor TILs, now clearly affirm the relevance of T_{RM} cells to tumor immunity. Preclinical immunotherapy studies further illustrate that established methods for vaccinating against tumor antigens can be highly effective at generating T_{RM} responses to cancer (93, 110, 114). Reaffirming studies of 15 years ago, vaccination route is crucial for generating the proper T_{RM} responses to tumors in various tissue locations (106). Treg depletion also generates T_{RM} responses against shared tumor/self-antigens, likely relating to the role of Tregs in controlling peripheral tissue autoimmunity (111). While correlative data support the notion that immune checkpoint inhibitor therapies act on phenotypically exhausted T_{RM}-like TILs in solid tumors, such therapies have not yet been shown to induce *de novo* T_{RM} responses in cancer patients. Regardless, the knowledge that ∼20% of anti-PD-1 treated melanoma patients develop vitiligo (115) may imply that melanoma-specific T_{RM} responses are generated or awakened in such patients. Indeed, skin immune-related adverse events of ICI therapy have excellent prognostic value for melanoma patients (116), further underscoring the idea that autoimmunity supports T_{RM} responses to cancer. The next section will discuss a role for T_{RM} cells in mediating autoimmunity; a class of diseases that has long instructed the field of tumor immunology.

ROLE OF T_{RM} CELLS IN AUTOIMMUNE DISEASE

Skin Autoimmunity

Skin autoimmune diseases provide the most compelling evidence of T_{RM}-mediated pathogenic responses against self. In accordance with the above studies in melanoma, three recent studies have demonstrated melanocyte Ag-specific CD8 T_{RM} cells in vitiligo-affected patient skin (non-melanoma associated) (117–119). The first of these showed that vitiligo-associated T_{RM} cells display a $CD8^+CD103^+CD49a^+$ phenotype, and become localized to both the epidermis and dermis of lesional patient skin (117). These cells are highly functional based on their production of perforin, granzyme B, and IFN-γ upon *in vitro* restimulation (117). T_{RM} cells in vitiligo-affected skin also expressed the tissue homing receptor CXCR3 (118), consistent with prior reports that vitiligo is mediated by IFN-γ induced ligands for this receptor; CXCL9 and 10 (120). Additional phenotypic analysis of vitiligo-associated T_{RM} cells revealed the expression of CD122, the alpha chain of IL-15R (119); a classic memory T cell marker that supports T_{RM} populations in viral infection models (20, 46). Importantly, mechanistic studies in a T cell receptor transgenic (TCR Tg) CD8 T cell-induced mouse model of vitiligo, revealed that anti-CD122 mAb treatment repigmented vitiligo-affected skin in a highly durable fashion (119). This study was the first to show that factors supporting T_{RM} cell maintenance can serve as targets to impair autoimmunity. Interestingly no differences were observed between T_{RM} responses in patients with active vs. stable disease (118, 119), further underscoring the concept that vitiligo is a disease of immune memory.

Corollary studies of CD8 T cell responses in psoriasis patients show that not all T_{RM} responses are created equal. Psoriasis, like vitiligo, occurs in lesional patches of skin, but is recognized as an IL-17-driven disease. Accordingly $CD8^+$ T_{RM} cells in psoriatic plaque skin preferentially produced IL-17 upon restimulation, exhibit a $CD103^+$ $CD49a^-$ CLA^+ $CCR6^+$ $IL\text{-}23R^+$ phenotype (117, 121), and lack expression of CXCR3 (118). Interestingly, cytokine production was maintained by psoriasis-associated T_{RM} cells even in patients that had undergone long term treatment and had resolved disease (117, 118), again supporting the highly durable nature of T_{RM} responses in autoimmune disease.

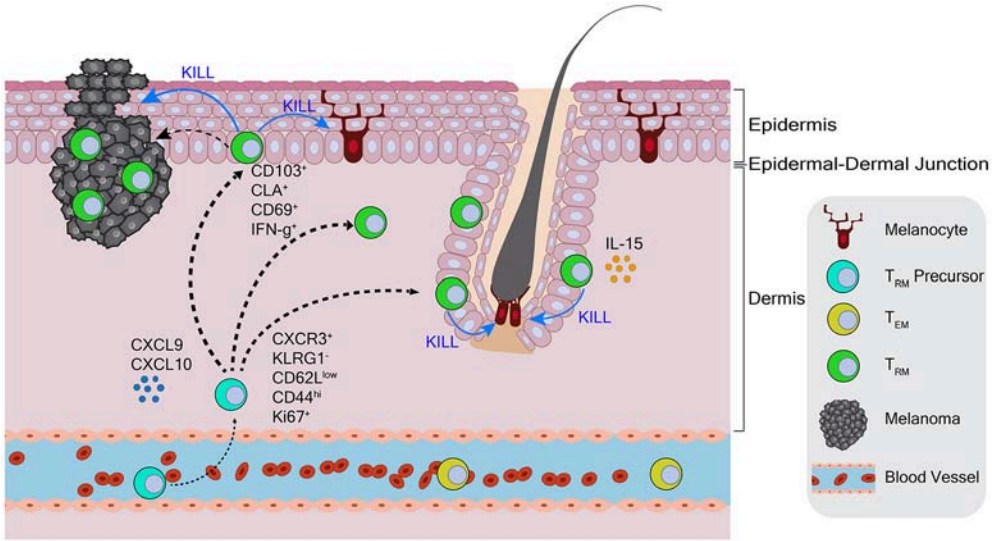

FIGURE 2 | Skin resident-memory T cell responses to melanoma in the context of autoimmune vitiligo**.** Tumor-specific T_{RM} precursor populations are recruited from circulation into the skin by chemokine signals. Following entrance into the skin, these T cells kill melanocytes, develop into T_{RM} cells, and produce IFN-γ. Tumor-specific T_{RM} cells populate the dermis, epidermis, and hair follicles, where IL-15 is produced. CD103-dependent T_{RM} cells mediate the durable recall response to melanoma in the dermis.

Contrary to the long-held belief that psoriasis is mediated by γδ T cells, active and clinically resolved psoriasis lesions were shown to contain oligoclonal T cell populations that were overwhelmingly of the αβ T cell lineage, and that produced both IL-17 and IL-22 (122). These studies collectively indicate a role for Tc17-like T_{RM} cells in the pathogenesis of psoriasis.

Although less well-studied, T_{RM} responses have recently been reported in conjunction with scleroderma—a disease of unclear etiology, characterized primarily by fibrosis of the skin. Analysis of skin from patients with early stage scleroderma revealed higher proportions of $CD8^+CD28^-$ T cells that expressed the T_{RM} marker CD69, and also overwhelmingly expressed CCR10, although largely lacked CD103 (123). Functional analyses indicated a proportion of restimulated cells that were capable of making granzyme B, IFN-γ and IL-13, a pro-fibrotic cytokine (123). This study, together with the above studies in vitiligo and psoriasis patients, highlights the diverse functional programs adapted by T_{RM} cells in the context of aberrant skin pathologies.

Other Autoimmune and Inflammatory Conditions

Several reports have provided evidence of T_{RM} responses in other autoimmune diseases and inflammatory conditions, particularly in diseases of the brain and CNS. With growing evidence that CD8 T cells may play a role in the pathogenesis of multiple sclerosis (124), work in mouse MS models has suggested that Ag-specific CD8 T_{RM} cells in the CNS could contribute to pathogenesis (125). The creation of TCR Tg mice expressing a TCR specific for an MHC-I restricted epitope of an astrocyte-expressed protein resulted in MS-like disease, with the brains of mice becoming infiltrated by CD8 T cells with a $CD44^+CD69^+CD103^+$ T_{RM} -like phenotype (125). Evidence of MS-associated T_{RM} cells also derives from lesional brain specimens of MS patients. In a small subset of patients with high inflammatory infiltrates, CD103-expressing CD8 T cells were identified, although these cells did not express CD69 (126). In addition to MS, a study of Rasmussen's Encephalitis, a rare pediatric neuroinflammatory disease of unknown etiology, also reported CD8 αβ T cells in seven out of seven RE brain surgery specimens, >50% of which expressed CD103 (127). Mouse studies show that T_{RM} cells may accumulate naturally with age in the CNS, evidenced by the appearance of a $CD8^+CD44^{hi}$ $CD62L^{low}$ $CD69^{hi}$ $PD1^+$ subset (128). In an ischemic stroke model in aged mice, restimulation of brain CD8 T cells induced the production of TNF-α, IFN-γ, and CCL2 (128). Despite this, the question remains of whether brain CD8 T_{RM} cells are pathogenic. $CD8^+CD103^+$ T_{RM} cells from brains of mice with MS-like disease did not produce cytokines (125). In lupus-prone mice, $CD8^+CD44^{hi}CD62^{low}CD69^+$ T_{RM}-like cells accumulated in the brain, but their ablation exacerbated neuropsychiatric lupus, suggesting that these cells might instead serve a regulatory role (129).

As of yet, few studies have convincingly identified T_{RM} cells in other autoimmune disease types. An investigation of lesional biopsies from recent-onset type-1 diabetes (T1D) patients reported $CD8^+CD69^+CD103^+$ T_{RM}-like cells in diseased islets (130), although a separate study showed a preponderance of CD8 T cells with similar phenotypes in normal, healthy human islets (131). Transcriptomes of T1D patient islets were more skewed to the production of inflammatory cytokines including IFN-γ IL-18, and IL-22 (130), although it is as yet unclear if these pathogenic cytokines are derived from T_{RM} cells. It has been speculated that T_{RM} cells mediate, or contribute to, a host of human autoimmune and inflammatory conditions (132). Advancements in our understanding of T_{RM} biology should guide further investigations of T_{RM}-mediated pathology.

T_{RM} responses against normal self-tissues can be instructive with regards to cancer immunity. First, they reveal that self Ag-specific T cells can be maintained as T_{RM} responses, in the peripheral tissues where carcinomas arise (**Figure 1**). Such T cells would seem ideally situated to provide surveillance against tumorigenesis, progression, and metastasis, and may naturally do so. Second, they illustrate that T_{RM} cells can adopt a number of stable immune effector states, producing IFN-γ, IL-17, IL-22, IL-13, and/or granzyme B, all of which have separately been shown to oppose tumor growth. In particular, vitiligo and psoriasis-associated T_{RM} cells maintain stable phenotypes and provide durable recall responses, with more apparent functional diversity than was originally recognized based on viral infection models. Our knowledge of mechanisms driving pathogenic self Ag-specific T_{RM} cells, coupled with our understanding of T_{RM} responses against foreign infections, can greatly inform our understanding of T_{RM} responses to cancer.

OPTIMIZING T_{RM} CELL RESPONSES FOR CANCER IMMUNOTHERAPY

The discovery that standard cancer vaccine and immunotherapy approaches provide long-lived protection through T_{RM} responses—rather than T_{CM} responses, as originally speculated—indicates that promoting T_{RM} responses is a worthwhile goal for the field. The challenge remains in generating T_{RM} responses when (and where) they don't naturally develop. The final section of this review will highlight current knowledge regarding T_{RM} cell behavior that can aid in promoting such responses in the setting of cancer immunotherapy.

Optimal T_{RM} Cell Precursor Populations

The ultimate goal of adoptive T cell therapy is to provide long-lived cancer cures. In patients with metastatic solid tumors, T cells must seek and destroy tumor cells in multiple peripheral tissue locations. T_{RM} cells seem ideal for this job, although successfully delivering these populations presents clear challenges. Because T_{RM} cells are, by definition, resident in peripheral tissue, their *in vitro* generation for intravenous delivery is a complex proposition. In considering the systemic administration of a T_{RM} response, one must consider the precursor populations that can optimally seed tissues in a receptive host.

T_{RM} Precursor Seeding

Studies in mouse models largely support the conclusion that timing is crucial for T_{RM} precursor seeding, and that this event must occur early during the course of T cell priming and differentiation. In LCMV infection, early effector cells migrated into intestinal epithelium, seeding a T_{RM} population within 7 days of the initial infection (41). Similarly, in HSV infection, a delay of 2 weeks did not alter T_{CM} formation, but it dramatically reduced T_{RM} cell seeding in the skin, resulting in a loss of protection against viral rechallenge (32). This window of opportunity is affirmed by studies of mucosal chlamydia vaccination, wherein T_{RM} precursors seeded uterine mucosa within 7 days of vaccination (24). Our own studies indicate that tumor-specific T_{RM} precursors are primed early in response to immunotherapy, as they could be isolated from melanoma-draining lymph nodes just 8 days following therapeutic depletion of regulatory T cells (111). These studies support the idea that T cells at an early differentiated state have the greatest propensity to form T_{RM}.

Despite this, some evidence supports the idea that T_{CM} cell plasticity can give rise to T_{RM}. In studies employing VACC-OVA as a dermal vaccine, sorted OVA-specific T_{CM} cells that were re-transferred *in vivo*, generated skin T_{RM} populations in response to VACC-OVA recall infection (110). On a per-cell basis, T_{CM} cells were shown to be more effective at generating T_{RM} cells compared with naïve T cells (110). Similarly, when administered to tumor-bearing mice, Ag-specific T_{CM} cells were better at accessing tumors and acquiring a T_{RM} phenotype, compared with their naïve counterparts. As T_{RM} and T_{CM} cells represent distinct lineages, it remains unclear how such memory T cell reprogramming occurs. While it has been shown that T_{RM} and T_{CM} cells have a common clonal origin (133), T_{CM} cells do not naturally convert to T_{RM} cells in the post-infection setting (17). Thus, factors relating to secondary infection with VACC-OVA, or tumor growth itself, may alter the plasticity of T_{CM} cells (110). Indeed prior preclinical approaches to melanoma adoptive immunotherapy with *in vitro* generated gp100-specific T_{CM}, T_{SCM}, and Tc17 subsets (12, 15, 134) may naturally give rise to T_{RM} cells in a tumor-bearing host. This is consistent with the observation that these treatments all induce overt vitiligo (12, 15, 134)—a disease of skin-resident memory.

T_{RM} Precursor Phenotypes

With regards to phenotype, epithelium-infiltrating T_{RM} precursors in infectious disease models have been shown to express CXCR3 and lack expression of the terminal effector T cell marker KLRG1 (20, 111). Deficiency in CXCR3 reduces the overall number of T_{RM} cells in the skin (20, 135, 136), and mAb-mediated CXCR3 blockade can prevent T_{RM} formation (34). Accordingly, we identified $CXCR3^{+}KLRG1^{-}$ T cells in tumor-draining lymph nodes, that were capable of seeding tumor-specific Trm responses in the skin following i.v. adoptive transfer (**Figure 2**) (111). CXCR3 is also reported as a mediator of T cell access to solid tumors (137), thus its role in the seeding of T_{RM} precursors further underscores the importance of CXCR3 expression on T cells in an immunotherapy setting.

In our melanoma model, we also observed CD103 expression on T_{RM} precursors in tumor-draining lymph nodes, and found that genetic loss of CD103 impaired early T cell lodgement in the skin (111). This is in contrast to viral models in which CD103 is only expressed upon T cell entry into the epidermis (20), although we also observed a further increase in CD103 expression following T cell entry into the skin. As our T_{RM} cells were primed in response to dermal melanoma growth, early CD103 expression in our model could result from tumor-derived TGF-β entering tumor-draining lymph nodes. Numerous infectious disease studies have shown that CD103 expression is required for the long-lived, antigen-independent maintenance and retention of T_{RM} cells (20, 47, 101, 138, 139). However, this unexpected role for CD103 in promoting skin lodgment indicates that CD103 may also be a useful feature of tumor-specific T_{RM} precursors.

T_{RM} Precursor Transcriptional Programs

In considering transcription factors that program T_{RM} development, whereas *Hobit* expression is restricted to late events in peripheral tissues (55), *Runx3* expression occurs earlier, and appears to program T_{RM} precursor behavior. Identified by an siRNA screen of factors that promote tissue residence *in vivo*, Runx3 was found to suppress *Tbet* expression, enforce *Itgae* expression, and suppress multiple genes associated with tissue egress (54). Runx3 expressing T cells were transcriptionally distinct from T_{CM} and T_{EM} precursor populations as early as 7 days post infection. Of therapeutic importance, Runx3 overexpression in LCMV-specific CD8 T cells promoted T cell access to viral antigen-expressing B16 tumors, enforced the acquisition of a T_{RM} phenotype in tumors, and enhanced T cell anti-tumor activity (54). These studies establish that Runx3 promotes T_{RM} characteristics and anti-tumor efficacy of transferred T cells (54), making it an attractive target for expression in the adoptive immunotherapy setting.

Optimal Host Tissue Microenvironment

Tumor cell dissemination from primary tumors to peripheral tissue locations is predicated on Paget's seed and soil hypothesis of 1898, which states that tumor cells (the seed) can only take up residence in suitable tissues (the soil) (140). This principle can also be applied to T_{RM} cell seeding in peripheral tissues. Indeed among T cells, T_{RM} cells are unique in their propensity for tissue. Providing the proper "soil" for precursor T cell residence will be a critical step toward supporting T_{RM} function, both in tumors and in cancer-prone tissues. Moreover, the properties of T_{RM} cells that enable them to function in diverse peripheral tissue niches, might imbue them with the unique ability to persist and function in solid tumors.

Metabolic Factors

Metabolic characteristics of T_{RM} cells that support their function in peripheral tissues may also support their function in tumors. In a VACV skin infection model, fatty acid binding protein 4 and 5 (*FABP4/5*) were shown to be among the highest expressed genes in T_{RM} cells, enabling the metabolism of exogenous free fatty acids in the skin (51). T_{RM} cells in the tumor microenvironment must compete for nutrients with tumor cells, which use high levels of glucose and glutamine (141). Indeed Tregs have been shown to function well in the TME due to their ability to utilize both glycolysis and fatty acid metabolism (142). Reliance on fatty acid catabolism has recently been shown to be essential for $CD8^+$ TIL function (143). The PPAR agonists fenofibrate, and bezafibrate, which both promote fatty acid oxidation, have each been shown to improve T cell anti-tumor activity (143, 144), although it remains to be seen if these effects are due to improved T_{RM} responses. Regardless, the metabolic requirements of T_{RM} cells may make them ideally suited to persist and function in a metabolically hostile TME.

Chemokines and Cytokines

Chemokine cues are crucial for the seeding of T_{RM} precursors, and can be used therapeutically to pull T_{RM} cells into peripheral tissues. Our finding that melanoma-specific $CXCR3^+$ precursors cells only induce T_{RM} responses in vitiligo-affected hosts (111), together with studies showing that the CXCR3/CXCL9/10 axis is crucial for vitiligo development (120, 145), underscores how an autoimmune tissue microenvironment can provide a hospitable niche for tumor-specific T_{RM} cell seeding (**Figure 2**). CXCL9 and 10 have also been used therapeutically to seed T_{RM} responses based on a "prime and pull" approach (146). In one study, subcutaneous vaccination elicited a systemic T-cell response against HSV, followed by topical CXCL9/10 application to the vaginal mucosa as a means to pull activated T cells into tissue (146). The resulting T_{RM} responses and long-term protection against HSV were comparable to that of mice that that had been immunized intravaginally (146). Similarly, when immunizing against mTB in the lung, parental vaccination followed by intranasal administration of CXCL16, pulled CXCR6 cognate receptor-expressing T_{RM} cells into the lung where they provided long-lived protection (147). CXCR10 has separately been shown to promote T_{RM} formation in the skin (135, 148), suggesting the possibility of a similar approach involving CCL27. These studies show that, even when precursors are generated in a systemic manner, chemokine signals in a specific tissue location can induce functional T_{RM}.

Inflammatory sensitizing agents can also modify peripheral tissue in a way that promotes robust T_{RM} responses. Using the chemical sensitizing agent dinitrofluorobenzene (DNFB), local inflammation in skin recruited effector T cells and converted them to a T_{RM} phenotype (32). Similarly, topical diphenyl cyclopropinone (DPCP) applied to human skin induced contact dermatitis and the subsequent formation of T_{RM} (133). Accordingly, DPCP application to human skin has been shown to induce high levels of cutaneous CXCL9 and 10 (149). Interestingly, DPCP recently received orphan drug approval for the topical treatment of cutaneous melanoma metastases (150), having demonstrating dramatic local efficacy against cutaneous melanoma metastases in one patient that received concurrent immune checkpoint inhibitor therapy (151). It is tempting to speculate that T_{RM} cells participated in this anti-tumor response, which was also accompanied by vitiligo (151).

Once T cells access peripheral tissues, both TGF-β and IL-15 are recognized as fundamentally important T_{RM} survival cues (20, 56). While TGF-β has come to be known for its roles in promoting tumor growth (152), suppressing T cell function (153), and enforcing Treg stability (154), its key role in T_{RM} cell generation suggests its value in certain immunotherapy contexts. On the other hand, IL-15 has long been recognized for its role in supporting anti-tumor T cell responses (155, 156). Studies show that T_{RM} cells preferentially accumulate at sites of high IL-15 production, such as hair follicles in the skin (46, 111). Accordingly, we found that melanoma/melanocyte (gp100)-specific T_{RM} cells cluster in hair follicles of vitiligo-affected skin (111). In conjunction with the finding that gp100-specific T cells express CD122 (IL-15Ra) and require IL-15 for their pathogenic role in melanocyte destruction (119), IL-15 may also enhance melanoma-specific T_{RM} maintenance. Although it is important to note exceptions to the requirement for IL-15, which are surprising considering its canonical role as a homeostatic memory cytokine (157). In fact, certain T_{RM} responses (i.e., in

the female reproductive tract) exhibit no dependence on IL-15 for proliferation or survival (157). T_{RM} cells that re-engage antigen in peripheral tissues appear to decrease their reliance on IL-15 (157), indicating that antigen also serves an important role in peripheral tissue.

Antigen and Costimulatory Requirements

As T_{RM} precursors seed tissue, their differentiation and maintenance has been shown to be shaped by the local engagement of Ag. Indeed following VACV infection, T_{RM} responses were increased ten to 50-fold if their cognate antigen had been engaged in the skin (158, 159). We also observed an important role for peripheral antigen in generating tumor-specific T_{RM} responses (111). Following priming in response to a B16-OVA tumor in the dermis, T_{RM} cells that re-engaged their target antigen on melanocytes in the skin (gp100-specific) were present in far greater numbers than those that could not (OVA-specific) (111). In vaccinating against a tumor-specific neoantigen, T_{RM} responses in peripheral (non-tumor) tissues might best be generated by a proposed "prime and trap" approach (34). This was demonstrated in the context of malaria vaccination, wherein the expression of cognate antigen on hepatocytes served to trap circulating CD8 effector T cells in the liver, where they underwent conversion to T_{RM} cells (34).

While memory T cells are defined by their ability to persist in the absence of Ag, in some settings chronic Ag exposure might also support T_{RM} cell persistence. Following HPV vaccination of patients with cervical intraepithelial neoplasia, $CD8^+$ T cells expressed CD69, and localized to tertiary lymphoid structures in neoplastic cervical tissue, where they expressed Ki67 as evidence of cognate antigen engagement (160). In considering T_{RM} responses against a tumor/self Ag, we showed that functional T_{RM} could be generated against a melanoma antigen (gp100), but only in conjunction with autoimmunity against normal host melanocytes (111). While this may reflect the chemokine/cytokine environment of the autoimmune tissue niche, it may alternately reflect a role for persistent antigen exposure. Interestingly, we identified melanoma-specific T_{RM} cells throughout the skin, although they were preferentially localized to hair follicles containing white hairs, suggesting an absence of local antigen (111). It remains possible that, in the context of autoimmune disease, T_{RM} cells develop into both Ag-dependent and independent subsets.

Although less well-studied, peripheral costimulation may also play a crucial role in programming the T_{RM} response. Recent studies of intranasal flu vaccination showed that boosting with 4-1BBL in a replication defective adenovirus vector generates a robust lung parenchymal $CD69^+$ $CD103^{+/-}$ T_{RM} population (161). Boosting required local (intranasal) 4-1BBL installation, and worked by recruiting additional Ag-specific T cells from circulation into the lungs (161). Lung T_{RM} responses generated in this fashion were highly durable and provided protection for at least 1 year after boost (161). Earlier work also suggests that agonistic OX-40 mAb treatment can promote lung T_{RM} responses, although these studies involved parenteral vaccination (162). Future work is needed to determine how and where to provide the most potent costimulatory signals for optimal T_{RM} formation.

CONCLUSIONS

The above findings represent a fundamental advance in our understanding CD8 T cell responses to cancer. While the success of ICI therapy has reaffirmed the long-held belief that CD8 T cells are crucial for tumor immunity, an evolving knowledge of memory T cell function in peripheral tissues has informed our understanding of the type of T cell that may be most needed. Innovations in single cell cytometry and RNA sequencing have rapidly been brought to bear on the characterization of tumor-infiltrating lymphocytes and, together with mechanistic studies in mice, provide compelling evidence that T_{RM} cells are players in the immune response to human cancer.

Several key questions remain. While T_{RM}-like TILs portend improved prognosis for a growing number of cancers, future studies should address how to fully revive such cells in the TME, and how to generate greater numbers of T_{RM} precursors through immunotherapeutic means. A role for T_{RM} cells in tumor immunosurveillance has not yet been established, and it will be interesting to learn if CD8 T_{RM} cells can also limit tumorigenesis. Finally, the field has focused heavily on $CD8^+$ T cells, although a role for CD4 T_{RM} populations is not yet understood. Local T_{RM} helper subsets might be greatly beneficial to tumor immunity, whereas T_{RM} regulatory cells might be particularly detrimental in the TME.

The knowledge that T_{RM} cells can be generated by cancer vaccine and immunotherapy regimens represents a paradigm-shift for a field that has long monitored tumor-specific T cells in the blood. Thus, going forward one must recognize a need to monitor T_{RM} responses in peripheral tissues and tumors of cancer patients receiving immunotherapy. Such peripheral T cell responses might provide the best indication of responsiveness to therapy, and long-term survival. Informed by studies in infectious disease models, and instructed by the involvement of T_{RM} cells in autoimmunity, future research efforts will hopefully overcome the barriers to promoting effective T_{RM} responses to cancer.

AUTHOR CONTRIBUTIONS

AM and MT both conceptualized, wrote, revised, and approved of the final submitted manuscript.

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Cheating the Hunger Games; Mechanisms Controlling Clonal Diversity of CD8 Effector and Memory Populations

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Effector and memory CD8 T cells have an intrinsic difference in the way they must approach antigen; effector cells need to address the pathogen at hand and therefore favor outgrowth of only high-affinity clones. In contrast, the memory pool benefits from greater clonal diversity to recognize and eliminate pathogens with mutations in their immunogenic epitopes. Effector and memory fates are ultimately the result of the same three signals that control T cell activation; T cell receptor (TCR) engagement together with co-stimulation and cytokines. Great progress has been made in our understanding of the transcriptional programs that drive effector or memory differentiation. However, how these two different programs result from the same initial cues is still a matter of debate. An emerging image is that not only the classical three signals determine T cell differentiation, but also the ability of cells to access these signals relative to that of other activated clones. Inter-clonal competition is therefore not only a selective force, but also a mediator of CD8 T cell fate. How this is regulated on a transcriptional level, especially in the context of a selective "hunger game" based on antigen-affinity in which only cells of high-affinity are supposed to survive, is still poorly defined. In this review, we discuss recent literature that illustrates how antigen-affinity dependent inter-clonal competition shapes effector and memory populations in an environment of antigen affinity-driven selection. We argue that fine-tuning of TCR signal intensity presents an attractive target for regulating the scope of CD8 T cell vaccines.

Keywords: immunity, CD8 T cell, affinity, memory, T cell receptor, differentiation, effector

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INTRODUCTION

CD8 T cells play a critical role in the protection of our body from the occurrence and recurrence of intracellular pathogens and tumors. To recognize the large number of potential threats, the naïve CD8 T cell pool consists of millions of clones, each unique based on its antigen receptor. To prevent an excessive use of resources for the maintenance of these cells, each clone is present at low frequency. Only upon activation do antigen-specific clones expand to form the effector and memory pools (1–4). Naïve CD8 T cells need three separate signals for optimal effector and memory generation: (1) antigen recognition by the T cell receptor (TCR), (2) co-stimulation, and (3) cytokines (5). These three signals are not hierarchically equal. Generally, only in case of TCR engagement do co-stimulation and cytokines contribute to T cell activation. Moreover, the affinity

of the TCR determines the capacity of an activated cell to access vital co-stimulatory molecules, cytokines and nutrients (6). Considering the vast diversity of the naïve CD8 T cell pool, statistical probability dictates that for any given antigen, many more low- than high-affinity clones exist. To mount an efficient CD8 T cell response, selection of activated clones based on antigen-specificity must take place (6, 7). We have therefore proposed a fourth factor that controls effector and memory T cell formation: "competitive fitness"—the ability to compete for extracellular signals with other activated T cell clones based on antigen affinity (8).

The parameters that determine competitive fitness differ between effector and memory cells, because of the difference in the way that these pools must approach antigen. Upon infection, the effector pool is faced with an actively replicating pathogen and therefore only the most efficient, high-affinity clones are selected into its ranks (6, 9). Immunological memory must protect the host against re-infection with a previously encountered pathogen. Due to selective pressure on the original pathogen as it moves through its host population, re-infections are more likely to occur with a variant carrying mutations in its immunogenic epitopes (10–12). Hence, selection of memory clones is a trade-off between specificity and diversity. Too much specificity restricts antigen-recognition, which precludes responsiveness against mutated pathogens. Too much diversity impairs efficiency of recall responses. In both mice and humans increasing the diversity of the memory pool enables recognition of a larger fraction of the potential pathogen-carried sequence space resulting in a higher probability of recognizing mutated pathogens (13, 14). How clonal selection within the effector and memory cell populations is regulated is only partially understood. Here, we propose a crucial role of TCR signaling in an affinity-based inter-clonal competition which shapes clonal diversity and regulates effector and memory differentiation.

THE IMPACT OF SIGNAL INTENSITY ON CD8 T CELL DIFFERENTIATION

The initiating event for CD8 T cell activation is recognition of an antigen embedded in the major histocompatibility complex (pMHC) on an antigen-presenting cell (APC) by the TCR. This results in the activation of a network of signaling cascades that mediate differentiation, proliferation, and survival (15, 16). Upon activation, a single naïve CD8 T cell has the potential to give rise to various effector and memory CD8 T cell subsets (17, 18). Divergent cell fates depend on the intensity of the cumulative signal activating an individual CD8 T cell (19). This signal strength represents the sum of different factors such as the affinity and avidity of TCR binding to antigen-pMHC complexes, co-stimulation, and cytokines (8, 20–22).

Initially, it was proposed that only a cumulative signal of high overall strength allows T cell activation and formation of effector and memory cells (23). This was based on the observation that only cells of high-affinity vigorously expand upon activation (24). The model was challenged by the finding that even very weak TCR-pMHC interactions promote proliferation and generation of functional memory (25, 26). In addition, even a brief 2 h priming phase was shown to be sufficient to induce the complete diversity of effector and memory CD8 T cell subsets (27–29). To analyze these processes more directly, SIINFEKL (Ova)-specific OT-1 cells were transferred to naïve recipient mice, which were subsequently infected with *L. monocytogenes* (LM) expressing Ova or altered peptide ligands (APL) that bind the OT-1 TCR with lower affinity. This revealed that even weak ligands are sufficient to activate naïve cells and mediate formation of both effector and memory T cells (30). This raised the question how the immune system prevents that clones of low specificity and efficiency expand and exhaust the limited amount of available resources. The answer came from the observation that the potency to induce effector cell proliferation positively correlates with the intensity of the TCR signal (24, 30–32). Decreasing the cumulative signal strength by pretreating mice with antibiotics before *L. monocytogenes* infection and thus lowering antigenic load resulted in reduced expansion of antigen specific effector T cells (33, 34). In addition to a proliferative advantage of high-affinity cells, activated effector CD8 T cells were shown to undergo negative selection of low-affinity clones based on a reduced capacity of these cells to access and thus outcompete other clones for limited resources (8). Upon activation T cells induce expression of the IL-2 receptor in an antigen-affinity dependent manner (6, 30). IL-2 mediates survival by triggering the PI3K signaling cascade and sustaining the pro-survival protein Mcl-1 (**Figure 1**). High-affinity effector cells therefore have a competitive survival advantage over low-affinity cells in their ability to access IL-2. This selection process narrows clonal diversity, since only highly specific clones are allowed to generate progeny and create an almost monoclonal effector CD8 T cell pool (6, 8). Animals lacking Noxa, a pro-apoptotic antagonist of Mcl-1, have a reduced survival threshold for effector cells and therefore showed reduced dependency on IL-2. As a result, these mice had an increased number of low-affinity clones contributing to the effector pool, which was of reduced anti-viral potential (6).

Co-stimulation and cytokines greatly contribute to the cumulative activating signal intensity and therefore have a major impact on TCR-affinity mediated selection of CD8 T cell clones. CD28-driven co-stimulation is essential for proper CD8 T cell responses after weak TCR-pMHC interactions. Conversely, high antigen doses and prolonged antigen stimulation can compensate for a lack of CD28 co-stimulation *in vivo* (35, 36). CD27-driven co-stimulation promotes production of IL-2 in activated T cells (37). Animals deficient for CD27 therefore have reduced access to IL-2, resulting in a less clonally diverse effector response of increased overall affinity (13). Notably, expression of CD70, the ligand of CD27, is regulated by antigen avidity (13, 38–40), but whether this contributes to the diversity of the effector response is unknown. Similarly, cytokines impact cell fate decisions and clonal selection mechanisms. CD8 T cells activated in the presence of high levels of IL-2 or IL-12 exhibit increased proliferation rates and superior effector functions (23, 30, 33, 41–43). Exogenous addition of IL-2 rescued survival of low-affinity cells (6), indicating that stronger inflammatory responses will allow for more clones to contribute to the effector response, though this does not necessarily promote their dominance.

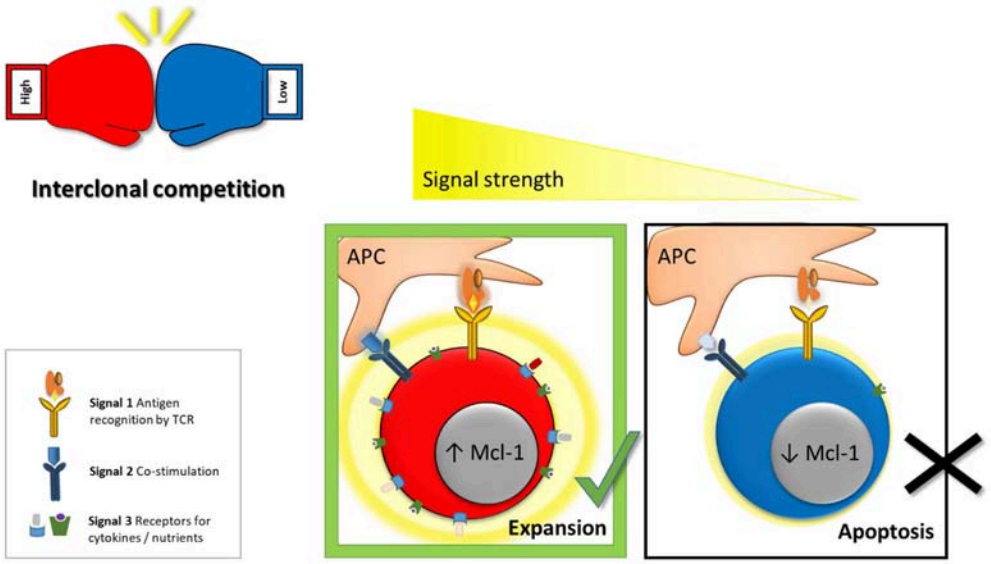

FIGURE 1 | Model for inter-clonal competition between effector cells based on antigen-affinity. For efficient activation and optimal effector CD8 T cell formation 3 signals are required (1) antigen recognition by the TCR, (2) co-stimulation, and (3) cytokines. We proposed as a fourth factor "competitive fitness"—the ability to compete for these signals with other activated T cell clones. Cumulative signal strenght (visualized by a graded yellow halo) is the main factor controling the capacity of activated lymphocytes to access vital co-stimulatory molecules, cytokines and nutrients (e.g., glucose, amino acids). Thus, high-affinity effector cells have a competitive advantage over low-affinity cells in their ability to access these signals. In addition, high-affinity cells take-up more IL-2 which in turn mediates survival of high-affinity clones by triggering the PI3K signaling cascade and sustaining pro-survival proteins such as Mcl-1. Hence, low-affinity clones undergo negative selection through apoptosis to ensure that only the fittest, high-affinity clones contribute to the antiviral response.

In summary, TCR signaling is not an on/off switch. Rather, it enables integration of signals with different intensities, which are further amplified by the right cytokines and co-stimulatory molecules. Fine-tuning of TCR signal intensity shapes T cell differentiation and clonal selection.

INTER-CLONAL COMPETITION IN THE CONTEXT OF EFFECTOR AND MEMORY FORMATION

Even though a naïve cell can generate both effector and memory cells (44), memory potential is associated with weaker activating signals. Very low affinity antigens are still able to induce memory formation but have a strongly reduced capacity to induce effector differentiation (30, 45). Exogenous factors such as IL-2, IL-12, or CD28 co-stimulation add to the cumulative activating signal and help activated cells to obtain an effector phenotype (43, 46). Very high levels of stimulation, in contrast, push T cells "beyond" an effector stage into exhaustion (47, 48). Various models have been proposed how activating signal strength regulates CD8 memory formation. The "decreasing potential" model suggests that memory formation is the "default" state of activated T cells and that effector memory or effector cell differentiation is only possible if a certain level of activation is reached(49, 50). Whether this level represents a binary threshold, or whether effector potential is gradually increased in response to increasing signal strength is a matter debate and appears to depend on the molecules that are used to determine threshold values(43, 51–55).

Mostly, the impact of affinity on effector and memory potential has been interrogated by presenting a single (TCR-transgenic) T cell clone, with high- or low-affinity ligands (30, 45). However, a biologically more relevant question is how signal strength is linked to memory formation, not at the level of a single clone but in the context of the entire antigen-responsive population. Statistical probability dictates that for a given antigen, many more high-affinity than low-affinity cells exist within the naïve T cell pool. Hence, molecular mechanisms are in place to ensure that preferentially cells of high-efficiency are selected into both the effector and memory cell pools (6–8, 13). The impact of cumulative signal intensity is therefore not only a checkpoint controlling effector vs. memory fate decisions, but also controls the competitive fitness of cells in a selective environment that regulates the diversity of antigen-experienced T cell populations. To shed more light on this concept, experiments were performed in which a pool of individually labeled OT-1 cells was transferred to a host which was subsequently infected with LM-Ova. Analysis of donor cells revealed that even within a monoclonal high-affinity population, a relatively small fraction of clones dominates the effector response (17, 18). This would suggest that only a small number of cells reaches the cumulative signaling threshold required for CD8 T cell expansion. When a sufficiently high number of monoclonal cells is transferred, stochastic effects are negated, which ensures that in experimental settings donor cells usually make a significant contribution to the effector response (18). However, in a physiological setting, each clone is present at very low frequency (3). This indicates that inter-clonal competition

becomes an important factor that controls shaping of the antigen-specific cell pool. Indeed, when mice were transferred with only a single OT-1 cell, in less than one third of animals these cells could be recovered after infection with LM-Ova (18). The recruitment of antigen-specific cells into the immune response is highly efficient and nearly complete (56), excluding limited antigen-exposure as a determining factor. Thus, considering the fact that effector cells are derived from a small number of precursors that is able to generate exponential expansion (17, 18), small differences in competitive fitness will ensure highly selective outgrowth of clones.

In the cell-tracing experiment, cells that did not undergo massive expansion generally adopted a memory-like phenotype (18). Together with the observation that low-affinity cells preferentially form memory, the question arises whether only the effector pool is selected for high-affinity clones and that the memory pool allows contribution of all activated cells. Studies in which the clonal diversity of effector and memory cells was directly compared showed that the effector cell pool is much more restricted in its clonal diversity than the memory pool directed against the same antigen (7, 13, 57). However, clones that dominate the effector pool are also dominant in the memory population, albeit to a lower degree (7, 13, 57). Low cumulative signal strength favors memory formation and is associated with reduced proliferation (30, 31). Why then, is the memory response not completely dominated by low-affinity cells? One possibility is that high-affinity cells have a selective advantage also during memory formation. Another option is that they preferentially use a different mechanism to form memory than low-affinity cells. These models are not mutually exclusive and experimental evidence for both exist (**Figure 2**). Mice deficient for the co-stimulatory molecule CD27 generate a memory pool of comparable size as wild type controls yet is almost devoid of low-affinity clones (13). Similarly, low-affinity cells have a higher dependence on TNF receptor signaling during recall (58). This indicates that low-affinity memory precursors have increased dependence on factors that contribute to the cumulative activating signal and thus have a survival disadvantage when competing for these factors.

Antigen-experienced cells can be subdivided based on different parameters, but a common segregation uses IL-7Rα (CD127) and KLRG1. Memory precursors (MPECs) are defined as $CD127^{+}KLRG1^{-}$ whereas short lived effectors (SLECs) have the converse phenotype. A recent study indicates, however, that with the $CD127^{+}KLRG1^{+}$ cell pool and even among SLECs, cells exist that form "$exKLRG1^{+}$" memory after clearance of a pathogen (59). Even though the frequency of cells with memory potential in these pools is much lower than amongst MPECs, the high number of $KLRG1^{+}$ cells formed during an immune response ensures that in absolute numbers $exKLRG1^{+}$ cells make a significant contribution to the memory pool (59). High-affinity cells preferentially form cells with a SLEC phenotype, whereas low-affinity cells more rapidly become MPECs (30, 45). Even though direct experimental evidence is still lacking, these findings indicate that low-affinity memory cells are formed directly, whereas high-affinity memory is also derived from $exKLRG1^{+}$ effector type cells (**Figure 2**).

Maintenance of CD8 memory cells is independent of antigen and predominantly depends on cytokines such as IL-15 and IL-7 (60, 61). Whereas expression of cytokine receptors differs between cells of high- and low-affinity early after activation, at later time-points these differences are lost (6, 30). In the first weeks after clearance of a pathogen, the avidity of the antigen-specific pool therefore still changes as long-lived effector cells undergo apoptosis (62). However, once the clonal composition of the memory pool is established it remains stable for months to years after initial infection, both in humans and mice (63–66). Thus, clonal diversity of the memory CD8 T cell pool appears to be a long-term investment of the immune system to counter viral mutants.

An open question is how memory cell formation is influenced by inter-clonal competition on a molecular level. Various factors important for effector cell formation are induced in a way that directly correlates with antigen affinity, such as T-bet, IRF4, and Blimp-1 and these suppress expression of memory-associated molecules, such as Eomes and Tcf7 (54, 67, 68). The transcription factor IRF4 was found to regulate expansion of effector cells by promoting the metabolic switch to aerobic glycolysis in a TCR affinity–dependent manner. IRF4 expression was higher in high-affinity clones, ensuring their preferential expansion and effector differentiation over low-affinity clones (69, 70). Surprisingly, both Eomes and Tcf7 are induced upon activation of T cells (71, 72) and expression of Eomes can even be higher in high- than in low-affinity cells, dependent on the level of stimulation (45, 70). Notably, both T-bet and Eomes are essential for CD8 T cells to obtain a normal effector cell phenotype (72). The ratio between these molecules, rather than their expression level therefore appears to determine whether a cell obtains a memory or effector cell phenotype (46). How this dynamic regulation of transcription factors is regulated in the context of affinity-based selection in effector and memory cell pools remains to be elucidated.

In summary, the impact of cumulative signal intensity on effector vs. memory cell differentiation should be viewed in the context of clonal selection strategies that shape the antigen-specific cell pools. The impact of affinity on cell fate decisions appears to have evolved in order to ensure selection of only highly specific cells in the effector cell pool, whilst allowing sufficient diversity of CD8 T cell memory in a pool that is still dominated by high-affinity cells.

THERAPEUTIC POTENTIAL OF CLONAL DIVERSITY

Enhanced diversity within the memory CD8 T cell pool is of particular benefit against re-infections with rapidly mutating viruses (73). For example, HIV patients appear to benefit from greater clonal diversity of their virus-specific T cell response (13, 14, 73–77). As an effective vaccine against HIV remains elusive, future strategies may involve manipulation of IL-2 levels and/or co-stimulatory molecules during priming to broaden the

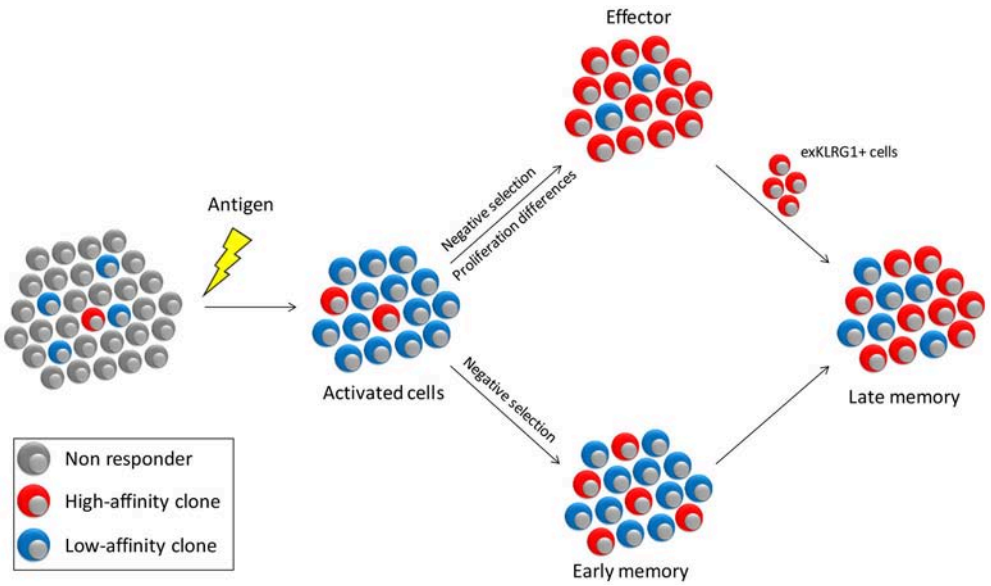

FIGURE 2 | Model for affinity selection within the effector and memory pools. Antigen encounter will activate a small number of high affinity cells and a much larger number of cells with lower affinity. The effector pool is stringently selected for cells of the highest affinity both through negative selection of low-affinity cells and through a proliferative advantage of high- over low-affinity cells. In contrast, affinity-based selection of early memory is less strong, since differences in proliferation between high- and low-affinity cells is less pronounced. Selection of high-affinity cells will therefore primarily occur due to competition for survival factors such as CD27 co-stimulation and cytokines. Late memory is supplemented by exKLRG1 cells, which are predominantly of high-affinity, thus increasing the overall affinity of the memory pool.

scope of the immune response. Evolutionary, increased clonal diversity of the memory pool compared to the effector pool is an acceptable strategy, as it does not appear to greatly reduce recall capacity against the original antigen. *Cd27*$^{-/-}$ mice, which generate a memory CD8 T cell pool that almost exclusively consists of high-affinity cells do not show an increased recall response following re-infection with a pathogen carrying high-affinity ligands (13). Similarly, co-transfer of high- and low-affinity memory cells directed against the same antigen does not result in a reduced ability of high-affinity cells to expand upon antigen re-encounter (45). In fact, re-encounter of the same antigen further skews the secondary effector pool in favor of high-affinity clones. In addition, re-infection with pathogens carrying a mutated immuno-dominant epitope promotes selective outgrowth of previously low-frequency clones that have now become of high-affinity (13, 65). Thus, clonal selection plays an important role both during primary and secondary responses, but does not affect functionality of subdominant clones. Increasing memory diversity of a vaccine against pathogens is therefore unlikely to reduce the overall effectiveness of protection.

Vaccination against tumors should target only transformed cells while avoiding unnecessary damage of healthy tissue. Reducing the number of targeted epitopes included in a vaccine lowers chances of off-target effects, but also limits the effectiveness of a vaccine and allows for more rapid outgrowth of cells with mutations in their immunogenic epitopes. Rather, anti-tumor vaccination in combination with a strategy that narrows the scope of the immune response per epitope holds promise for a more efficient and specific treatment. A better understanding of the molecular mechanisms that control the diversity of the T cell response are therefore of crucial importance (8, 78, 79).

The degree of heterogeneity within the CD8 T cell response depends on the ability of activated clones to integrate signals from the TCR, co-stimulatory molecules and cytokines, but also their relative fitness in an environment of rapidly expanding cells competing for the same resources. Recent studies demonstrate the importance TCR signal strength in regulating T cell differentiation, but much remains unknown about the molecular mechanisms that control the clonal selection strategies that shape the diversity of the effector and memory pools. Deeper insight in the transcriptional network underlying affinity-based clonal selection therefore holds great promise for the development of novel, more efficient CD8 T cell vaccines with an altered scope.

AUTHOR CONTRIBUTIONS

IK and FMW wrote the manuscript. IK, FMW, and BP participated in drafting and editing the text and figures. All authors gave final approval to the version submitted.

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REVIEW
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Resident-Memory T Cells in Tissue-Restricted Immune Responses: For Better or Worse?

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Tissue-resident-memory CD8+ T cells (T_{RM}) have been described as a non-circulating memory T cell subset that persists at sites of previous infection. While T_{RM} in all non-lymphoid organs probably share a core signature differentiation pathway, certain aspects of their maintenance and effector functions may vary. It is well-established that T_{RM} provide long-lived protective immunity through immediate effector function and accelerated recruitment of circulating immune cells. Besides immune defense against pathogens, other immunological roles of T_{RM} are less well-studied. Likewise, evidence of a putative detrimental role of T_{RM} for inflammatory diseases is only beginning to emerge. In this review, we discuss the protective and harmful role of T_{RM} in organ-specific immunity and immunopathology as well as prospective implications for immunomodulatory therapy.

Keywords: resident memory T cells, chronic, inflammation, infection, autoimmune

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INTRODUCTION

During an infection, our immune defense operates in a sensitive balance in which the eradication of an invading pathogen should take place efficiently with the least possible damage to the body's own structures. For this, different subsets of immune cells have evolved, which form several lines of defense and are equipped with different functional specializations. Various leukocyte subsets—from broadly acting innate immune cells to antigen-specific and specialized lymphocytes—act together to constitute a joint defense reaction against infectious intruders. CD8+ (so-called cytotoxic) T lymphocytes are essential executors of the adaptive immune system and are particularly specialized in eliminating aberrant cells that are either infected with an intracellular pathogen or of tumorous nature. Regional and functional specialization can also be observed among CD8+ T cells, especially among memory T cells that provide long-term protection against reinfection with a previously encountered pathogen (1). While central memory (CM) T cells home to secondary lymphoid organs (SLO) where they provide a stem cell-like pool of highly-proliferative antigen-specific memory T cells, effector memory (EM) T cells lack homing receptors for SLOs and patrol the body, charged with effector molecules (2, 3). In the last decade, a third memory T cell subset, referred to as resident-memory (RM) T cells, has emerged as an important guardian providing potent local immune surveillance at sites of previous infection, especially at barrier sites in the body (4, 5). T_{RM} procure superior protective immune memory in comparison to circulating memory T cells (6, 7) and presence of T_{RM} in tumors is associated with enhanced tumor control and survival (8). The generation and maintenance of this non-circulating, "sessile" immune subset is therefore the focus of intensive research efforts, for example with the aim of developing more

potent vaccines (9). Conversely, more and more reports start to emerge linking the presence of T_{RM} with chronic inflammation and autoimmune diseases (10). Consequently, we need to deepen our understanding of T_{RM} biology in order to consider protective and possible harmful functions of T_{RM} into our strategies for new therapeutic approaches. There is currently a tendency to generalize the observed T_{RM} functions across different organs, although some reports suggest that besides sharing a common differentiation program, T_{RM} generation seems to be influenced by multiple factors and also adapt to the environment of their tissue of residence. In this review, we will focus on the presumed role of T_{RM} in protective immunity, chronic inflammation and organ-specific autoimmune diseases. In particular, we will place special emphasis on CD8+ T_{RM}, as they are the best studied T_{RM} population so far. However, other resident lymphocyte populations have also been described. The latter include resident CD4+ memory T cells (11) and several resident invariant lymphocyte populations, such as liver NKT cells, gut-associated intraepithelial lymphocytes [including CD8αα T cells and (mucosal-associated invariant T) MAIT cells], and skin- and gut-resident memory γδ T cells (12–15). Moreover, resident innate lymphocyte (ILC) populations have been reported (16). Although we do not discuss these populations further in this review, some of our considerations might also apply to these cell subsets.

T_{RM} GENERATION AND MAINTENANCE

The principal hallmark of bona fide T_{RM} is their long-term persistence in non-lymphoid tissues (NLT) as a stable memory T cell pool independent of input from circulating T cells. T_{RM} are often identified by a combination of surrogate markers (see **Table 1**), the most commonly used being CD69 and CD103, which are associated to their persisting and resident phenotype. Phenotypically, T_{RM} resemble a mixture of T_{CM} cells and effector T cells expressing markers associated with homeostatic proliferation and survival, such as Ki-67 and Bcl-2, and effector function, such as Granzyme B and co-inhibitory molecules (6, 32). **Table 1** summarizes frequently used T_{RM} markers in mice and humans. However, a mere phenotypical identification without functional analysis might include circulating T cell subsets that can transiently express e.g., CD69 and CD103 (33, 34). In order to unequivocally identify T_{RM}, besides phenotypical analysis, functional experiments assessing T_{RM} tissue egress, persistence, and their disequilibrium with peripheral T_{CM} and T_{EM} cells are usually performed (6, 35). T_{RM} demonstrate a strong disequilibrium (>90%) in parabionts (36, 37) and remain stable in numbers even when recruitment of circulating T cells to NLT is inhibited (20, 27). In most NLT, with exception of the liver (25), T_{RM} are anatomically separated from the blood and therefore not accessible to intravenously applied antibodies (32, 38, 39).

In humans, T_{RM} and T_{RM}-like cells are mostly identified in a descriptive manner based on the homology with mouse T_{RM} (17, 40) and by differential gene expression when compared to circulating memory T cell subsets (19, 24, 41). While functional analyses in humans are obviously more limited, studies in patients treated with immune-ablative regimens (42, 43), or transplantations of human tissue (44) indicate that human T_{RM} -like populations identified on this basis likely constitute a similarly stable persisting T cell pool. T_{RM}-like populations in human NLT vastly outnumber T cells in circulation (17, 30, 45, 46), something that cannot be found in mice housed in a specific-pathogen free (SPF) environment, but in pet shop mice (47). Human T_{RM} will probably remain challenging to study, due to limited access to these cells and the lack of an *in vitro* culture system to this point. However, since not all aspects of human T_{RM} biology can be reproduced in SPF mice, a combined approach of mouse and human research will be instrumental to extend our knowledge about the role of T_{RM} in human health and disease.

T_{RM} Differentiation and Maintenance Program

T_{RM} mostly arise from CD127(IL7Rα)+KLRG1- memory precursor cells (22, 48, 49). Their differentiation into a long-term stably persisting and non-circulating cell population is based on two main requirements: the inhibition of tissue egress (residency) as well as longevity and/or homeostatic proliferation (maintenance). Once T cells have been recruited to the site of infection, T_{RM} precursor cells probably receive local signals from their future tissue of residence that guide the timely activation and inhibition of specific transcriptional programs. The most common mechanism is the upregulation of CD69, which antagonizes sphingosin-1-phosphate-receptor-1 (S1P1)-mediated tissue egress, and thereby confers early tissue retention until T_{RM} differentiation is complete (50–52). Most T_{RM} express CD69 constitutively and in the absence of CD69, T_{RM} generation in organs is strongly impaired (22). However, CD69 might be dispensable for long-term maintenance of fully-differentiated T_{RM}, as has been described in the lung and the thymus (53, 54). Thus, temporary CD69 expression may be sufficient for T_{RM} generation and may explain the absence of CD69 expression on a subset of long-term persisting T_{RM} in the pancreas, salivary gland and female reproductive tract (37). Loss of S1P1, and potentially other tissue egress receptors, e.g., mediated by downregulation of the transcription factor KLF2 (31), together with expression of specific adhesion molecules, confers long-term tissue residency. Further, a combination of gene expression programs otherwise involved in the differentiation of both peripheral T_{CM} and effector T cells ensure maintenance of a stable population of T_{RM} by conserving proliferative capacity as well as acquisition of constitutive expression of effector molecules (49, 55). The transcription factors known to be involved in this process have been reviewed in detail recently (56, 57). T_{RM} and T_{CM} are probably generated from the same naive precursors (58), however, the gene expression profile of T_{RM} is clearly distinct from peripheral memory T cells in mice (22, 59) and in humans (19, 24, 41). In mice, particularly the expression of transcription factors Blimp1, Hobit, and Runx3 in T_{RM} precursors seems to be essential to acquire tissue residency (49, 59). For the maintenance of stable T_{RM} population, a combination of signals stimulating longevity and homeostatic

TABLE 1 | Frequently used T_{RM} markers in mice and humans.

Marker	Expression	Proposed function	Mouse	Human
CD69	Almost all	Antagonisation of S1P1-mediated tissue egress	(4)	(17–19)
CD103	Subset *	Epithelial location via binding to E-Cadherin	(4)	(18)
CD44	All	Binding to hyaluronic acid	(20)	
Bcl-2	Subset	Longevity	(21, 22)	(23)
CD49a	Subset	Binding to Collagen and Laminin, specialization of effector function	(4, 22)	(19, 24)
CD101	Subset	Inhibition of T cell activation and proliferation	(25)	(19)
GrB	All	Cytotoxicity	(21, 26)	(24)
CD127	Subset	Homeostatic proliferation	(27–29)	(30)
S1P1low	All	Low sensitivity to tissue egress signals	(31)	(19)
S1P5low	All	Low sensitivity to tissue egress signals	(22, 26)	
CD62L low	All	Low sensitivity to tissue egress signals	(4, 21)	(19)
Ccr7low	All	Low sensitivity to tissue egress signals	(26)	(18)
CX3CR1low	Subset	Low sensitivity to tissue egress signals	(26)	(19)
KLRG1low	All	High memory potential	(22, 29)	

**Mucosal sites and skin. GrB, Granzyme B.*

proliferation seems to be necessary. Most T_{RM} express CD127 (IL7Rα), while expression of CD122 (IL2rβ), which can bind IL-2 as well as IL-15 when paired to CD132 (common γ chain, γc), seems to be more variable (22, 60). Previous studies have shown that IL-7 and IL-15-dependent longevity and homeostatic proliferation are maintaining T_{CM} by Stat5 signaling (61–63). Likewise, both cytokines have been implied to contribute to T_{RM} survival and maintenance (22, 64) and phosphorylation of Stat5 has been observed in a subset of brain T_{RM} (32). However, the sources providing homeostatic signals assuring T_{RM} long-term survival are so far still not completely known.

Tissue-specific Influences on T_{RM} Differentiation and Maintenance

The gene expression program of T_{RM} generated in different tissues is largely overlapping (19, 22, 59, 65), but some variations of this program as well as particular requirements for T_{RM} differentiation seem to exist in different experimental settings, organs and even show inter-individual variability. A particular T_{RM} phenotype and its functional characteristics are thus likely to be due to pathogen- and tissue-specific cues as well as the genetic background of the host (see **Figure 1A**). Moreover, most T_{RM} markers are not homogeneously expressed in the whole resident population (18, 68), suggesting further specialization of a particular T_{RM} population into functional subsets—even if they have been generated by one definite infection and harbor the same antigen-specificity. Differential gene expression programs and surface receptor expression on putative T_{RM} subsets are likely to confer different tissue locations and functionality, as we will further discuss below. More detailed analysis, probably using single cell-based approaches will soon identify possible T_{RM} subsets on a phenotype and functional basis.

One of the major incongruities of T_{RM} differentiation in different organs is the dependency on local antigen expression. While T_{RM} in the gut, skin and some mucosae can be generated and maintained independently of local antigen presentation (69–71), expression of local antigen seems to be required for the generation of T_{RM} in the brain (29, 32). In theory, local antigen expression serves various purposes: In a very basic manner, local antigen expression will enhance recruitment and local expansion of T_{RM} precursor cells and thereby increase the resulting T_{RM} population (72). For some organs, local antigen expression might be strictly required for tissue entry of antigen-specific T cells, as suggested for the brain (73), and thereby be essential for T_{RM} generation. In general, however, inflammatory cues, such as certain cytokines and chemokines, seem to be sufficient to promote T_{RM} differentiation, such as evidenced by so-called "prime-and-pull" and "prime and trap" vaccination approaches, which efficiently generate T_{RM} in skin, mucosae and the liver (25, 60, 70). Local antigen expression, and thus the local reactivation of T_{RM} precursor cells by antigen-presenting cells (APC), might also serve the expression of cytokines and chemokines required to guide T_{RM} differentiation and localization (55, 74), which could explain why in some experimental settings antigen is required (75), but not in others (22).

T_{RM} heterogeneity is particularly evident with regard to their expression of adhesion molecules. T_{RM} in different organs (and even further, different subsets of T_{RM}) show sometimes combined, sometimes exclusive expression of adhesion molecules such as CD103 (IntegrinαE), CD49a (Integrinα1β1), LFA-1 (IntegrinαLβ2), and E-Cadherin (22, 24, 28, 37, 46, 76, 77). Depending on their interaction partner, adhesion molecule expression on a specific T_{RM} subset probably serves its specific retention and positioning in their tissue of residence (68). CD103 mediates epithelial localization and T_{RM} retention in the skin and gut by interacting with E-Cadherin (4, 22, 69, 78), while CD49a expression anchors T_{RM} to the collagen matrix (79). Besides T_{RM} localization, expression of adhesion molecules has also been linked to T_{RM} functionality. CD103 expression has been

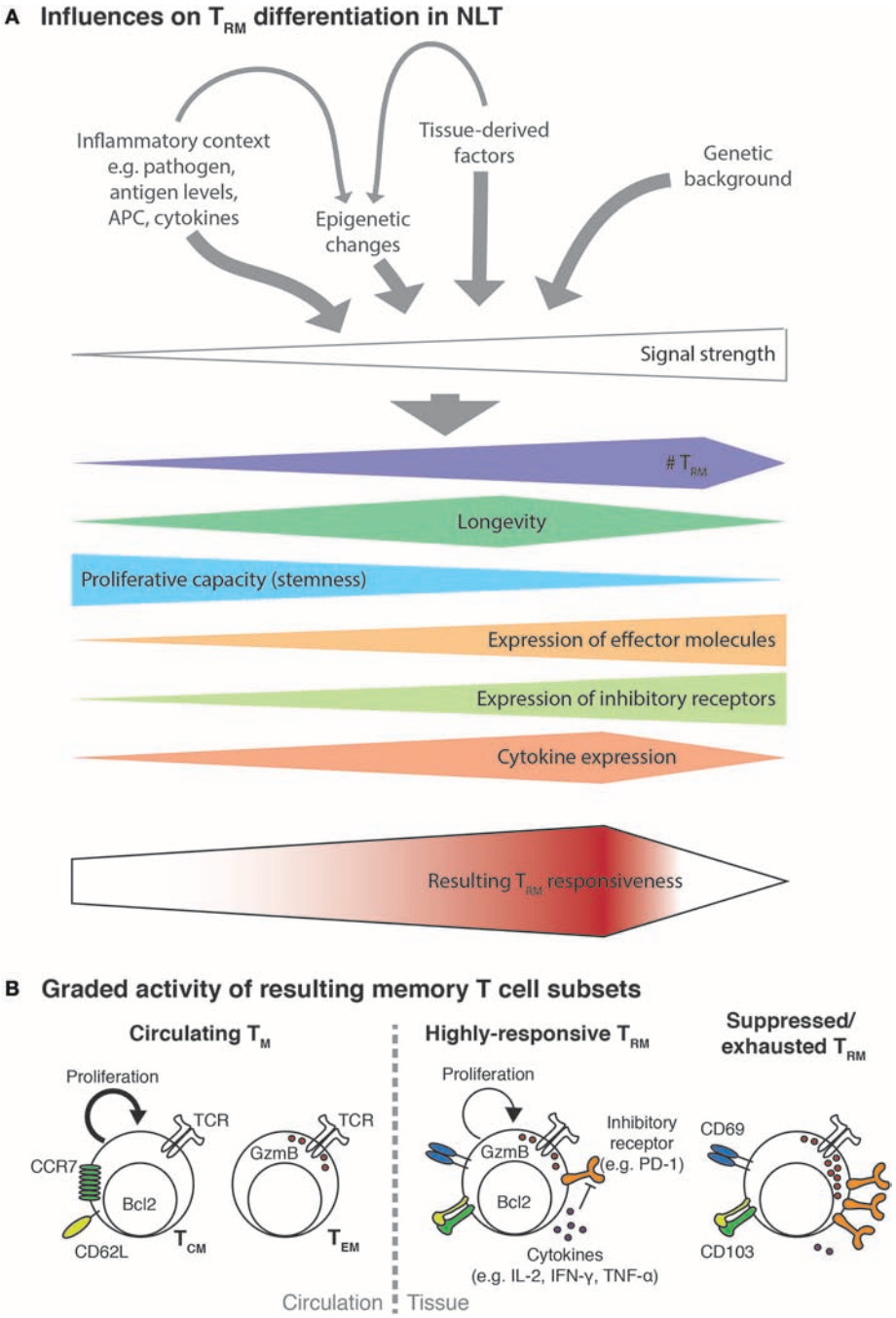

FIGURE 1 | Multiple factors influence T_{RM} functionality. **(A)** Activated T cells recruited to NLT will encounter an inflammatory environment shaped by the nature and extent of infection. The encountered signals will consist of different cytokines (e.g., Il-2, IL-12, IL-15, type I and type II interferons) potentially in concert with varying levels of cognate antigen presented on professional APC and infected cells as well as tissue-derived trophic factors, metabolites or the microbiome. Probably additionally influenced by the genetic background of the infected individual, the strength of the resulting signal to activated T cells will direct their expansion and differentiation into T_{RM}. In analogy to the signal strength model of CD8+ T cell differentiation (66), higher signal strength will result in higher T_{RM} numbers and be associated with more terminal differentiation, which manifests with progressive loss of proliferative capacity, acquisition of expression of effector molecules and increasing levels of inhibitory receptors. Encounter of very strong signals, such as during chronic infection, might lead to dysfunctional and exhausted T_{RM} and even to their elimination. The combined effect of all these factors will then determine the responsiveness of the resulting T_{RM} population to a secondary antigenic challenge or other inflammatory stimuli. **(B)** As a result of T cell activation and tissue-derived signal, circulating and resident memory T cells of different responsiveness will be generated. Circulating memory cells, namely central memory T cells (T_{CM}) and effector memory T cells (T_{EM}) show a delayed recruitment to the infected site. In addition, those cell subsets seem to specialize in either proliferative potential or immediate effector function. In contrast, a moderately strong T_{RM} differentiation signal will result in high numbers of highly-responsive T_{RM} that combine features of both T_{CM} and T_{EM} cells. Even though T_{RM} may express inhibitory receptors such as

(Continued)

FIGURE 1 | PD-1 to some degree, they can overcome this regulation e.g., due to their high expression of inflammatory cytokines (67). Highly-responsive T_{RM} can efficiently protect against re-infection but due to their low threshold for reactivation they could be prone to drive immunopathology or be involved in aberrant immune responses such as in allergies and autoimmune diseases. Alternatively, T_{RM} can be subject to regulation by regulatory T cells and other mechanisms, which may impair their longevity and/or induce a suppressed phenotype.

associated with an enhanced cytotoxic capacity of CD8+ T cells toward E-Cadherin-expressing target cells (80). Likewise, CD49a expression by human skin T_{RM} seems to discriminate between IFN-γ- and IL-17A-producing cells (24). We are however only beginning to understand how the exposure of T_{RM} precursors to their specific inflammatory context affects T_{RM} differentiation and functionality.

Cytokine redundancy (the common use of receptors and receptor subunits by different cytokines) and pleiotropy (multiple different functions exerted by one cytokine) are possible explanations for some of the observed variations in the dependency of T_{RM} generation on cytokines in different experimental contexts. Interestingly, resting non-activated T cells share a common receptor (CD122/γc) for IL-2 and IL-15. It seems therefore likely that in conditions in which T_{RM} precursors are exposed to e.g., high levels of IL-2 during the acute inflammatory response, IL-15 signaling becomes redundant for T_{RM} generation. As mentioned above, both IL-7 and IL-15 can mediate pro-survival as well as homeostatic proliferation, and a certain functional redundancy might occur between these two cytokines, depending upon which receptors predominate on T_{RM} or their precursors and which cytokine is available in the tissue niche occupied by T_{RM}. Consistent with this idea, IL-15 dependency of T_{RM} varies considerably between different organs and might be differentially required for T_{RM} differentiation, survival and/ or homeostatic proliferation (81). This could also explain why expression levels of anti-apoptotic signaling molecules in T_{RM}, such as Bcl-2, vary between organs, as do the rates of their spontaneous proliferation (22, 29, 32). Thus, it seems possible that for maintaining a stable T_{RM} population, T_{RM} longevity and potential for self-renewal can partly substitute for each other and the signals driving either process might therefore be functionally redundant to some extent. Similarly, transcriptional programming of T_{RM} precursors might vary between one tissue to another. Hobit and Blimp1 have been described to play a partially redundant role during T_{RM} differentiation, but depending on the tissue, T_{RM} generation is more dependent on one of these transcription factors than the other (59). This indicates that transcriptional regulation of T_{RM} differentiation could be incited in a different manner depending on the tissue niche and inflammatory context, possibly giving rise to T_{RM} of different reactivity and functional potential (**Figure 1A**). In support of this concept, a recent study describes that the presence of pro-inflammatory cytokines like type I interferons and IL-12 drive differentiation of CD103– T_{RM} (74), in contrast to the TGF-β-dependent differentiation of CD103+ T_{RM} (22, 55, 78).

During their differentiation and long-term maintenance, T_{RM} have to adapt to the metabolic environment of their tissue of residence. In most NLT, nutrients such as glucose and certain amino are more limited than in the circulation, and invading T cells need to adapt their metabolic processes to match their energy demands in this environment (82). While glucose plays a central role as energy source for all T subsets, activated T cells show especially high glycolysis rates and also fuel glucose-derived carbons into anabolic pathways such as fatty acid synthesis (83). Further, T cells are dependent on amino acid uptake and metabolism for full activation and differentiation (84–86). However, memory T cells critically rely on fatty acid oxidation (FAO) as an energy source (87–89), for which they synthesize long-chain fatty acids as substrates from glycolytic intermediates intracellularly (90). By contrast, T_{RM} in the skin and adipose tissue rely on uptake of fatty acids from the extracellular space (91, 92), possibly due to the limited amount of glucose available for *de novo* fatty acid synthesis. However, it remains to be determined if T_{RM} in more nutrient-rich organs such as the gut, liver and brain might show distinct tissue-specific metabolic adaptations.

Despite providing the energy for T cell expansion and survival, the metabolic environment also dictates T cell differentiation and effector function (93). Cytokine production, cytotoxicity, migration, and tissue invasiveness as well as the differentiation of memory T cells are instructed by metabolic changes (87, 94–97). One central regulator of this so-called metabolic reprogramming is mammalian target of rapamycin (mTOR) (98). mTOR is phosphorylated in response to TCR ligation, cytokine signaling as well as intracellular energy state. In turn, mTOR regulates CD8+ T cell differentiation via T-bet and Eomesodermin (99) as well as via the regulation of fatty acid metabolism (87). Inhibition of mTOR leads to a higher number of memory precursors and circulating memory T cells (94), by contrast, formation of long-lived T_{RM} in mucosal tissues is impaired (100). Interestingly, activation of mTOR (together with phosphoinositol-3-kinase) induces downregulation of KLF2 and S1P1 in activated T cells (101), indicating that mTOR activation during T_{RM} differentiation could contribute to establish tissue retention. In line with this, upregulation of CD69 on γδ T cells has been shown to enhance uptake of the amino acid tryptophan, which in turn enhanced mTOR- and arylhydrocarbon receptor (AhR)-dependent signaling pathways (102). AhR has been shown to be required for generation of T_{RM} in the skin (103), further corroborating the idea of a mechanistic link between the metabolic, possibly tissue-specific, environment encountered by T_{RM} precursors and the successful formation of a tissue-resident and long-lived T cell population.

Altogether, it seems likely that the combination of antigen load, inflammatory signals and nutrients in a tissue-specific niche creates a specific environmental context for T_{RM} differentiation and maintenance (**Figure 1**). Given that some T_{RM} niches, especially mucosal tissues and epithelial layers, undergo constant turnover and replacement of cells, it seems likely that the inflammation-induced T_{RM} niche undergoes certain changes in

cellular composition and expression of T_{RM}-maintaining factors. To date, the exact sources of these determining factors still remain largely unknown. It might even be possible that the T_{RM}-maintaining niche in some organs or under certain circumstances has only a limited lifespan, which could explain why T_{RM} are not maintained long-term in some experimental settings (104). The environmental context probably determines not only the functional features of T_{RM} residency and maintenance but also T_{RM} responsiveness toward new inflammatory stimuli during a secondary infection (see **Figure 1A**). Future studies are needed to reveal more context-dependent variations in T_{RM} generation and functionality, discovering new targets, potentially in a tissue-specific manner, for experimental and therapeutic manipulation of T_{RM}.

T_{RM} IN ACUTE-RESOLVED INFECTION

T_{RM} serve as a front-line defense against viral re-infection in various tissues. Due to their unique positioning, often directly at barrier surfaces, they can rapidly detect invading pathogens and provide immediate immune function. In comparison, immune surveillance by circulating memory T cells is slower and often allows virus spread for several days before sufficient recruitment, local expansion, and differentiation of peripheral memory T cells takes place to confine and successfully combat infection (27, 32). This notion is supported by a breadth of experimental models, that demonstrate accelerated pathogen control in the presence of T_{RM} at the pathogen entry site in comparison to circulating memory T cells alone (9). Protective functions of T_{RM} have been described for barrier tissues such as the skin (4, 27, 70, 105), the lung (106–109), the gut (48), and the reproductive tract (110). T_{RM} localized to body surfaces may thus play an important role to prevent systemic infection by recurring pathogens invading via the skin and mucosae and to limit extensive tissue damage and scarring at the entry sites. As a consequence, T_{RM} of a multitude of epitope specificities accumulate with age at these pathogen entry zones in free-living mice and humans (17, 47, 111). Interestingly, a protective role of T_{RM}-mediated immune defense has been described also for internal organs such as the liver and the brain (25, 32), which display unique immune-regulatory functions (112, 113). As such, immune cell activation is impeded in these organs, e.g., due to low expression of MHC molecules, and often occurs with considerable delay, which increases the risk of persistent and widespread infection. The latter in turn can contribute to more severe immunopathology once an immune response is finally triggered. Similar to their positioning at epithelial surfaces in barrier tissues, T_{RM} in the brain and liver are also preferentially located at potential pathogen entry sites, be it in meninges and close to brain blood vessels (32) or liver sinusoids (25). This enables T_{RM} to quickly react and eliminate invading pathogens and thereby protect these vulnerable organs from potentially harmful inflammation.

Upon re-encounter of their cognate antigen, T_{RM} employ two main paths to assure protection against the recurring pathogen. Firstly, they instantly provide highly potent cytotoxic effector functions that can eliminate the initially infected cells (barrier immunity) (27, 32). Indeed, a subset of T_{RM} constitutively expresses Granzyme B, and perforin-mediated elimination of infected cells contributes to their protective effect in the brain (32). Secondly, T_{RM} trigger a variety of local and recruited innate and adaptive immune mechanisms that can even provide bystander resistance to unrelated pathogens (39, 105, 110). T_{RM}-derived interferon-γ (IFN-γ) plays an important role by stimulating the expression of adhesion molecules and chemokines that facilitate endothelial transgression of peripheral memory T and B cells (39). Further, the expression of IFN-γ-responsive genes—many of them with direct anti-viral functions—in uninfected bystander cells limits pathogen spread (105). Moreover, Granzyme B can deactivate a viral protein in neurons during latent HSV infection without inducing neuronal apoptosis (114) and IFN-γ can even purge viruses from infected cells in a non-cytolytic manner, a process that seems important to maintain tissue homeostasis in non-regenerative tissues such as the brain (115, 116). It is important to note that the protective capacity of T_{RM} related to their cytotoxic activity and cytokine production requires the presentation of cognate antigen on MHC-I molecules, even though T_{RM} can show signs of bystander activation in an inflammatory environment (32).

The protective capacity of T_{RM} makes their generation a new objective for the development of vaccines. Indeed, skin vaccination and scarification during small pox vaccination that has now been associated with the generation of T_{RM} has been shown to provide superior protective immunity than hypodermal injection (117). Alternatively, the above-mentioned "prime and pull" and "prime and trap" vaccination strategies, in which systemic administration of a vaccine is combined with local application of chemokines or antigen, improves immunological barrier functions through T_{RM} generation (25, 60, 70). Interestingly, upon recruitment and activation in skin and mucosae, some T cells exit and give rise to SLO-associated T_{RM} (118). Being positioned at entry sites for draining peripheral antigen, these SLO T_{RM} provide a second line of defense and extend T_{RM}-mediated immune memory to the draining lymphoid tissue (119). During antigenic re-challenge, T_{RM} are the predominant population undergoing secondary expansion and together with recruited circulating T cells give rise to new generations of T_{RM} (120, 121). This implies that protective immunity mediated by T_{RM} can be boosted by repeated local immunizations. Further, infections with different pathogens can lead to a persisting T_{RM} population that contains multiple specificities at once, which provide broader and more efficient protection (122). Future vaccination approaches implementing these new insights could thus improve T-cell-mediated protection at external and internal anatomical barrier sites.

T_{RM} AND CHRONIC INFLAMMATION

Chronic inflammation results from repeated or continuous immune cell activation by recurrent or persisting antigens. Such responses are desirable to control latent-reactivating or

TABLE 2 | T_{RM} in human chronic inflammatory diseases.

Diseases	Phenotype	References
Allergic contact dermatitis	CD3+	(58)
DED	CCR7– CD45RO+/– CD69+ CD103+/–	(124)
Chronic rhinosinusitis	CD69+ S1P1–	(125)
FDE	CD69+ GrB+; CD45RA+ CD62L–CCR7– CD103+	(126, 127)
Psoriaris	CD103+; CD103+/– CD45RO+; CD103+ CD49a+ GrB+	(128–131)
Systemic sclerosis	CD69+ CD103+/–	(132)
Type I diabetes	CD69+ CD103+; CD69+ CD103+/–	(133, 134)
Multiple sclerosis	CD69+ CD103+/– GrB+/– S1P1–	(135)
HIV-1	CD69+ CD103+/– S1P1–	(136)
HBV	CD69+ CD103+/–; CD69+ CD103+/– GrB+/–	(137, 138)
HCV	CD69+ CD103+/– GrB+/–	(138)
Chronic pancreatitis	CD103+	(139)
Rasmussen's encephalitis	CD103+	(140)
HSV-2	CD69+ CD103+/–	(141)
EBV	CD103+	(142)
Breast cancer	CD69+ CD103+ GrB+	(143)
Lung cancer	CD62L– CD69+ CD103+; CCR7– CD62L– CD69+ CD103+ CD49a+ S1P1–	(144, 145)
Ovarian cancer	CD103+/–	(146)
Colorectal cancer	CD69+ CD103+/– CD49a+/–	(147)

DED, dry eye disease; FDE, fixed drug eruption; HIV-1, human immunodeficiency virus-1; HBV, chronic Hepatitis B virus; HCV, chronic hepatitis C virus; HSV-2, herpes simplex virus-2; EBV, Epstein-Barr virus.

persistent infections and to eliminate neoplastic cells. However, aberrant inflammation caused by environmental or self-antigens carries the risk of developing chronic inflammatory diseases, such as allergies and autoimmune diseases (AD). Indeed, T_{RM} have been detected in several human inflammatory diseases (10, 123) (see **Table 2**). In principle, two main roles for T_{RM} in chronic inflammatory settings can be envisaged. T_{RM} can be drivers of chronic inflammation, thereby providing a compartmentalization of the immune response. And in a not necessarily exclusive scenario, T_{RM} could trigger the bystander activation of allergen-reactive or self-reactive T cells and thereby serve as contributing triggers to chronic inflammatory diseases.

T_{RM} Functionality in the Context of Persisting Antigen

T_{RM} in Chronic Infection

One of the earliest reports on resident T cell responses came from latently-infected sensory ganglia, in which HSV reactivation was controlled by a non-circulating T cell population (148, 149). Together with the above-mentioned observations during prime-and-boost vaccinations (122), this demonstrates that T_{RM} may retain their inflammatory activity over repeated rounds of antigen stimulation. In the best scenario, this will prevent virus reactivation and ensure continuous virus latency and limitation of virus spread. Indeed, T_{RM} can be detected in sanctuaries of persistent viruses such as human and mouse Cytomegalovirus (CMV) (150, 151), Hepatitis B virus (HBV) (67), Hepatitis C virus (HCV) (138), and Human Immunodeficiency Virus (HIV) (136, 152). Interestingly, high T_{RM} numbers in HBV-infected liver and HIV-infected gut as well as clonal expansion of SLO T_{RM} have been inversely correlated to virus loads and associated with spontaneous resolution of chronic infection (67, 136, 152), provoking interest in T_{RM}-directed therapeutic approaches (137). Infection with most persisting viruses leads to chronic immune activation over time, including accumulation of a large virus-specific T cell population, a process referred to as "memory inflation" (153). Inflationary T cells can acquire a T_{RM}-like phenotype and become resident, e.g., in the salivary gland, despite being probably an ontogenically-different T cell subset (154). Chronic inflammatory tissue damage is the common long-term consequence of persisting virus infection. Since HBV-specific T_{RM} overcome immunosuppressive mechanisms in the liver and have high expression of pro-inflammatory cytokines like IL-2, IFN-γ, and TNF-α (67), it remains possible that T_{RM} are also drivers of tissue damage in the context of chronic virus infections. Therefore, a potential harmful role of T_{RM} in persisting infections merits further investigations, especially at the chronic stage of HBV and HCV infection.

So far, we understand very little about how and whether functional T_{RM} can be generated in conditions in which their cognate antigen is continuously present. Chronic high levels of antigen in some persistent infections, such as Lymphocytic choriomeningitis virus (LCMV) clone 13 or latent CMV, seem to hamper *de novo* generation of T_{RM} (69, 151). It is therefore likely that virus levels have to significantly contract after initial infection to allow for efficient T_{RM} generation, even in the context of chronic infection. Interestingly, when T_{RM} retention is impaired by lack of TGF-β signaling during chronic LCMV

clone 13 infection in the gut, a stable population of anti-viral CD8+ T cells is maintained by continuous recruitment (78), indicating that impaired T_{RM} formation can be compensated for. This suggests that depending on the cytokine milieu present during chronic infection, the local T cell pool might consist of variable proportions of T_{RM} and recruited T cells.

T_{RM} and Cancer

Tumors can be a source of neo-antigens stimulating anti-tumor CD8+ T cell responses (155, 156) and T cell infiltration is a prognostic marker for a beneficial outcome in some cancers (157, 158). Recent studies demonstrate, that a subset of tumor-infiltrating lymphocytes (TIL) in solid tumors resemble bona fide T_{RM} and are associated with its epithelial layers (159–161). T_{RM}-like TILs, in particular when they express CD103, have been associated with better prognosis (143–145, 162), a fact that could be explained by an enhanced cytotoxic efficiency upon interaction of CD103 on T_{RM} with its ligand E-Cadherin on tumor cells (80). Accordingly, experimental strategies inducing tumor-specific T_{RM} show superior tumor control in comparison to approaches that solely generate circulating tumor-specific effector T cells (163–165).

Tumor cells rely heavily on the uptake and metabolism of glucose and other nutrients, resulting in a metabolically-deprived tumor microenvironment (TME) (166, 167). Tumor-infiltrating T lymphocytes (TIL) are further subject of active immunosuppression by myeloid-derived suppressor cells (MDSC) and regulatory T cells (168). MDSC express ligands for immune checkpoint inhibitors (e.g., PD-L1 and PD-L2) and can also contribute to nutrient deprivation in the TME by uptake and metabolism of arginine. As a consequence of the increase of lactate in the TME, TIL lose cytotoxic effector functions and show impaired motility (169). T_{RM} adapt to the metabolic environment of their tissue of residence by utilizing free fatty acids (92), and are under certain circumstances resistant to checkpoint blockade (67). This indicates that tumor-specific T_{RM} might be better adapted to the immunosuppressive tumor microenvironment than their circulating counterparts (8). This opens new avenues for cancer immunotherapies. T_{RM} already present in the tumor could be functionally enhanced by checkpoint inhibitors, potentially together with increasing their catabolic fatty acid metabolism. Indeed, administration of a PPAR-α agonist or free fatty acids increases the functionality of TIL in a melanoma model, especially in combination with anti-PD-1 treatment (170). Moreover, one could envisage to genetically engineer T cells for cell therapy with the aim to promote T_{RM} generation. Recently, such an approach has been realized by modifying chimeric antigen receptor (CAR) T cells to express orthogonal IL-2 receptors allowing for a specific targeting of the transferred cell population (171). A better understanding of T_{RM} differentiation and maintenance could inform a similar strategy aiming at increasing T_{RM} differentiation, maintenance of functionality during CAR T cell therapy. In addition, reprogramming of tumor-infiltrating dendritic cells with β-glucan curdlan in a humanized mouse model of breast cancer enhances the differentiation of CD103+ TIL via DC-derived TGF-β production, resulting in rejection of an established tumor (172), highlighting how adoptive cell therapy can target T_{RM} differentiation.

It remains unknown, how tumor-associated T_{RM} are generated. Analogous to persistent infections (69), tumor-specific T_{RM} are chronically exposed to their cognate antigen, which could impair their successful differentiation. Otherwise, one may speculate that tumor-associated T_{RM} are already generated after development of the first tumor cells when there is still little cognate antigen present. This would imply that these T_{RM} could also play a role in the control of tumor transformation, since they might co-reside with a primary tumor for years. Therapeutic induction of tumor-specific T_{RM}, together with other resident populations, could enforce local anti-tumor immune response to the cancer (173) and may help to eradicate tumor cells from the body as well as reduce systemic side effects. To take advantage of this therapeutically, application of e.g., viral vectors that efficiently generate local T_{RM} with only limited numbers of peripheral tumor-specific effector cells could be envisaged. The constitutive expression of checkpoint inhibitors by T_{RM} (19) also harbors the hope that the anti-tumor activity of endogenous or therapeutically-induced T_{RM} could be further enhanced by checkpoint inhibitor blockade (174). However, one has to keep in mind, that enhancing T_{RM} activity and expansion might come with undesired side effects, and disinhibiting T_{RM} might in turn give rise to tumors. Due to their localization and non-circulating behavior, T_{RM} are refractory to most immune ablative therapies, as evidenced by mycosis fungoides, a human T_{RM}-like skin lymphoma (42).

T_{RM}-driven Chronic Inflammatory Diseases

T cells specific for self-antigens or environmental antigens are considered active drivers of diverse allergic reactions such as food and drug allergies, asthma, and diabetes, as well as autoimmune diseases such as psoriasis, inflammatory bowel disease, and multiple sclerosis (MS). In the past, these diseases were considered to be driven by effector or effector memory T cells that infiltrate the affected organ, however, several lines of evidence suggest that some of these chronic inflammatory diseases or some disease stages are instead predominantly driven by resident immune cells (10, 123). Therapeutic inefficacy of drugs inhibiting T cell recruitment indicates a putative T_{RM} involvement in disease progression since T_{RM} reside behind the blood-tissue barrier and are often refractory to systemic blockade or ablation. This applies, for example, to psoriasis (128) and progressive stages of MS (135). Interestingly, new exacerbations in fixed drug eruption (FDE) and psoriasis frequently occur at sites of previously-resolved skin lesions, indicative of an involvement of localized immune memory (175). Likewise, so-called smoldering lesions characterized by activated macrophages/microglia together with T cells at their fringes are almost exclusively observed in progressive MS (176, 177). Evidence of T_{RM} persistence has been found in resolved psoriatic skin lesions (178) and in chronic MS lesions (135), in which they constitute the dominant T cell subset (our own unpublished observations). Indeed, psoriatic normal-appearing skin contains all immune components necessary to elicit lesion formation upon an environmental trigger (44) and compartmentalized

inflammation has been correlated to cumulative brain damage during progressive MS. Beyond that, indications for T_{RM} involvement were found in many other chronic inflammatory diseases (see **Table 2**), suggesting possible common mechanisms for their development and involvement in disease pathogenesis.

One hallmark of allergic exacerbations is the short time frame (24–48 h) after exposure to the environmental trigger, in which exacerbations occur as is observed in FDE, a T cell-driven allergic local cutaneous reaction to certain administered drugs (179). This short time interval between trigger and reaction cannot be explained by recruitment of circulating T cells, which typically takes longer (58). Evidently, analog time intervals are difficult to determine for flares of autoimmune diseases, where the trigger of an exacerbation is unknown. T_{RM} generally have a low threshold for re-activation, and presence of their cognate antigen (e.g., by exogenous administration of peptide) is usually enough to elicit expression of cytolytic effector molecules and cytokines (39, 105, 110, 180). Likewise, induction of antigen expression in non-hematopoietic cells, without concomitant danger signals, is sufficient to elicit activation of resident T cells in the skin (180), probably via intermediary presentation of antigen on professional APC (180, 181). One may speculate that the reactivation threshold of T_{RM} depends upon the residence organ and/or the T_{RM}-generating stimulus, which could in turn result in organ-dependent susceptibility to allergic and self-reactive T_{RM} responses (see **Figure 1B**).

Allergen- or self-reactive T cell responses are usually considered to be elicited by preceding sensitizing events, which are hypothesized to occur in two main ways. Sequence similarities between a pathogen and an allergen- or self-antigen (also called molecular mimicry) can elicit pathogen-specific T cells that may cross-react to allergens or self-antigen upon future exposure (182). In case these T cells differentiate into T_{RM}, a compartmentalized allergic or autoreactive T cell response is the consequence. Alternatively, exposure of allergen- or self-reactive T cells to a pro-inflammatory environment, such as that generated during an infection, could elicit their bystander activation and recruitment (183). As mentioned above, non-specific inflammation can be sufficient to allow for T_{RM} differentiation in the absence of their cognate antigen (25, 60, 69, 70), making this a plausible scenario for the generation of T_{RM} specific for environmental or self-antigens. Moreover, bystander activation could also explain how T_{RM} could serve as triggers for chronic inflammatory diseases. Antigenic challenge of T_{RM} not only induces a new generation from the pre-existing T_{RM} pool, but can also activate and recruit bystander T cells of unrelated antigen-specificity, which give rise to newly formed T_{RM} (120, 121). Accumulation of T_{RM} can further lead to the displacement of other pre-existing resident immune cells (47, 103). Although this might serve to replace the more resident innate immune system by a more specific and efficient resident adaptive immune system, it carries the risk of replacing mostly "naive" immune cells by more trained, and possibly more pro-inflammatory immune cell components. Indeed, T_{RM} are often observed in clusters together with mature professional APCs and often CD4+ T helper cells as well as other immune cells, indicative of organ-associated lymphoid tissues (19, 29, 64, 184, 185). These specific niches are speculated to contribute to chronic inflammation, since they provide an optimal environment for T cell re-stimulation (186–188). Such structures have been shown to contribute to T_{RM} maintenance by chemokine and cytokine production (64, 184), however, whether T_{RM} actively sustain these immune cell clusters is not clear.

Altogether, this supports the idea that pathogen-specific T_{RM} generated during an infection could trigger and/or drive chronic inflammatory diseases. A possible connection between T_{RM} and chronic inflammation could also provide a mechanistic explanation for the observed epidemiological association of infections and the development or exacerbation of allergic and autoimmune diseases (189). In many chronic inflammatory diseases, not only CD8+ T_{RM}, but also other immune cells such as T helper (Th) cell subsets, regulatory T cells, APCs and innate lymphocytes can probably become resident and thereby contribute to a compartmentalized immune response that is resistant to many systemic immunomodulatory therapies. Thus, more research efforts are needed to understand the requirements for the differentiation and maintenance of resident immune cells in order to be able to functionally impair or even deplete T_{RM} in chronic inflammatory diseases. By identifying signaling pathways involved in T_{RM} retention and maintenance, we are currently undertaking the first steps toward a specific targeting of T_{RM} without global immunosuppression. One possibility could be to interfere with T_{RM} metabolism. Pharmacological treatments with Rapamycin (100) or inhibitors of FAO such as Trimetazidine and Etomoxir (92) have already been shown to decrease T_{RM} numbers in experimental models.

Immunoregulatory mechanisms are in place to prevent extensive T_{RM} accumulation in some organs or their over-activation. T_{RM} generation is intimately linked to TGF-β (55, 78), a cytokine associated to resolution of infection. This indicates that T_{RM} differentiation might not occur in presence of chronic antigen exposure, thereby preventing extensive T_{RM} generation in chronic inflammatory settings. Further, T_{RM} can express inhibitory receptors, such as PD-1, Lag3, and Tim3 (19, 29, 49, 107), in principle making them susceptible to checkpoint inhibition. Although T_{RM} have the possibility to overcome PD-1-associated inhibition (67), exhausted T_{RM} have been detected in immune privileged sites such as the eye (190). T_{RM} are also susceptible to regulatory T cell-mediated immunosuppression (191, 192). For the lung, liver and brain and other immune privileged sites, even mechanisms of natural suppression of T_{RM} accumulation have been suggested (104) that could assure tissue homeostasis by prevention of T_{RM} accumulation.

CONCLUSION

Vaccine strategies inducing T_{RM} against recurring infections are promising approaches to improve immunological protection. Equally, tumor-specific T_{RM} might help to eradicate aberrant tumor cells from the body and enforce a localization of this

response, thereby minimizing systemic side effects. However, several challenges have to be overcome to realize these goals, which are firstly of a technical nature. T_{RM} generation cannot be monitored in peripheral blood and therefore requires the taking of biopsies from target organs, which might not always be easily performed. Further, suitable vaccination vectors need to be designed that allow the efficient local induction of specific T_{RM} and that do not result in unwanted side effects such as bystander-induced self-reactive T_{RM}. Until now, most research studies have focused on the overwhelmingly positive role of T_{RM} acting against infected or tumorous cells, however we still lack an appropriate understanding of the possible physiological consequences of T_{RM} persistence. Further research efforts are warranted to better understand the role of T_{RM} in chronic inflammatory diseases in order to identify the risks in amplifying T_{RM} numbers or function. So far, we are lacking appropriate mouse models allowing specific genetic targeting of T_{RM} and are not able to completely deplete already-established T_{RM}. It is therefore instrumental to perform detailed preclinical and clinical studies to gain more insight into T_{RM} biology and its adaptation during different experimental regimens and in different tissues to allow for a safe and efficient therapeutic tissue targeting of T_{RM}.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Transcriptional and Epigenetic Regulation of Effector and Memory CD8 T Cell Differentiation

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Immune protection and lasting memory are accomplished through the generation of phenotypically and functionally distinct CD8 T cell subsets. Understanding how these effector and memory T cells are formed is the first step in eventually manipulating the immune system for therapeutic benefit. In this review, we will summarize the current understanding of CD8 T cell differentiation upon acute infection, with a focus on the transcriptional and epigenetic regulation of cell fate decision and memory formation. Moreover, we will highlight the importance of high throughput sequencing approaches and single cell technologies in providing insight into genome-wide investigations and the heterogeneity of individual CD8 T cells.

Keywords: CD8 T cell, memory differentiation, cell fate decision, transcriptional, epigenetic, single cell sequencing

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INTRODUCTION

During an acute viral or bacterial infection, pathogen-specific T cells robustly proliferate, acquire effector functions, and migrate to the site of infection to eliminate the pathogen. The majority (>90%) of antigen-specific CD8 T cells die via apoptosis upon pathogen clearance, leaving behind distinct memory subsets with unique phenotypic and functional properties. However, the molecular and genetic mechanisms that guide how these cell fate decisions are made remains incompletely understood. Additionally, although it is well-appreciated that antigen specific memory CD8 T cells can persist for extended periods of time in a functionally quiescent state, and that this is important for conferring long-term protective immunity against previously encountered pathogens, the underlying mechanisms that endow memory CD8 T cells with this longevity remain unclear. Moreover, the molecular pathways that help maintain the phenotypic and functional heterogeneity of memory subsets, and enable memory CD8 T cells to remain poised to quickly recall their effector function are still incompletely understood. Current evidence suggests that multiple signals, such as T-cell receptor (TCR), co-stimulation, inflammation, and metabolic signals can orchestrate CD8 T cell fate decisions, with some of these commitment choices occurring early in the immune response (1, 2). As the incorporation of multiple distinct signals received by individual T cells likely triggers diverse transcriptional programs, it is important to discuss the key transcription factors that have been known to orchestrate CD8 T cell fate decision. Moreover, we highlight the field's current understanding of CD8 T cell differentiation on the epigenetic and single-cell level, and provide a brief discussion on how modern technologies may help to refine the CD8 T cell differentiation paradigm.

MEMORY CD8 T CELL DIFFERENTIATION AND CELL FATE DECISION

The process of memory CD8 T cell selection is not entirely stochastic, as originally proposed (3), as effector cells can display inherently distinct memory cell potential, with some CD8 T cells being intrinsically better at persisting and populating the memory pool. It was previously identified that a small subset of effector T cells survive the contraction phase and serve as the precursors of the memory CD8 T cell compartment (4–8). This minor population of effector cells, termed memory precursor effector cells (MPECs), can be distinguished based on their high expression levels of CD127, the IL-7 receptor alpha (IL-7Rα), and their decreased expression of killer cell lectin-like receptor G1 (KLRG1) (5, 6). Other surface proteins that co-segregate with increased IL-7Rα expression on MPECs include CD27, CD28, CD62L, and CXCR3 (1). By contrast, a larger proportion of effector CD8 T cells display high expression of KLRG1 and low expression of IL-7Rα and are more terminally differentiated than their MPEC counterparts. This subset of KLRG1^{hi} effector CD8 T cells is collectively referred to as short-lived effector cells (SLECs). Of note, although MPECs and SLECs were observed in various infectious settings in different species including humans, these phenotypic distinctions are not exclusive criteria for forming memory T cells nor do they represent universal markers for memory precursor cells across all types of immune response (9–11). Furthermore, several studies previously demonstrated that MPECs can give rise to both T central memory (T_{CM}) and T effector memory (T_{EM}) populations (1, 5–7) and recent evidence further indicates that the precursors of tissue resident memory cells (T_{RM}) in the skin and small intestine are also derived from less differentiated KLRG1^{lo} memory T cell precursor cells (12, 13). It is important to note however that these phenotypic distinctions are not exclusive criteria for memory T cell formation, as cell death may also occur among IL-7Rα^{hi} effector T cells following infection, and many long-lived KLRG1^{hi}IL-7Rα^{hi} memory CD8 T cells have been observed following secondary infections (14–17). Moreover, the frequency of KLRG1^{hi} cells can vary widely depending on the type of infection or vaccination. Indeed, a recent study further highlighted the limitations of these markers and elegantly demonstrated that some KLRG1^{hi} cells can downregulate KLRG1 during the contraction phase and differentiate into all memory T cell lineages (18). Thus, a higher degree of developmental plasticity than previously appreciated may exist during the effector to memory CD8 T cell transition phase. Importantly, however, these cell surface markers do offer a useful framework of determining the relative memory cell potential of effector CD8 T cells in several circumstances, and they have become invaluable for identifying molecular pathways that regulate these effector-to-memory cell fate decisions.

Heterogeneity of Memory CD8 T Cell Subsets

As CD8 T cells transition from naïve to effector to memory cells, their overall gene expression profiles changed, resulting in phenotypic and functional variations among the different populations. As such, several fundamental studies have demonstrated that memory CD8 T cells can be compartmentalized into at least 3 distinct subsets on the basis of their effector function, proliferative potential, migration patterns and transcriptional program (19–23). For well over a decade, the population of circulating memory CD8 T cells has been broadly categorized into two distinct subsets, conventionally designated T_{CM} and T_{EM} (20, 24). These two subsets can be distinguished based on their differential expression of CCR7 and CD62L (L-selectin), with T_{CM} cells expressing both of these lymph node homing receptor molecules which facilitates their trafficking to and retention within secondary lymphoid tissues (19, 20). By contrast, T_{EM} cells lack expression of CCR7 and CD62L and are most commonly found in the blood and in non-lymphoid tissues (e.g., lung, liver, intestine) (20, 21, 25). Compared to T_{EM}, T_{CM} cells display an enhanced proliferative potential and an increased capacity to produce the cytokine IL-2, but are unable to immediately produce effector molecules until they undergo secondary proliferation and differentiate into effector cells (20, 26–28). Conversely, T_{EM} cells constitutively display effector functions such as cytolytic activity and IFN-γ production (1, 21, 29, 30). Notably, within the past 10 years, T_{RM} have emerged as the third major memory CD8 T cell subset and have been identified to permanently reside in peripheral tissues after pathogen clearance and provide site-specific protection upon re-infection (22, 23). The specific anatomical location of where T_{RM} cells develop and are maintained can depend on the nature or route of the infection and the inflammatory signals experienced during the effector phase of the T cell response (31). T_{RM} cells can generally be distinguished from T_{EM} cells infiltrating non-lymphoid tissues based on their high expression of CD69 and the integrin CD103 (12, 32–35), although not all T_{RM} cells constitutively express CD103 (31, 34). An important component of T_{RM} differentiation is the migration of T effector cells to target sites (such as the skin or intestines) and their subsequent downregulation of tissue egress receptors, such as S1PR1 (35) and upregulation of adhesion molecules, such as CD103 (12). Other distinguishable features of T_{RM} cells are their sustained expression of granzyme B (that may vary by location) and their maintained high levels of mRNAs encoding TNFα, IFNγ, and IL-2 (31), which allow them to eliminate any re-occurring microbial threat at portal entry sites. Whether T_{RM} undergo homeostatic proliferation to maintain a stable population has not been clearly demonstrated. T_{RM} cells from brain, skin and mucosal sites showed much lower homeostatic proliferation ability and turnover rate compare to their circulating counterparts (22, 31, 36, 37). Interestingly, T_{RM} cells in the lung airway may require constant replenishment from recirculating memory cells (38). As T_{RM} and T_{EM} subsets display constitutive effector functions and occupy the frontline sites of pathogen entry, they are uniquely positioned to be among the first responders of the adaptive recall response. Conversely, T_{CM} recall is critical for the rapid generation of a pool of secondary effector cells that may help contain pathogens that breach the initial containment. In humans and mice, there is a newly defined subset, called T memory stem (T_{SCM}) cells.

The characteristic of T_{SCM} is stemness. T_{SCM} cells represent increased proliferative, self-renewal and long-term persistence capacity (39, 40). Additionally, only naive T cells and T_{SCM} cells were able to reconstitute the entire heterogeneity of memory T cell subsets, indicating that T_{SCM} cells are multipotent (39). In patients undergoing haploidentical hematopoietic stem cell transplantation (HSCT), T_{SCM} cells are preferentially generated from naïve cells and the dominant long-term clonotypes appeared to preferentially originate from infused T_{SCM} rather than T_{CM} clones (41, 42). Gene expression data also showed that there is a progressive change moving from naïve to T_{SCM} to T_{CM} and T_{EM} cells (39). These evidences indicate that T_{SCM} cells are at the apex of the hierarchical tree of T cell differentiation and at a hierarchically superior level over the T_{CM} cells (40). Moreover, memory T cells can be further subdivided based on differential expression of additional phenotypic markers. As one example of such endeavor, CX_3CR1 has been recently used to identify a peripheral memory (T_{PM}) subset that possesses high cytotoxicity and provides global immune surveillance (43, 44). Collectively, the formation of these distinct memory CD8 T cell subsets and their division of labor likely ensures optimal protective immunity upon pathogen re-challenge. However, a key question that remains to be addressed is whether these distinct memory subsets are maintained by signals from the tissue microenvironment or preprogrammed by cell-intrinsic mechanisms, such as transcription profiles and the chromatin landscape.

The Impact of Signal Strength on CD8 T Cell Fate

During infection or vaccination, naïve CD8 T cells engage with antigen-presenting dendritic cells (DCs) and are presented cognate peptide in a major histocompatibility complex (MHC) class 1-restricted manner (45, 46). Upon TCR-mediated recognition of the MHC-peptide complex, antigen-specific CD8 T cells will start to rapidly proliferate and acquire effector functions and the ability to migrate to sites of infection. During this process of T cell priming, newly activated T cells will integrate multiple signals in the form of TCR signaling, co-stimulation, cytokine, chemokine, and metabolic signals, all of which can have a major impact on the accumulation, survival, and cell-fate decision of effector T cells (1, 2, 47) (**Figure 1**).

TCR signaling is one of the initiating signals that helps shape T cell memory. The strength and quality of TCR signaling, which is determined by the affinity of the TCR for peptide–MHC molecules (pMHC), the dose of antigen presented by APCs, the duration of the TCR–pMHC interaction, and the timing of TCR recognition (early or late during infection phase) have been shown to partially contribute to memory commitment, function and the diversity of the memory pool (48). The balance between co-stimulatory and co-inhibitory signaling is not only required for effector T cell activation and expansion, but also determines the size and quality of the memory T cell pool (49). Co-stimulatory molecules, such as CD28, 4-1BB, CD27 and OX40 have been shown to promote memory formation as well as contribute to secondary responses (50, 51). As for co-inhibitory signals, it has previously been reported that lower PD-1 expression may drive T cell differentiation away from a SLEC fate and skew toward T_{EM} memory generation (52). TIM-3 is another inhibitory receptor and blockade of TIM-3 increases transcription of genes involved in T cell effector function and differentiation but decreases expression of genes associated with memory T cell formation (52, 53). Further studies are required to determine how co-stimulatory and co-inhibitory signaling pathways coordinately regulate memory T cell development.

Several studies have identified that exposure to certain inflammatory signals can play a major role in regulating the differentiation of effector and memory CD8 T cell subsets

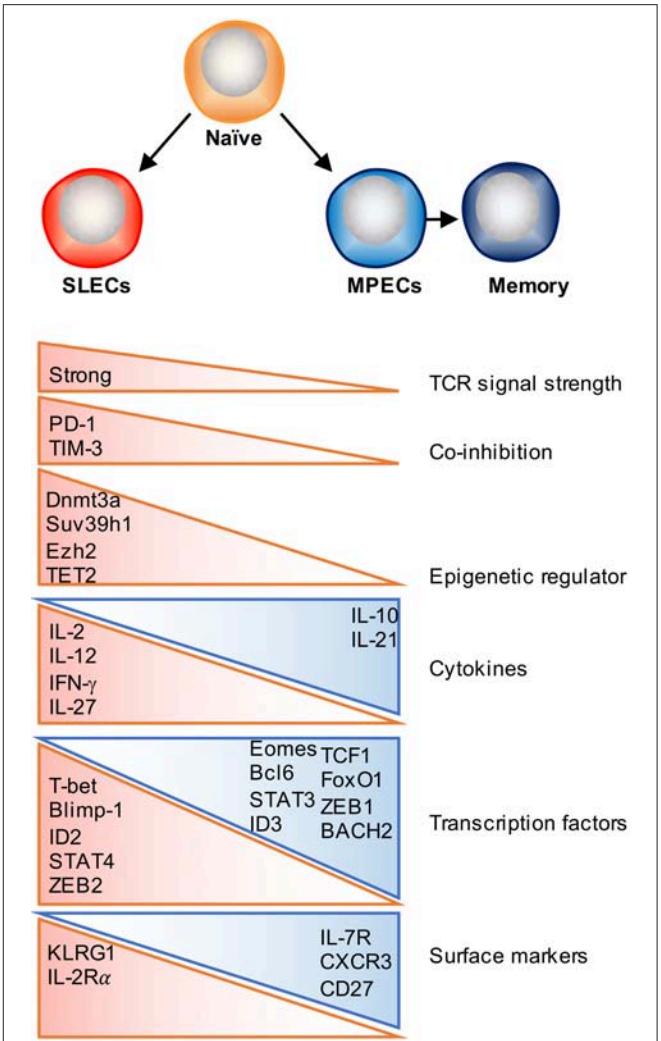


FIGURE 1 | Factors that regulate effector and memory cell fate decision. Following activation, antigen-specific naïve CD8 T cells proliferate and differentiate into a heterogeneous pool of effector T cells that consist of two major subsets: SLECs and MPECs. Majority of SLECs die by apoptosis during contraction phase, whereas MPECs survive and become long-lived memory cells. Numerous factors as depicted can contribute to this cell fate decision process, which include TCR signal strength, co-stimulatory/co-inhibitory molecules, cytokines, transcription factors, and epigenetic regulators.

in a context dependent manner. For example, IL-12 or IL-27 enhances SLEC formation during acute bacterial or viral infection, whereas type I and type II interferons can either promote memory or enhance SLEC differentiation under different settings (17, 54–59). Other studies have further identified that exposure to IL-15 helps skew CD8 T cells along a memory pathway (60–62), whereas IL-2 signaling is implicated in promoting the differentiation of short-lived effector T cells (63, 64). However, the effects of IL-2 signaling on CD8 T cell memory formation may be regulated in a temporal manner, as administration of recombinant IL-2 (rIL-2) during the expansion phase diminishes T cell survival, whereas treatment with rIL-2 during the contraction phase promotes T cell proliferation, survival, and memory formation (65). By contrast, IL-10 and IL-21 signaling through a STAT3-SOCS3 pathway was found to promote memory formation, potentially by insulating T cells from excessive inflammatory stimuli (66). Recent studies have also begun to shed light on potential cytokine signaling pathways that contribute to T_{RM} development, with recent findings elucidating an important role for the cytokine transforming growth factor β (TGF-β) and IL-15 in facilitating T_{RM} differentiation by inducing CD103 expression on T_{RM} precursor cells infiltrating the skin, lung, and small intestine (12, 13, 34, 67, 68).

TRANSCRIPTIONAL REGULATION OF EFFECTOR AND MEMORY CD8 T CELL DIFFERENTIATION

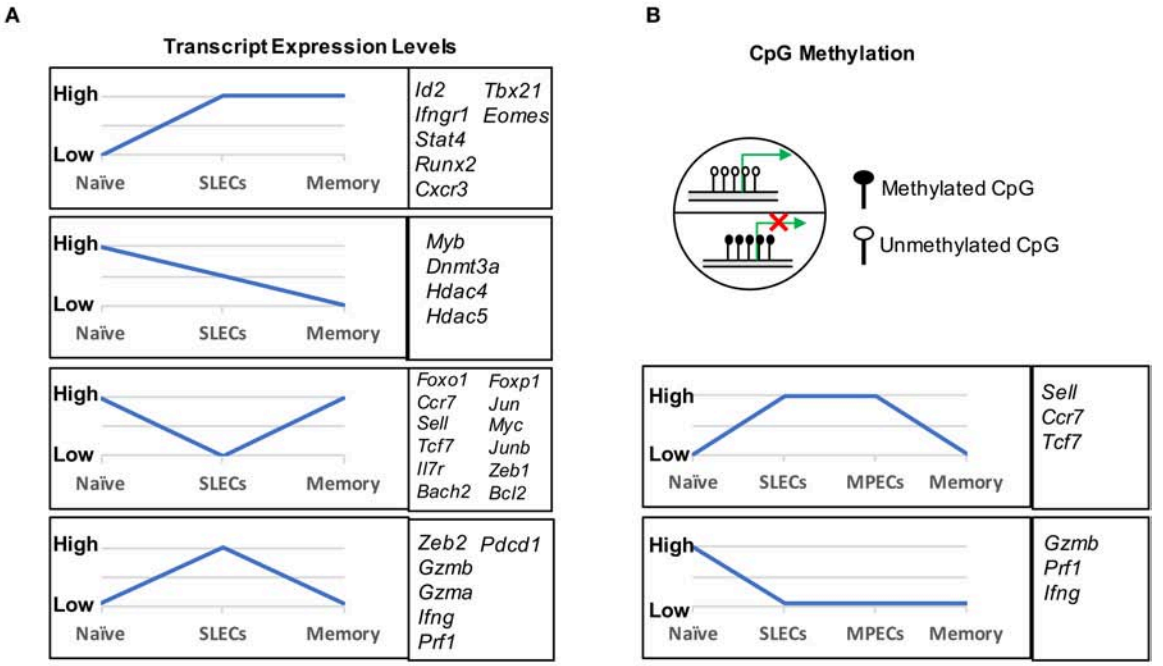
Pioneer Transcription Factors Initiate Effector Differentiation

During naïve to effector transition, dynamic changes occur at both the transcriptional and epigenetic level (**Figures 2A**, **3**). Learning from CD4 differentiation (69), the fundamental identity of these heterogeneous effector CD8 T cells can generally be established by upstream "pioneer transcription factors" that regulate the entire transcriptional network to initiate early effector differentiation. Additionally, current evidence suggests that the majority gain-of-methylation and loss-of-methylation events, which represent a repressed and active transcription state respectively, happen within the first 4 days of activation, and more than half of these differentially methylated regions (DMRs) were similarly acquired in both SLECs and MPECs (70) (**Figure 2B**). Furthermore, effector and memory CD8 T cells have been found to share a more similar pattern of chromatin accessibility as compared to naïve CD8 T cells (71). Among these shared accessible regions, the binding motifs for bZIP, IRF, and T-box transcription factors are highly enriched (71–73) (**Figure 2D**). This then brings to a question, which transcription factors are initiating this early effector differentiation? Among naïve CD8 T cells, bivalency ($H3K4me3^{+}H3K27me3^{+}$) was observed at the promotors of transcription factors that are known to be crucial for initiating an effector program, such as T-bet, Eomes, Blimp1, and IRF4 (74) (**Figure 2C**). This finding indicates that these transcription factors may remain poised in naïve T cells but rapidly start transcription by acquiring a permissive histone methylation signature upon TCR stimulation within 24 h (74). Indeed, it has been demonstrated that IRF4 cooperates with BATF (belongs to AP-1 family) to serve as "pioneer transcription factors" that promote chromatin accessibility and gene expression associated with various aspects of effector CD8 T cell differentiation (75–78). In addition, Runx3 is another potential "pioneer transcription factor" that can initiate changes in chromatin accessibility after CD8 T cell activation, especially at the binding sites of IRF, bZIP transcription factors, and Blimp1 (73) (**Figure 2D**).

Transcriptional Regulation of CD8 T Cells Fate Decisions: Terminal Differentiation or Memory Formation?

After the initial expansion phase, effector T cells can be bifurcated into two distinct effector populations, SLECs and MPECs. Early on in the effector phase, the chromatin landscape has already been universally prepared by "pioneer transcription factors," and now lineage-specifying transcription factors start to take effect. Considering that TCR signal strength is negatively associated with memory formation (2, 48), it is possible that TCR-induced transcription factors can influence the type of progeny derived from a single T cell. One such transcription factor is IRF4, expression of which is highly dependent on the signal strength of TCR signaling (79). Indeed, IRF4 has been found to be crucial for initial expansion and promoting SLEC formation (79). In addition, the expression level of memory associated transcription factors, Eomes and TCF1 appear to be highly sensitive to graded expression levels of IRF4 both in acute and chronic viral infection, indicating potential mechanisms by which IRF4 may regulate CD8 T cell fate decision (79, 80). Furthermore, Nuclear receptor subfamily 4 group A member 1 (NR4A1) supports formation of MPECs and T_{CM} via inhibiting the expression of IRF4 by directly binding to its promoter region (81). Similarly, the transcription factor BACH2 represses genes associated with terminal differentiation by binding to their enhancer regions and attenuating the availability of AP-1 binding sites (82). In this manner, BACH2 suppresses the differentiation of SLECs and tips the balance in favor of generating memory cells (82). Collectively these findings indicate that TCR-responsive transcription factors, such as IRF4 and AP-1 family members establish effector differentiation while NR4A1 and BACH2 suppress effector-associated genes. Thus, these transcription factors may cooperatively or antagonistically regulate cell fate decisions in response to different TCR signal strength intensities.

Importantly, there is an ever-expanding list of transcription factors known to orchestrate various signals experienced during the effector phase to polarize terminal differentiation or memory formation. STATs are cytokine-induced lineage-specifying transcription factors. Inflammatory cytokines, such as IL-2, IL-12, IFN-γ, and type I IFNs, signal through STAT1, 2, 4, and 5 respectively, and direct effector CD8 T cell proliferation and differentiation by inducing T-bet and Blimp-1 expression, and downregulating Bcl6, TCF1, and IL-7Rα expression (6, 83, 84). Conversely, STAT3 activation, which is induced by IL-10



C

Histone modifications

Activating	
H3K4me1	Active enhancer regions. Enhancers associated with transcriptionally poised genes.
H3K4me3	Activating H3K4me3 and repressive H3K27me3 at gene promoters represents a bivalent chromatin signature.
H3K9ac	Lost in effector cells at memory associated genes
H3K27ac	Active enhancer regions
Repressive	
H3K27me3	Increased in SLECs at genes required for survival and memory
H3K9me2	
H3K9me3	

D

Transcription Factors Motifs Enrichment

	Naïve	SLECs	MPECs	Memory
TCF1	■			■
E2A	■			■
FoxO1	■			■
AICE (BATF/IRF4)		■	■	
AP-1 (bZIP)		■	■	
T-bet		■	■	
Blimp-1		■	■	
RUNX3		■	■	

FIGURE 2 | Transcriptional and epigenetic profiling during naïve to effector to memory transition. **(A)** Gene clusters defined by the mRNA expression levels in naïve, SLECs and memory cells. Four major expression patterns emerged: genes that were up- or downregulated during the effector stage and persisted into the memory phase, and genes that were up- or downregulated during the effector phase and then reverted to the naive state. **(B)** CpG methylation levels of different genes in naïve, SLECs, MPECs and memory cells. Naïve/memory genes are similarly acquired CpG methylation in both SLECs and MPECs. MPECs, not SLECs, have the capacity to erase their newly acquired methylation programs and re-express naïve/memory genes as they develop into memory CD8 T cells. SLECs and MPECs both show demethylation of several effector-associated genes which remain demethylated in memory cells for a long period of time. **(C)** Histones posttranslational modification (PTM) and their functions that are essential for CD8 T cell differentiation. For example, the epigenetic bivalency for H3K27me3 and H3K4me3 represent an epigenetic state from which a gene can be rapidly activated or repressed depending on the differentiation pathways. **(D)** Differentially enriched motifs of transcription factors in naïve, effector and memory cells. Motif analysis identified the cell-subsets specific transcription factors binding sites in enhancer or promoter regions. Gray depicts highly enriched motifs.

and IL-21, is necessary for memory formation by promoting the expression of memory related transcription factors, such as Bcl6, Eomes, the SOCS3 (66, 85, 86). Moreover, T-bet/Eomes, Id2/Id3 and Blimp-1/Bcl6 (1), and the newly defined ZEB1/ZEB2 axis (87, 88) have reciprocal expression patterns in SLECs and MPECs, and drive differentiation toward opposing cell fates (**Figure 1**). As cell identity determined by complicated gene regulatory networks, further studies should focus on how these networks cooperatively regulate downstream target genes and cell fate decisions.

Transcription Factors Promote Memory Maintenance

Transcription factors that are critical for naïve T cell homeostasis have also been identified to promote memory CD8 T cell self-renewal and maintenance. For example, FoxO1, TCF1, and LEF1 are all highly expressed in naïve CD8 T cells, downregulated in effector cells, and re-acquired in memory cells (84, 89–93) (**Figures 2A,D**). Their expression is continuously required for the long-term survival and homeostatic proliferation but not the initial activation and clonal expansion, or effector function (91, 94–96). FoxO1 promotes the expression of pro-memory and pro-survival genes, such as *Il7r, Bcl2, Sell, Ccr7, Eomes, Tcf7, Bach2, Zeb1, and Socs3,* potentially by shielding these genes from deposition of repression associated histone 3 lysine 27 trimethyl (H3K27me3) chromatin modifications (91, 93). TCF1 and LEF1 are downstream factors of the Wnt-signaling pathway and their downregulation in effector cells is due to cell cycle and IL-12-dependent CpG methylation at the TCF1 promoter (84). Intriguingly, TCF1 and LEF1 can induce deacetylation at effector genes regions, such as *Prdm1*, to favor memory formation (97).

Transcriptional Regulation of Tissue-Resident Memory CD8 T Cells

In parallel with circulating memory cell subset differentiation, T_{RM} acquire a unique transcriptional program during differentiation and adaptation to a particular microenvironment (98–101). As early as 7 days after acute infection, a unique transcriptional signature and chromatin landscape is already established in intestinal intraepithelial lymphocytes (IELs) (102). The transcription factor Runx3 has been identified as a central regulator for T_{RM} specification by controlling a core tissue-residency gene-expression program in barrier tissues (such as lung, skin, and small intestine) and non-barrier tissues (such as salivary glands and kidney), as well as in tumors (102). Blimp-1 and its homolog protein, Hobit, establish a universal transcriptional program of tissue-residency in lymphocytes, and they have been shown to be required for T_{RM} retention in the gut, skin, liver, kidneys and lung by promoting CD103 expression while repressing *Klf2, S1pr1,* and *Ccr7* expression (99). In addition, Notch controls T_{RM} maintenance by promoting CD103 expression and regulating metabolic programs (98). Recently, NR4A1 was shown to be critical in regulating the tissue residence and function of human T_{RM} (103), and AhR was also shown to be required for skin T_{RM} (104). By contrast, the transcription factors ZEB2, T-bet (87), and KLF2 (100) have been demonstrated to inhibit T_{RM} formation by promoting tissue egress. Although T-bet and Eomes can inhibit T_{RM} formation, certain levels of T-bet expression are required for CD122 expression and IL-15 mediated T_{RM} survival (105).

THE ROLE OF EPIGENETICS IN THE CELL FATE DECISION OF CD8 T CELLS

A critical feature of memory CD8 T cells is their ability to rapidly re-acquire effector functions upon secondary challenge with the same pathogen. We are now learning that changes in the epigenetic landscape of memory CD8 T cells, including DNA methylation, histone modifications, and chromatin accessibility, play a substantial role in this phenomenon. In this section, we will discuss how these epigenetic changes shape the effector and memory fate decision as well as memory T cell formation and function (**Figure 3**).

Differences in the Epigenetic Landscapes of SLECs and MPECs Underlie Their Divergent Cell Fate Decisions

DNA methylation occurs primarily at CpG dinucleotides with the cytosine being methylated. Genomic regions with high frequencies of these CpG dinucleotide sequences are known as CpG islands and are often found in promoters. DNA methylation is commonly thought of as a repressive epigenetic mark, exerting its downstream effects by influencing transcription factor binding and acting as a docking site for various histone modifying enzymes (**Figure 2B**). In CD8 T cells, the DNA methyltransferase Dnmt3a has been shown to reduce MPECs formation by catalyzing DNA methylation at sites such as the promoter of *Tcf7*, a critical transcription factor for memory CD8 T cells (106). TET2 is methylcytosine dioxygenase and mediates active DNA demethylation. TET2 gene expression is rapidly and transiently induced by TCR signaling. TET2-deficient CD8 T cells rapidly acquired memory associated surface markers such as CD62L, CD27, and CXCR3 to promote memory formation (107). Interestingly, while naïve genes become methylated and effector genes become demethylated in both MPECs and SLECs, MPECs erase these DNA methylation marks at naïve genes as they develop into long-lived memory CD8 T cells, indicating that epigenetic repression in the form of DNA methylation can be reversed (70) (**Figure 2B**).

Genomic DNA is packaged in nucleosomes, comprised of DNA wrapped around histone octamers made up of two copies each of the histones H2A, H2B, H3, and H4. Each histone has a flexible N-terminal tail that is subject to post-translational modifications that subsequently influence transcription of nearby genes. These modifications can affect gene expression by recruiting other transcriptional regulators or, in the case of acetylation, by neutralizing the positively charged histone N-terminal tail and decreasing its interaction with negatively charged phosphates on DNA. Large-scale genomic studies have found patterns of histone modifications that can identify cis-regulatory elements such as promoters and

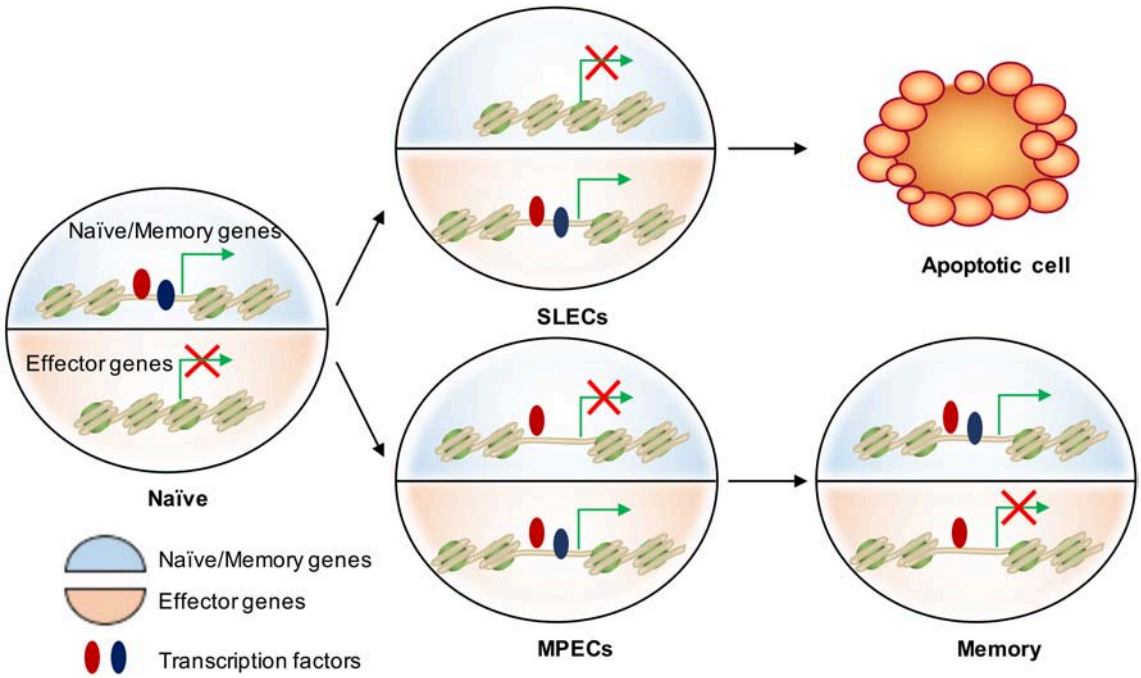


FIGURE 3 | Epigenetic regulation of cell fate decision during acute infection. There are two major transcriptional circuits in regulating CD8 T cell differentiation: one of them associated with effector function and another one is essential in naïve/memory cells controlling T cell quiescence and homeostasis. In naïve CD8 T cells, naïve/memory genes are open (epigenetically by TFs in red) and on (transcriptionally by TFs in blue), while the effector genes are closed and off. When naïve cells are activated, effector genes are turned on mainly by "pioneer TFs" (in red) in both SLECs and MPECs. These genetic regions remain open but poised as MPECs develop into memory cells. Transcriptional repression of naïve/memory genes in MPECs cells can be reversed in memory CD8 T cells through recruiting additional TFs (in blue) to restart gene expression. In contrast, SLECs lose the accessibility at these TF-bound cis-regulatory elements and therefore permanently turn off the naïve/memory gene expression. This leads to their loss of memory potential and long-term survival.

enhancers, as well as provide information regarding their activity (108–111) (**Figure 2C**). Additionally, active promoters and enhancers tend to have a central region that is depleted of nucleosomes, where transcription factors can more easily access their binding sites. It is therefore reasonable to suspect that a combination of histone modifications and accessible regions also contribute to the enhanced function of memory CD8 T cells. From studies investigating chromatin accessibility using assay for transposase-accessible chromatin (ATAC)-seq (112) and the deposition of histone modifications (H3K4Me1, H3K27Ac, H3K27Me3) by chromatin immunoprecipitation (ChIP)-seq in CD8 T cells during acute infections with *Listeria monocytogenes* and lymphocytic choriomeningitis virus (LCMV), we now have a genome-wide overview of the epigenetic changes accompanying memory CD8 T cell differentiation (71, 72, 113). These studies provide important insights into the epigenetic differences between MPECs and SLECs and through which their differentiation is regulated. Regulatory regions that are more open in MPECs than SLECs are genetic loci regulate feature genes related to naïve and memory T cell properties. However, these regulatory regions are less open or permanently silenced in terminally differentiated SLECs or exhausted CD8 T cells, suggesting that MPECs keep their memory potential through maintaining accessibility at critical memory-related cis-regulatory elements (71). Terminally differentiated SLECs have increased levels of the repressive histone modification H3K27Me3 at genes required for survival and memory cell formation, and deposition of this mark is catalyzed by the polycomb repressive complex 2 (PRC2) (93). The histone methyltransferase Suv39h1 also promotes terminal differentiation by trimethylating histone H3 lysine 9 at memory-related genes, repressing their expression (114). These differences in the epigenetic landscape between the two subsets of effector CD8 T cells provides a potential mechanism for their divergent gene expression profiles and cell fate decisions.

Epigenetic Changes in Memory CD8 T Cells Allow for Rapid Activation

The chromatin accessible regions of memory CD8 T cell are quite similar to effector cells, especially around effector gene regions (115). Moreover, their promoter regions remain demethylated from effector to memory transition (70, 115). Much work has been done investigating DNA methylation at the *Ifng* locus in CD8 T cells, which encodes the important cytokine IFNγ that is rapidly expressed by memory cells (116–120). Naïve CD8 T cells possess substantial DNA methylation at the *Ifng* promoter, at least in part due to the activity of the DNA methyltransferase Dnmt1 (117). After activation, effector CD8 T cells have this site demethylated and turn on the expression of *Ifng*. Despite no longer expressing *Ifng*, memory CD8 T cells maintain a

demethylated state at the *Ifng* promoter, thereby decreasing the number of steps required before gene expression. Help from CD4 T cells during initial activation appears to play a role in this process (119). Similar patterns seem to exist at the sites of other critical CD8 T cell effector molecules, including *Gzmb* and *Prf1*, which were found to maintain their demethylated state for at least 12 years in humans who received the yellow fever virus (YFV) vaccine (115). Therefore, regulation of DNA methylation provides a mechanism for the ability of memory CD8 T cells to quickly respond to infection.

Levels of histone H3 acetylation (119, 121) and, more specifically, H3 lysine 9 acetylation (H3K9Ac) contributes to the rapid reactivation in memory CD8 T cells (122–124). Furthermore, several studies have characterized a number of different histone modifications and chromatin accessibility at a genome-wide level over the course of a CD8 T cell response to infection or vaccination (71, 74, 93, 113, 115, 125–128). In the same study mentioned earlier, YFV-specific CD8 T cells in vaccinated humans maintain open, accessible chromatin at the promoters of the effector molecules *Ifng* and *Gzmb* (115). Overall, the establishment of specific patterns of DNA methylation, histone modifications, and chromatin accessibility prime memory CD8 T cells to more rapidly produce effector molecules and clear the pathogen.

Transcription Factors Regulating the Epigenetic Landscape of CD8 T Cells

Individual transcription factors can affect the epigenetic landscape through the recruitment of chromatin modifying enzymes or their own intrinsic activity. Blimp-1, for example, directly binds to the genes *Il2ra* and *Cd27*, recruits the histone methyltransferase G9a and the histone deacetylase HDAC2, and leads to increased deposition of the repressive marks H3K9Me2, H3K9Me3, and H3K27Me3 and decreased levels of permissive marks H3Ac and H3K4Me3 (129). The AP-1 factor BATF has been proposed to act as a pioneer transcription factor, in cooperation with its binding partner IRF4, by directly binding to tightly packed chromatin and promoting its accessibility to other transcription factors (130). Runx3 was recently shown to drive memory CD8 T cell formation by regulating chromatin accessibility of memory cell *cis*-regulatory elements (73). While TCF7 has not yet been shown to affect the epigenetic landscape during the differentiation of activated mature CD8 T cells, it establishes critical regions of open chromatin during T cell development in the thymus (131). Additionally, studies performed in thymocytes have shown that TCF7 has intrinsic histone deacetylase activity (97). Given its importance in memory CD8 T cell formation (94, 95, 132), it is likely that TCF7 uses a combination of these two methods to regulate the memory differentiation process. Other transcription factors will likely continue to be identified that either directly or indirectly lead to epigenetic changes in activated CD8 T cells, and untangling this complex network of transcription factors and the epigenetic changes they induce will help decode the differentiation of memory CD8 T cells.

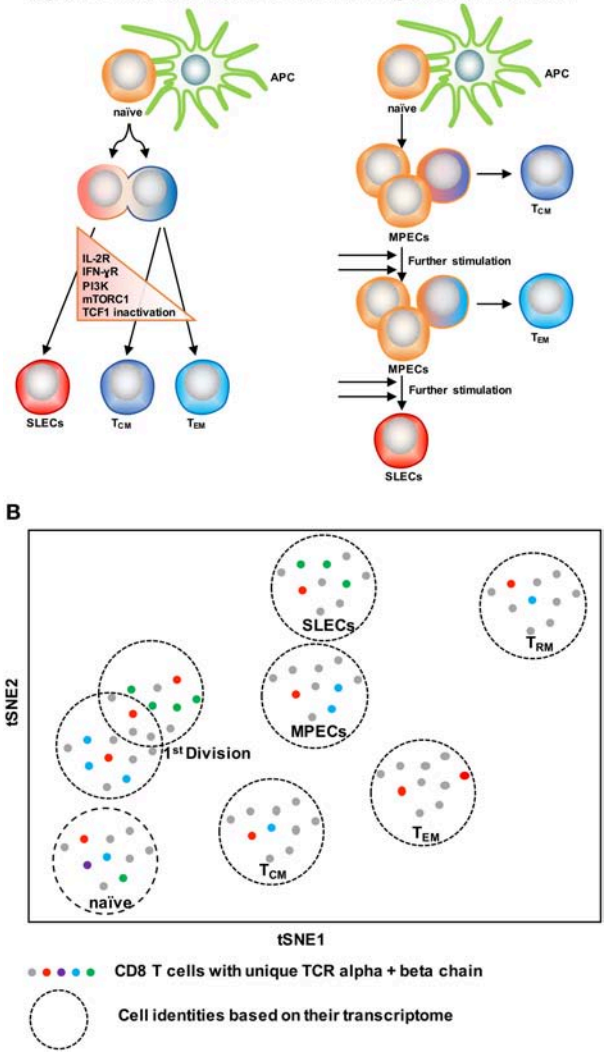


FIGURE 4 | Two CD8 T cell differentiation models. **(A)** The asymmetric division model emphasizes the significance of asymmetric segregation of cytokine receptors and signals pathways as early as the first division in dictating the memory or effector potential of T cells. The proximal daughter cell (red) inherits molecules that make it more likely to become an effector cell, while the distal daughter (blue) inherits more memory-related molecules. The progressive differentiation model is a linear model in which the cumulative history of encounters with antigen and inflammation dictate the cell fate from a memory-like stage to terminally differentiated cells. **(B)** A method to depict "one cell, one fate" and "one cell, multiple fates" models. Single cell identity of a T cell can be profiled based on their transcriptome using scRNA-seq. TCRs are nature molecular tags to track T cells. Integration of TCR clonotypes to a gene expression profile on a single-cell level can monitor the dynamics of effect and memory CD8 T cell fate decision during infection.

CELL FATE DETERMINATION OF CD8 T CELLS AT SINGLE CELL LEVEL

Previous studies show that there can be anywhere from ∼80 to 1,200 naïve CD8 T cells or from ∼20 to 200 CD4 T cells specific for a particular epitope in one mouse (133, 134). Following

infection, each antigen specific CD8 T cell can interpret and integrate signals in a distinct way to create differential responses in the generation of terminally differentiated effector cells and self-renewing memory T cells (6, 20). However, when and how this fate decision is made following infection has been a topic of research for many years (127, 135–139).

Different Experimental Approaches to Study the Cell Fate of Single CD8 T Cells

In terms of fate specification from a single T cell, two obvious possibilities can happen: (1) one T cell can give rise to two daughters cells with each being capable of choosing multiple fates or (2) one T cell can give rise to daughter cells with only one fate (140). Different experimental approaches have been applied to understand the *in vivo* fate of single CD8 T cells following acute viral or bacterial infections. Using an OT-I TCR transgenic adoptive cell transfer model, it has been demonstrated that diverse cellular progeny, including both effector and memory T cells, could develop out of a single naïve T cell following infection with *L. monocytogenes* (135). Similar results have been found using tetramer enrichment to isolate antigen specific naïve CD4 T cells followed by a single cell adoptive transfer approach for the *in vivo* fate mapping for CD4 T cells (141). Surprisingly, in both cases single naïve T cells displayed diverse patterns of differentiation, yet when combined together, they resembled the endogenous T cell response in the same individual mouse. Although these studies were instrumental in developing our understanding of T cell fate decision at the single cell level, a limitation of these approaches is that they only allow for deciphering the fate of one T cell at a time per mouse. To overcome this hurdle and to facilitate the analysis of multiple T cell families at the same time, one elegant study performed adoptive transfer experiments using barcoded TCR transgenic CD8 T cells. Upon bulk transfer of single barcoded naïve CD8 T cells the authors demonstrated that individual naïve T cells have multiple fates and can differentiate into both effector and memory subsets during acute infection (136). This approach offers the opportunity to analyze large numbers of barcoded TCR transgenic single naïve CD8 T cells and their fates at the same time. However, this experimental strategy is limited by its dependency on using indirect approaches (microarray, sequencing) for barcode identification and by its inability to conduct a functional assessment of T cells at the protein level. Notably, other powerful tools have emerged that help alleviate some of these pitfalls. To serve the purpose of analyzing multiple T cell families simultaneously, adoptive transfer experiments have been accompanied with the use of a matrix co-expressing congenic markers, followed by their breeding to TCR transgenic mice (137). This innovative approach allowed for the transfer and assessment of eight naïve TCR transgenic CD8 T cells at a time and revealed differential subset diversification by each single cell resulting in broad and vigorous CD8 T cell immunity. To rule out any TCR-based influence, a limiting dilution strategy has been developed with the aim of transferring a single naïve antigen specific CD8 T cell into recipient mice, which is plausible mathematically but *in vivo* difficult to prove (138). With this approach, single naïve CD8 T cells have been found to exhibit differential cell fates as well as display some extreme bias toward a particular cell fate. Importantly, using the latest powerful technology- single-cell RNA sequencing (scRNA-seq), it has recently been demonstrated that virus-specific CD8 T cells display vast transcriptional heterogeneity and can give rise to multiple cell fates, which unexpectedly was found to occur as early as the first cell division (127). This study highlights the power of using scRNA-seq and computational analyses to elucidate cell-fate decisions at the singe-cell level.

Two Models of CD8 T Cell Differentiation

With the knowledge of possible cellular fates (single fate vs. multiple fates) at the single cell level, the next question to ask is: how does this subset diversification occur following infection? There are two possibilities to support this: the model of asymmetric division driven differentiation vs. progressive differentiation model (140, 142) (**Figure 4A**). According to the asymmetric division model, the generation of long and short-lived progenies from a single precursor T cell occurs at the immediate onset of response, i.e., as early as the first cell division (143). Asymmetric segregation of cytokine receptors like IL-2Rα and IFN-γR and intracellular signaling pathways like PI3K and mTORC1 during mitosis (92, 139, 143–146) can cause proximal and distal daughter cells to have differential cytokine signaling that may lead them toward an effector or memory cell differentiation process, respectively. Supporting this, three groups (127, 138, 139) have found that at the single cell level, cellular bifurcation is possible during early rounds of cellular division in response to acute infection. On the other hand, the progressive differentiation model supports the subset diversification process from a single cell via a gradual differentiation process, from a memory-like stage to terminally differentiated cells, which is affected by the signaling strength of the signals that are received in the priming phase (115, 142). This model has been supported by a study (147) using unbiased mathematical model and probabilistic framework. It has been shown that a linear developmental pathway is responsible for cell fate diversification, that progresses from slowly proliferating memory precursors to the rapidly expanding effector population. However, none of these models alone can explain why during differentiation some cells take multiple fates while some show extreme bias toward a singular fate. On this note, it is important to consider that cellular differentiation is a dynamic process and can be accompanied by encountering stochastic initial priming events, which can make a difference in the fate of every single cell, depending on their reception and interpretation of various signals. In this respect, both T cell intrinsic factors like: signaling strength, co-stimulation, amount of cell intrinsic signaling molecules, the epigenetic landscape, and cellular metabolism and also cell extrinsic factors like: anatomical location to interact with APC, and inflammation can affect the fate of a single T cell undergoing differentiation (1, 148–150). Intriguingly, a recent finding that showed, depending on the developmental origin of naïve CD8 T cells: either fetal derived CD8 T cells or adult bone marrow derived CD8 T cells, can give rise to either memory-like

CD8 T cells in adulthood or in the generation of naïve-like CD8 T cells, respectively (151). The diverse *in vivo* response generated at the single cell level during acute infections may potentially be a result of the recruitment of heterogeneous naïve CD8 T cells, a topic, which demands further research. In terms of technological advancement, it is now possible to do *in vivo* fate mapping of single naïve CD8 T cells in a way which was limited previously with the usage of cell number, while simultaneously accounting for the influence of TCR and more importantly to recapitulate an *in vivo* natural infection scenario without the reliance on adoptive transfer strategies.

ScRNA-seq has been emerged as an innovative platform to understand the cellular development and differentiation process (127, 152, 153). With the power of computational analysis, it also offers an assessment of the subset diversification and developmental trajectory in an unbiased manner without reliance on the preexisting knowledge of cellular types (153, 154). To understand single T cell fate and its kinship with subsequent progenies, it is ideal to trace the cell fate decision by using a natural T cell lineage barcode, the TCR sequence (155) (**Figure 4B**). Using TCR sequencing to uncover the identity of single T cells was limited with the determination of both TCR alpha and beta chain information in a single cell (156–158). With the use of more powerful algorithms, it is now possible to reconstruct TCR alpha-beta gene information from single cell RNA sequencing data and to couple the cellular identity of a T cell with its transcriptomic profile at the single cell level (159, 160). This approach can overcome the usage of TCR transgenic T cells and can allow for *in vivo* single cell fate mapping by observing and tracing thousands of single T cells simultaneously in a natural infection setting (152–154).

CONCLUDING REMARKS

Current studies on genome-wide transcriptional and epigenetic changes during infection have revealed that DNA methylation, histone modifications and transcriptional signatures define CD8 T cell subsets and regulate CD8 T differentiation. Eventually, an identification of a core set of transcription factors or epigenetic regulatory molecules that can regulate memory formation could potentially be sufficient to help reprogram terminally differentiated CD8 T cells. Such findings will undoubtedly have an impact on T cell-based therapies and vaccine designs. Although the epigenetic patterns associated with distinct T cell subsets are starting to be unraveled, additional functional analyses are needed to further reveal the role of epigenetic modifying proteins and their relationship to key transcription factors that coordinately work together to determine cell-fate decisions. Moreover, as naïve CD8 T cells go through tremendous changes in their cell cycle, metabolism, cell signaling, and genetic landscape, it is starting to become well-appreciated that individual effector cells may acquire distinct cell fates, that as a whole results in the generation of a heterogeneous pool of memory T cells. While our current understanding of CD8 memory formation is derived from investigations using pooled cell populations to study cell fate decisions, recent technological advances in scRNA-seq and computational approaches hold great promise for deciphering the true transcriptional heterogeneity of individual CD8 T cells.

AUTHOR CONTRIBUTIONS

YC, RZ, DS, and AK wrote the manuscript. YC, RZ, and WC edited the manuscript.

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REVIEW
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Dendritic Cells and CD8 T Cell Immunity in Tumor Microenvironment

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Dendritic cells (DCs) play a central role in the regulation of the balance between CD8 T cell immunity vs. tolerance to tumor antigens. Cross-priming, a process which DCs activate CD8 T cells by cross-presenting exogenous antigens, plays a critical role in generating anti-tumor CD8 T cell immunity. However, there are compelling evidences now that the tumor microenvironment (TME)-mediated suppression and modulation of tumor-infiltrated DCs (TIDCs) impair their function in initiating potent anti-tumor immunity and even promote tumor progression. Thus, DC-mediated cross-presentation of tumor antigens in tumor-bearing hosts often induces T cell tolerance instead of immunity. As tumor-induced immunosuppression remains one of the major hurdles for cancer immunotherapy, understanding how DCs regulate anti-tumor CD8 T cell immunity in particular within TME has been under intensive investigation. Recent reports on the Batf3-dependent type 1 conventional DCs (cDC1s) in anti-tumor immunity have greatly advanced our understanding on the interplay of DCs and CD8 T cells in the TME, highlighted by the critical role of $CD103^+$ cDC1s in the cross-priming of tumor antigen-specific CD8 T cells. In this review, we will discuss recent advances in anti-tumor CD8 T cell cross-priming by $CD103^+$ cDC1s in TME, and share perspective on future directions including therapeutic applications and memory CD8 T cell responses.

Keywords: $CD103^+$ cDC1s, CD8 T cell immunity, anti-tumor immunity, cross-priming, tumor microenvironment, cancer immunotherapy

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INTRODUCTION

Cancer is characterized by the accumulation of genetic mutations and the loss of normal cellular regulatory functions (1). The identification of tumor-associated antigens (TAAs) that separated cancerous cells from non-transformed healthy cells, and the observation of tumor antigen-specific CD8 T cells in cancer patients have greatly advanced our understanding on tumor immunology and formed the basis for antigen-specific immunotherapy (2). The first human tumor antigen recognized by CD8 cytotoxic T lymphocytes (CTL) was identified in melanoma and was designated melanoma-associated antigen (MAGE)-1 (3). The isolation of tumor-specific CTL from peripheral blood or tumor tissue of patients from various cancer patients provided evidence for existence of CD8 T cell-mediated anti-tumor immunity (4–7). The detection of TAA-specific CD8 T cells in spontaneously regressing tumors further supported the importance of tumor-specific CD8 T cell responses (5). It is well accepted now that CD8 T cells play a central role in mediating anti-tumor immunity, and their effector CTLs eliminate tumor cells by recognizing tumor-associated antigens presented on major histocompatibility complex class I (MHCI) by their expressed T cell receptor (TCR). Indeed, studies have shown that infiltration of T cells, especially CD8 T cells into tumor microenvironment, correlates with better prognosis in multiple malignancies such as breast, lung, melanoma, colorectal, and brain cancer (8, 9). However, even when tumor-specific CD8

T cell responses were observed, they rarely provided protective immunity as tumors often evade immune surveillance by dampening T cell effector and memory functions (10, 11). Promising cancer immunotherapies that aim to boost CD8 T cell-mediated anti-tumor immunity include DC cancer vaccines, adaptive cell transfer (ACT) of tumor-reactive T cells, either native (CTL clones or Tumor infiltrated lymphocytes–TIL) or engineered to express tumor antigen-specific TCR or chimeric antigen receptors (CAR), and immune checkpoint blockade (ICB) such as anti-PD-1, anti-PD-L1, and anti-CTLA-4 (2). Among them, immunotherapies with ICB and CAR T cells have achieved unprecedented clinical efficacy leading to a number of drugs being approved by the FDA. However, a majority of patients still fail to respond to these checkpoint or CAR T cell therapies, and many patients that do respond often experience relapse (12). While direct presentation of tumor antigens onto their MHCI by tumor cells play an important role in effector function of CD8 T cells, cross-presentation by professional antigen presenting cells in particular DCs are required for prime naive CD8 T cells and sustaining the cytotoxic immune responses (13). Thus, increasing efforts has been made to repair and enhance insufficient T cell priming by DCs to further improve the efficacy of immunotherapies with ICB and CAR T cells due to DCs' critical role in priming and directing CD8 T cells to target tumor cells (12, 14). Indeed, the ability of DCs to cross-present exogenous tumor-associated antigens onto MHCI molecule to prime CD8 T cells is the foundation of the "Cancer-Immunity cycle" proposed by Chen and Mellman (11). Thus, better understanding the interaction of CD8 T cells and DCs would be critical to improve the efficacy of current cancer immunotherapies.

DENDRITIC CELLS AND TUMOR MICROENVIRONMENT

Ralph Steinman was awarded the 2011 Nobel Prize for Medicine or Physiology for his pioneering work on DCs (15). As the sentinel of the immune system, DCs play a central role in linking innate and adaptive immune responses (16). Known as the most potent professional antigen presenting cells (APCs), DCs initiate all adaptive immune responses by uptaking, processing and presenting antigens including tumor antigens to activate naive antigen-specific CD4 and CD8 T cells (17). Since their identification in 1973 (18), DC development and the regulation of their function have been under intensive study. DCs originate in bone marrow from macrophage/DC progenitors (MDP) that give rise to common DC progenitors (CDP), which then differentiate into two major DC subsets: classical/conventional DCs (cDCs) and plasmacytoid DCs (pDCs) (19–26). Murine cDCs consist of two subtypes currently described as cDC1s ($XCR1^{hi}CD24^{hi}CD26^{hi}CD11c^{hi}MHCII^{hi}CD11b^{lo}CD172a^{lo}F4/80^{lo}CD64^{lo}Lin^{lo}$, type 1 cDCs) and cDC2s ($CD11b^{hi}CD172a^{hi}CD26^{hi}CD11c^{hi}MHCII^{hi}XCR1^{lo}F4/80^{lo}CD64^{lo}Lin^{lo}$, type 2 cDCs), and their human counterparts are $CD141^{+}$ DCs (also known as $BDCA3^{+}$) and $CD1c^{+}$ DCs (also known as $BDCA1^{+}$), respectively (27, 28). These two subtypes of cDCs differ in their transcriptional factor dependency, function and phenotypes (23, 24). cDC1 cells include lymphoid tissue $CD8\alpha^{+}$ cDC1s and migratory $CD103^{+}$ cDC1s (29). cDC1 cells rely on interferon regulatory factor 8 (IRF8) and basic leucine zipper transcriptional factor ATF-like 3 (Batf3) for their development, and are specialized in presenting internalized exogenous antigens onto MHCI to prime CD8 T cells by cross-presentation (30). cDC2s depend on interferon regulatory factor 4 (IRF4) for their development and comprise a heterogeneous population that are very efficient in presenting internalized antigens on MHCII to activate CD4 T cells (31–34).

pDCs are a multifunctional population best known for their specialized ability in producing and secreting large amount of type I interferons (IFNs) (35–37). pDCs also express high level of IRF8 similar to cDC1s, but require the E2-2 transcription factor for their development (38). E2-2, encoded by TCF4, is a member of the E family of basic helix–loop–helix transcription factors (39). In both mice and humans, E2-2 is required for the differentiation of pDCs from CDPs (38). Induced deletion of E2-2 in mature pDCs results in the acquisition of cDC-like properties, such as dendritic morphology, MHCII and CD8α expression, and the ability to induce proliferation of allogeneic CD4 T cells (40). Deletion of E2-2 in pDCs also induces the expression of ID2, which is required for cDC1 development. Murine pDCs express Siglec-H, B220, Ly6c, PDCA1 (CD317) and intermediate level of CD11c, and human pDCs express HLA-DR, CD123, BDCA2 (CD303), and BDCA4 (CD304) but not CD11c (36, 41). Initially reported as IFN-producing cells (IPCs), pDCs have been extensively studied for their function in sensing viral RNA and DNA by toll-like receptor (TLR)-7 and−9 (42, 43). In addition to their function in producing IFNs, pDCs have also been shown to play an important role in immune tolerance. In autoimmune diseases, aberrant activation of pDCs has been implicated in the pathogenesis of psoriasis, systemic lupus erythematosus (SLE), and IFN-related autoimmune diseases (36, 44, 45).

Monocytes that arise from MDPs could also differentiate into another DC subset named Monocyte-derived inflammatory DCs (inf-DC) under conditions such as inflammation, cancer and infection (46). The inf-DCs have been shown to both activate antigen-specific CD4 T cells and cross-present tumor antigens to activate CD8 T cells, and their presence has been found to be important for the efficacy of cancer immunotherapy (47–49). Recently, TNF/iNOS-producing DCs (TIP-DCs), a novel type of inf-DCs that produce TNF-α and nitric oxide (NO) was shown to be critical for tumor growth control upon treatment with adaptive CD8 T cell transfer (50).

The TME is a specialized niche composed of tumor cells, fibroblasts, endothelial cells, infiltrating leukocytes, and extracellular matrix components. TIDCs have been found in many cancer types including breast, lung, renal, head and neck, gastric, colorectal, bladder and ovarian cancers (51). However, in general within the TME tumor cells are able to adapt their environment to favor tumor growth, evade immune surveillance and confer resistance to immunotherapies

(52, 53). A key mechanism in achieving tumor immune evasion is through modulation of DC function by tumors and tumor-associated cells/factors in the TME. Thus, despite the presence of DCs in TME and their potential in generating anti-tumor immunity, TIDCs often exhibit impaired or defective function, thus might mediate immunosuppression instead (41, 54). Indeed, the TME employs a variety of mechanisms to modulate DCs to suppress their ability to induce anti-tumor responses.

DC-SUPPRESSIVE MOLECULES IN TME

A number of factors such as IL-6, Macrophage colony-stimulating factor (M-CSF), IL-10, Vascular endothelial growth factor (VEGF), and Transforming growth factor beta (TGF-β) that are present in TME have been shown to negatively regulate DC functions (55, 56). IL-6 and M-CSF, cytokines secreted by tumor cells, have been shown to switch the differentiation of $CD34^+$ progenitors from DCs to $CD14^+$ monocytes that failed to mediate allogeneic T cell proliferation (57, 58). Tumor-derived IL-6 has been shown to negatively regulate DC function by inhibiting their maturation and migration, affect the differentiation of hematopoietic progenitor cells from DCs to macrophage, and induce tolerogenic phenotypes of DCs (59–61). In the TME, a variety of cells such as tumor cells, myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), DCs, and Tregs, have been shown to produce IL-10 (62). IL-10 has been shown to suppress DC function by inhibiting different aspects of DC biology, such as DC maturation, their ability to secret IL-12, their capacity in antigen presentation and priming of T cells (63, 64). IL-10 has also been shown to convert immunogenic DCs into tolerogenic DCs leading to the induction of anergic cytotoxic CD8 T cells (65). In addition, IL-10 derived from tumors has also been shown to switch differentiation from monocytic precursors to immunosuppressive TAMs rather than DCs (66). VEGF has been shown to inhibit differentiation and maturation of DCs (67, 68). Tumor-derived TGF-β significantly suppresses DC function and their ability to initiate anti-tumor immune responses by inhibiting DC maturation (69, 70).

Several factors such as VEGF, TGFβ, IL-1β, IL-13, Granulocyte-macrophage colony-stimulating factor (GM-CSF) and prostaglandins, that are produced by tumor cells and other cells in the TME, have been shown to inhibit DC differentiation from progenitors and promote their differentiation into immunosuppressive cells such as MDSCs and TAMs (71).

Another mechanism used by TME to evade immune detection is by modulating DC function to skew T cell differentiation. Factors in TME, such as Matrix metalloproteinase 2 (MMP-2) and Thymic Stromal Lymphopoietin (TSLP) have been shown to modulate DC function to induce detrimental Th2 responses (72, 73). Tumor-produced TSLP has been shown to up-regulate OX40L expression on DCs, thus inducing the generation of Th2 cells that produce IL-4 and IL-13 that have been shown to promote tumor growth in breast and pancreatic cancer (74, 75).

Several signaling pathways such as β-catenin, MAPK and STATs that are active in cancers also play critical roles in crosstalk between tumor cells and DCs in the TME (76, 77). β-catenin signaling in melanoma cells has been shown to inhibit the recruitment of T cells and DCs into tumors (78). Melanoma-derived Wnt ligand Wnt5α has been shown to increase the production of Indoleamine 2,3-dioxygenase (IDO) by TIDCs via β-catenin signaling, leading to increased generation of T_{reg} cells (79). Conditional knockout of Wnt co-receptors LRP5 and LRP6 on DCs, on the other hand, enhanced DC-mediated anti-tumor immunity leading to delayed tumor growth (80). In addition, activation of β-catenin in DCs from tumor-bearing mice exhibited a more tolerogenic phenotype and mediated the suppression of DC vaccine-induced cross-priming of anti-tumor CD8 T cells through IL-10 (81, 82).

REGULATORY T CELLS

Regulatory T cells (Tregs), working in concert with tolerogenic DCs, play critical roles in the establishment and maintenance of an immunosuppressive TME to inhibit anti-tumor immunity (83). Tregs are comprised of a heterogeneous population of T lymphocytes that have shared the ability to suppress immune responses, with the $CD4^+CD25^+Foxp3^+$ Tregs being most studied. These Tregs express the inhibitory receptors CTLA-4, Tim-3, PD-1, GITR, LAG3, and BTLA that exert their suppressive function on DCs through different mechanisms. For example, Tregs have been shown to inhibit DC maturation by down-regulating the expression of co-stimulatory molecules such as CD80 and CD86 through CTLA-4 (84). Engagement of CTLA-4 on Treg by CD80/CD86 on DCs has been shown to up-regulate both human and murine DCs' production of IDO (85), which then activate antigen-specific regulatory T cells to induce potent suppressor activity (86, 87). In turn, IDO-activated Tregs have been shown to induce the up-regulation of the inhibitory PD-L1 on DCs (88). In addition, Tregs secrete IL-10 and TGF-β, two of the main immunosuppressive cytokines that are known to induce DC dysfunction (89, 90).

EXPRESSION OF INHIBITORY LIGANDS

The expression of inhibitory molecules, such as PD-L1, PD-L2, Tim3, LAG3 contributes to the suppressed function of DCs in tumors and tumor-draining LNs. It has been reported that tumor-derived factors up-regulate Tim3 expression in tumor DCs (91). TIM-3 on DCs then inhibits anti-tumor responses and reduces the efficacy of cancer treatments by binding to high-mobility group box 1 protein (HMGB1), a damage-associated molecular pattern molecule involved in cytosolic nucleic acid recognition in the TME. In addition, signaling via TIM-3 on both BMDCs and splenic DCs has been shown to inhibit DC activation and maturation (92). For PD-L1, $CD103^+$ DCs from tumor-draining

LNs have recently been shown to have increased expression of PD-L1 compared to non-draining LN DCs (93). PD-L1 and PD-1 blockade has been shown to reverse DC dysfunction leading to enhanced T cell immunity (94, 95), suggesting that PD-L1/PD-1 signaling negatively regulates DC function.

INHIBITION OF ANTIGEN PRESENTATION FUNCTION OF TIDCs

Tumor cells escape immune surveillance by disabling the process of tumor antigen presentation. Recent studies have shown that DCs in TME often exhibited impaired capacity in cross-presentation (25, 96). The TME can specifically modulate DCs' antigen presentation function by targeting the molecules and machinery directly involved in antigen presentation, for example, decreasing the expression of their MHCI and MHCII molecules and their regulators such as CIITA, down-regulation of genes such as ER-resident aminopeptidases (ERAP) and transporter associated with antigen processing (TAP) (97). Abnormal accumulation of lipids in DCs has emerged as an important mechanism for DC dysfunction, as TIDCs from multiple tumor models and cancer patients exhibited reduced capacity in cross-presentation because of lipid accumulation (98, 99). Supporting this notion, a recent study has shown that the accumulation of lipids in TIDCs was involved inplay arole in blunting inhibiting anti-tumor T cell responses in ovarian cancer (100).

pDCs IN TME

Recruitment of pDCs to the tumor microenvironment has been reported in a variety of cancers, however, these tumor-infiltrated pDCs are often tolerogenic, favoring tumor progression. High tumor infiltration by pDCs has been associated with poor prognosis in melanoma, head and neck, breast, and ovarian cancers (45, 101–103). pDCs have been shown to induce the generation of Tregs in the TME and tumor-draining LNs (88, 104). pDCs can also stimulate the generation of Tregs by their expression of ICOS-L, and ICOS-L expression on pDCs has also been shown to be associated with breast cancer progression (105, 106). On the other hand, pDCs have also been shown to promote immunogenic anti-tumor responses if properly stimulated, as therapeutic activation of pDCs have shown efficacy in melanoma, basal cell carcinoma, and T cell lymphoma (25, 41, 103, 107).

DENDRITIC CELLS IN CROSS-PRIMING OF ANTI-TUMOR CD8 T CELLS AND BEYOND

Cross-priming, a process which DCs activate CD8 T cells by cross-presenting exogenous antigens (108, 109), plays a critical role in generating anti-tumor CD8 T cell immunity (110–115). Anti-tumor CD8 T cell responses are induced in three sequential steps: (1) tumor antigen uptake and cross-presentation; (2) tumor antigen-specific CD8 T cell priming by DCs, and (3) elimination of tumor cells by effector CTLs (116). However, TME-mediated suppression and modulation of TIDCs often leads to their dysfunction, resulting in failure in cross-priming (step 1 and 2) and suppressed anti-tumor CD8 T cell immunity. Indeed, DC-mediated cross-presentation of tumor antigens in tumor-bearing hosts often induces T cell tolerance instead of immunity (110). However, not all TIDCs within TME exhibit suppressive and/or regulatory functions. For example, the infiltration of BDCA3$^+$ cDC1s in the TME has been shown to correlate with increased T cell infiltration and improved prognosis in cancer patients and better efficacy of cancer immunotherapies, highlighting the critical positive role of cDC1 in generating anti-tumor immunity in the TME (78, 117). Thus, recent discoveries on the critical role of cDC1s in particular CD103$^+$ cDC1s in CD8 T cell cross-priming in tumors have generated much interest, and have offered opportunities for improved cancer immunotherapies (96).

The generation of Batf3$^{-/-}$ mice that selectively lack cDC1s has greatly advanced our understanding of their function in CD8 T cell cross-priming in tumors (30). Batf3$^{-/-}$ mice exhibited defective cross-presentation and impaired anti-tumor immunity, suggesting that cDC1s play a critical role in initiating CD8 T cell-mediated anti-tumor immunity through cross-presentation (30). The mechanisms that make cDC1s superior in cross-presentation are only being uncovered recently. While cDC1s exhibit high efficiency at endocytosis of cell-associated antigens, their superior capacity in cross-presentation is thought to due to their specialized capability in processing antigens (96). In addition, the cross-presentation capacity of cDC1s is further enhanced by their expression of Clec9A, which facilitate the cross-presentation of antigens from dead cells by binding filamentous actin (118–120).

Examining the TME, Broz et al. have identified CD103$^+$ cDC1s as the only population with the capability to induce proliferation of both naive CD8 T cells and established CTLs, suggesting that CD103$^+$ cDC1s are the APCs that cross-prime CTLs in the TME (117) (**Figure 1**). More importantly, analysis of The Cancer Genome Atlas (TCGA) database indicated that the CD103$^+$/CD103$^-$ gene ratio correlates strongly with increased patient survival across 12 different cancer types (117). Consistently with cDC1s' critical role in anti-tumor immunity, a recent study has shown that activation of β-catenin signaling in melanoma cells reduces the numbers of intratumoral CD103$^+$ cDC1 cells, thus preventing tumor-specific T cell priming, suggesting that CD103$^+$ cDC1s might not only promote anti-tumor immunity but also be suppressed by cancer cells for immune evasion (78). In both B16 and Braf-mutant mouse melanoma models, CD103$^+$ cDC1s have been shown to play a critical role in the efficacy of immunotherapy with PD-L1 and Braf inhibition (93). A combined treatment of systemic FMS-like tyrosine kinase 3 ligand (FLT3L) and poly I:C at the tumor sites, which induced the expansion and maturation of CD103$^+$ cDC1s, improved the efficacy of BRAF and PD-L1 blockade, suggesting that combined FLT3L and poly I:C therapy might be a promising approach that could improve the efficacy of current ICB immunotherapy in cancer patients (93). Similarly, efficacy of immunotherapy using PD-1 and CD137 blockade has been shown to depend on

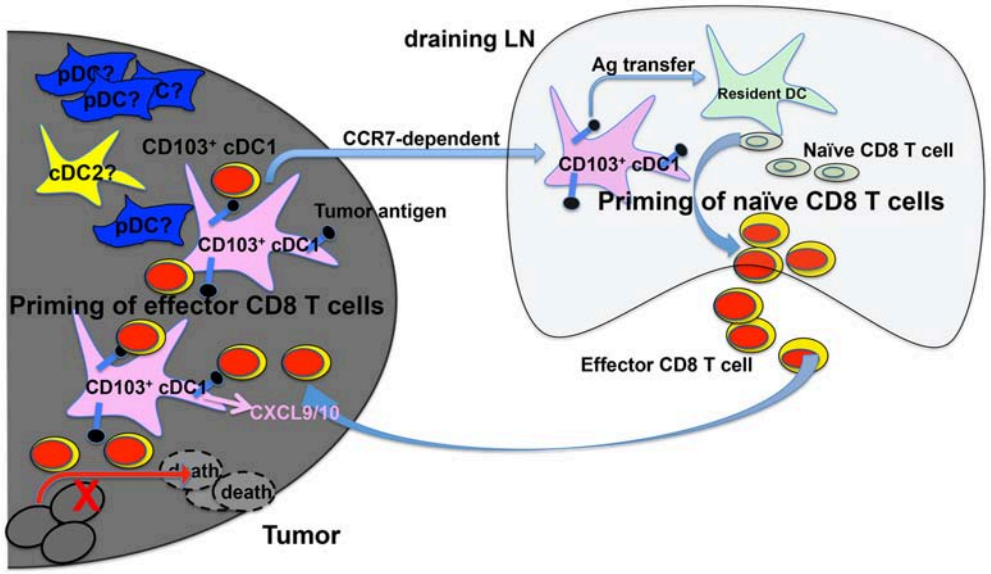

FIGURE 1 | cDC1s and priming of tumor-antigen-specific CD8 T cells in the tumor microenvironment (TME) and tumor-draining lymph nodes (tdLNs). Migratory $CD103^+$ cDC1s in the TME take up tumor antigens (black dots), and transport tumor antigens to tdLN by migrating to the tdLN in a CCR7-dependent mechanism. Once in the tdLN, cross-presenting $CD103^+$ cDC1s prime naive tumor antigen-specific CD8 T cells to become effector CD8 T cells. Cross-presenting $CD103^+$ cDC1s also transfer tumor antigens to other resident myeloid cells including $CD8\alpha^+$ cDC1s that are also likely involved in priming naive CD8 T cells in tdLN. cDC1s in the TME produce CXCL9/10 to recruit primed effector CD8 T cells into TME, where they are re-stimulated by $CD103^+$ cDC1s leading to the efficient killing of tumor cells. The function of other DCs such as pDCs and cDC2s in CD8 T cell priming is less understood.

$CD103^+$ cDC1s, likely due to their function in cross-priming (121).

Recent studies have also shown that $CD103^+$ cDC1s are the only population that mediates the transport of solid tumor antigens from TME to tumor draining lymph nodes for cross-priming of CD8 T cells (93, 122) (**Figure 1**). DC migration to LNs is mediated by the CCR7 chemokine receptor expressed on $CD103^+$ cDC1s, as $CCR7^{-/-}$ $CD103^+$ cDC1s exhibited reduced function in migration and T cell priming (122). In addition, intratumoral $CD103^+$ cDC1s also play a critical role in the trafficking of tumor-specific effector T cells into tumors, as effector T cell recruitment into tumors depends on the presence of CXCL9/10-producing $CD103^+$ cDC1s (123, 124) (**Figure 1**). Importantly, the expression of Batf3-dependent DC transcripts in human melanoma tumors correlates with CXCL9/10 expression and CD8 T cell infiltration, suggesting that Batf3-dependent cDC1s might regulate T cell recruitment to tumors in both mice and human (124).

The role of cDC2s and pDCs in CD8 T cell cross-priming in tumors are less well understood. cDC2s isolated from the TME have been shown to engulf tumor antigens and induce T cell proliferation *in vitro*, suggesting cDC2s may play a role in cross-priming CD8 T cells in TME (117). Given the dominant role of cDC1s as described above, however, cDC2s likely play a minor role in promoting anti-tumor immunity. pDCs can present antigens to activate CD4 T cells as well as activate CD8 T cells through cross-presentation (125, 126). Recruitment of pDCs to the TME has been reported in a number of cancers, although high tumor infiltration of pDCs has been shown to correlate with poor prognosis in melanoma, head and neck, breast, and ovarian cancers (45, 101–103). Activation of pDCs has been shown to promote anti-tumor immunity, likely through the production of type 1 IFNs (127, 128). The role of tumor infiltrated pDCs in the cross-priming of tumor-specific CD8 T cells, however, remains underinvestigated and poorly understood. Interesting, several recent reports have shown the cooperation of pDCs and cDCs in achieving optimal cross-priming (129, 130), suggesting that pDCs could play a positive role in generating anti-tumor CD8 T cell immunity.

STRATEGIES TARGETING DC FUNCTION IN CD8 T CELL PRIMING TO IMPROVING THE EFFICACY OF CANCER IMMUNOTHERAPIES

It's worth noting that the best efficacy for anti-CTLA-4 blockade was achieved in combination with $GM\text{-}CSF^+$ tumor cell vaccination two decades ago by the lab of the newly Nobel laureate Dr. James Allison (131, 132). In the 1998 PNAS paper, the authors suggested that "the most effective and synergistic vaccine strategy targets treatments that enhance T cell priming at the level of host-derived antigen-presenting cells" (131), which quite accurately predicted the direction of cancer immunotherapy as combining ICB with DC-based cancer immunotherapy. In light of the recent discovery of the critical role of cDC1s in priming tumor-specific CD8 T cells, repairing

and/or enhancing DC-mediated CD8 T cell priming represents an exciting approach to improve the efficacy of current T cell-based cancer immunotherapies including ICB and ACT (12, 14, 96). Indeed, Spranger et al. have shown that vaccination with *in vitro*-generated DCs improved the efficacy of anti-PD-L1 and anti-CTLA-4 immunotherapy (78). Similarly, treatment of FLT3L/poly I:C, which expands and induces the maturation and activation of $CD103^+$ cDC1s at the tumor sites, has been shown to enhance anti-tumor responses and improve efficacy when combined with BRAF and PD-L1 blockade (93). Recently, we have genetically engineered tumor-specific CD8 T cells with a second T-cell receptor (TCR) that recognizes a *Listeria* antigen. And we have shown that *Listeria* infection led to the eradication of primary tumors and development of immunological memory against tumor re-challenge in combination with adoptive cell transfer (ACT) of these dual-specific T cells, likely due to the substantially enhanced T cell priming involving DCs (133). *In vivo* DC-targeted vaccines that deliver tumor antigens to cross-presenting DCs with monoclonal antibodies carrying tumor antigens is another attractive approach to enhance cross-priming of tumor-specific CD8 T cells. As multiple clinical trials with human anti-DEC-205 monoclonal antibody fused with antigens such as tumor antigen NY-ESO-1 have shown promising results (134–137), it will be interesting to combine *in vivo* DC-targeted vaccines with T cell-based cancer immunotherapies such as ICB and ACT to further improve their efficacy. Another intriguing approach is the manipulation of pDCs. While tumors are known to prevent the infiltration of cDCs exemplified by recent reports involving β-catenin signaling pathway (78), accumulation of pDCs has been reported in multiple tumors including melanoma, head and neck, breast, and ovarian cancers (45, 101–103), thus offering an opportunity to manipulate these pDCs to generate anti-tumor immunity in the tumor microenvironment (TME). Indeed, therapeutic activation of pDCs have been reported to induce immunogenic anti-tumor responses and shown efficacy in multiple human cancers (25, 41, 103, 107). While the roles of cross-priming by pDCs *in vivo* are still under debate (29, 138–140), recent studies have shown that the co-operation of pDCs and cDCs was required to achieve optimal cross-priming of CD8 T cells (129, 130, 141). Thus, studies are warrantied to further understand the contribution of other DC subsets including pDCs and cDC2s in CD8 T cell priming in TME and tumor-draining LN, which will help develop better strategies to improve efficacy of cancer immunotherapies by enhancing DC function in CD8 T cell priming.

MEMORY CD8 T CELLS

Generation of durable memory CD8 T cells responses that are capable of protection from recurrence and relapse is the ultimate goal of cancer immunotherapy. Memory CD8 T cells are heterogeneous populations that include both circulating memory CD8 T cells and non-circulating tissue resident memory CD8 T cells (T_{rm}) (142). Circulating memory CD8 T cells can be further divided into stem cell memory (T_{scm}), central memory (T_{cm}) and effector memory (T_{em}). Tumor infiltrated Tcm and Tem cells have been reported in multiple cancers such as colorectal and breast cancer (143–145). However, memory CD8 T cells in tumors often exhibit dysfunctional phenotypes and their dysfunction correlates with cancer progression (142). Highlighting their role in anti-tumor immunity, intratumoral expansion of T_{em} cells in patient samples have been associated with improved responses to anti-PD-L1 therapy (146). For the recently identified T_{rm} cells, tumor infiltrated $CD8^+CD103^+$ T_{rm} cells have been reported in tumor samples of ovarian, endometrial, breast and lung cancer patients, and their number correlates with prolonged survival and better prognosis (147–152). While the presence of the memory CD8 T cells in tumors is clear, whether and how TIDCs in particular $CD103^+$ cDC1s regulate the generation and function of memory CD8 T cells remains largely unexplored. Under certain conditions, cross-priming of CD8 T cells by $CD103^+$ cDC1s in TME does lead to memory CD8 T cell responses. For instance, Salmon et al. have shown that FLT3L/poly I:C treatment synergized with PD-L1 blockade to prevent the secondary melanoma lesions after Braf inhibition, as well as provide protection against tumor re-challenge, indicated the generation of memory CD8 T cell responses after CD8 T cell priming (93). Thus, further studies on memory CD8 T cells in TME are warrantied to understand how to better achieve memory CD8 T cell responses in TME.

CONCLUSION

DC-mediated cross-priming of tumor-specific CD8 T cells plays a critical role in initiating and sustaining anti-tumor immunity (110–115). TME employs an array of mechanisms to modify the phenotype and function of TIDC to transform them into immunosuppressive DCs. Insufficient T cell priming likely contributes to cold tumors (no T cell infiltration in TME) and unresponsiveness to immune checkpoint blockade (ICB) therapy, and is under intensive investigation (12). Recently, a number of studies have shown that $CD103^+$ cDC1s in TME are critical in cross-priming CD8 T cells to generate anti-tumor immunity. These $CD103^+$ cDC1s mediate cross-presentation and transport tumor antigens from tumors to draining LN to prime naive CD8 T cells, have the capacity to prime tumor-reactive CTLs in TME, play a critical role in trafficking of effector CD8 T cells to tumors, thus impact all three steps of anti-tumor CD8 T cell responses required for tumor eradication (78, 93, 117, 121–124, 153). In addition, the presence of $CD103^+$ cDC1s has been shown to be critical for efficacy of multiple ICB therapies (93, 121). Thus, manipulating CD8 T cell cross-priming by cDC1s, by employing strategies to increase the number of cDC1s and enhancing their capacity of cross-priming in tumors and tumor draining LNs, represents an exciting approach to enhance anti-tumor CD8 T cell immunity and improve the efficacy of current cancer immunotherapies including ICB and ACT (see reference 13 for an excellent recent review on DC-based cancer immunotherapy). Of note, combination treatment of FLT3L/poly I:C, which expands and induces the maturation and activation of $CD103^+$ cDC1s at the tumor sites, has already been shown to enhance anti-tumor responses to BRAF and PD-L1 blockade (93).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Blimp-1 Rather Than Hobit Drives the Formation of Tissue-Resident Memory CD8$^+$ T Cells in the Lungs

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Tissue-resident memory CD8$^+$ T (T_{RM}) cells that develop in the epithelia at portals of pathogen entry are important for improved protection against re-infection. CD8$^+$ T_{RM} cells within the skin and the small intestine are long-lived and maintained independently of circulating memory CD8$^+$ T cells. In contrast to CD8$^+$ T_{RM} cells at these sites, CD8$^+$ T_{RM} cells that arise after influenza virus infection within the lungs display high turnover and require constant recruitment from the circulating memory pool for long-term persistence. The distinct characteristics of CD8$^+$ T_{RM} cell maintenance within the lungs may suggest a unique program of transcriptional regulation of influenza-specific CD8$^+$ T_{RM} cells. We have previously demonstrated that the transcription factors Hobit and Blimp-1 are essential for the formation of CD8$^+$ T_{RM} cells across several tissues, including skin, liver, kidneys, and the small intestine. Here, we addressed the roles of Hobit and Blimp-1 in CD8$^+$ T_{RM} cell differentiation in the lungs after influenza infection using mice deficient for these transcription factors. Hobit was not required for the formation of influenza-specific CD8$^+$ T_{RM} cells in the lungs. In contrast, Blimp-1 was essential for the differentiation of lung CD8$^+$ T_{RM} cells and inhibited the differentiation of central memory CD8$^+$ T (T_{CM}) cells. We conclude that Blimp-1 rather than Hobit mediates the formation of CD8$^+$ T_{RM} cells in the lungs, potentially through control of the lineage choice between T_{CM} and T_{RM} cells during the differentiation of influenza-specific CD8$^+$ T cells.

Keywords: hobit, blimp-1/PRDM1, lung T cell, T cell differentiation, influenza virus infection, central memory CD8(+) T cells, tissue-resident memory CD8(+) T cells, TCF-1

INTRODUCTION

Long-term memory of previously encountered pathogens is crucial to enable enhanced responses of our body's immune defenses in future encounters with the same pathogen. Antigen-specific memory CD8$^+$ T cells form an essential aspect of immunological memory. Particularly in the context of local infections, tissue-resident memory CD8$^+$ T (T_{RM}) cells situated in the originally infected tissues are important for protective immunity (1). Their position in barrier tissues, including skin, intestine, female reproductive tract and lungs, places non-circulating CD8$^+$ T_{RM} cells at sites of pathogen entry, where they provide efficient early protection against local reinfection

(2–5). The superior protective capacity of $CD8^+$ T_{RM} cells is mediated through direct effector functions and by promoting the activation and recruitment of other immune cells. Upon antigen encounter, $CD8^+$ T_{RM} cells rapidly release pro-inflammatory cytokines, including interferon-γ (IFN-γ), which induce a tissue-wide state of alert and initiate the recruitment of circulating B cells and memory T cells (4, 6, 7). In some tissues, $CD8^+$ T_{RM} cells can also directly lyse target cells and limit pathogen spread by employing cytotoxic mechanisms (8, 9). Given their potency in limiting pathogen spread, insights into the mechanisms regulating the development and maintenance of $CD8^+$ T_{RM} cells may contribute to improved strategies to induce protective immunity via vaccination (10, 11).

The localization of $CD8^+$ T_{RM} cells to different organs suggests the existence of tissue-specific adaptions, which, in turn, may influence the local development and maintenance of $CD8^+$ T_{RM} cells. Compared to $CD8^+$ T_{RM} cells in the skin and intestine, lung $CD8^+$ T_{RM} cells are distinct at the transcriptional level (12), indicating specific adaptions to the local microenvironments. Lung $CD8^+$ T_{RM} cells share common features with $CD8^+$ T_{RM} cells from other tissues, including the expression of tissue-retention molecules such as CD69 and the α_E integrin CD103 (13, 14). Importantly, lung $CD8^+$ T_{RM} cells provide robust protection against heterosubtypic influenza virus infection (5, 15). However, $CD8^+$ T_{RM} cells in the lungs are divergent from $CD8^+$ T_{RM} cells at other peripheral sites in terms of their maintenance. While $CD8^+$ T_{RM} cells in most tissues are long-lived and self-sustaining (2, 16), the virus-specific $CD8^+$ T_{RM} cell population in the lungs declines over time after pulmonary infection, which coincides with waning of heterosubtypic immunity to influenza virus (5, 14, 17). The mechanisms underlying this limited longevity of lung $CD8^+$ T_{RM} cells are not fully understood. After pulmonary infection, $CD8^+$ T_{RM} cells localize to specific niches at sites of tissue regeneration in the lung, and it has been suggested that the disappearance of these niches over time may account for the limited longevity of lung $CD8^+$ T_{RM} cells (18). The preservation of the resident population in the lungs may additionally require continuous replenishment from a circulating effector memory $CD8^+$ T (T_{EM}) cell pool (17, 19). The gradual decline in the capacity of circulating memory $CD8^+$ T cells to form $CD8^+$ T_{RM} cells may thus contribute to the demise of the lung $CD8^+$ T_{RM} cell population (17). Consequently, tissue residency might be differentially regulated at the transcriptional level for $CD8^+$ T cells in the lung compared to other organs.

We have recently found that the transcription factor Hobit and its homolog Blimp-1 control the generation and/or maintenance of $CD8^+$ T_{RM} cells across several tissues, including skin, small intestine, liver, and kidney (20). These transcription factors instruct a universal program of tissue-residency, in part by directly suppressing the tissue egress receptors CCR7 and S1PR1 (20). Here, we investigated the role of Hobit and Blimp-1 in the development of lung $CD8^+$ T_{RM} cells after pulmonary influenza virus infection. $CD8^+$ T_{RM} cells in the lungs exhibited high expression of Hobit and Blimp-1 at the transcript level. However, we found that Blimp-1, but not Hobit, is essential for the formation of lung $CD8^+$ T_{RM} cells. Blimp-1 also limited the formation of central memory $CD8^+$ T (T_{CM}) cells. These findings highlight the unique transcriptional regulation of $CD8^+$ T_{RM} cells in the lung, which may have implications for future influenza vaccination strategies.

RESULTS

Lung $CD8^+$ T_{RM} Cells Arising After Respiratory Influenza Virus Infection Express Hobit

Infection of mice with influenza virus induces differentiation of virus-specific $CD8^+$ T cells into $CD8^+$ T_{RM} cells, which persist in the lung and provide protection against subsequent reinfection (5, 21, 22). To investigate lung $CD8^+$ T_{RM} cells arising after influenza virus infection, mice were infected intranasally with HKx31 influenza A virus and $CD8^+$ T cells were isolated and analyzed in the memory phase (day 30+ p.i.). Influenza virus infection gave rise to a substantial $CD69^+$ $CD8^+$ T_{RM} cell population in the lung, which partially expressed CD103 (**Figure 1A**). This population was nearly absent in lungs from naïve mice, indicating that the vast majority of $CD8^+$ T_{RM} cells were a direct result of influenza virus infection. In mice, a core signature of gene-expression has been determined in $CD8^+$ T_{RM} cells (12). Transcriptional profiling of $CD69^+$ and $CD69^-$ memory $CD8^+$ T cells isolated from lungs of HKx31-immune mice (day 30+ p.i.) by RNA sequencing confirmed the resident phenotype of the $CD69^+$ $CD8^+$ T cell population arising after influenza virus infection (**Figure 1B**). When compared to the T_{RM} core signature, the obtained transcriptional profiles of lung $CD8^+$ T_{RM} cells showed a good congruency. Genes associated with tissue-residency, including *Cdh1*, *Itga1*, *Itgae*, *Rgs1,* and *Rgs2*, were specifically upregulated in the $CD69^+$ population, while genes associated with circulating memory $CD8^+$ T cells, including tissue-egress factors (e.g., *S1pr1* and *S1pr5*), were substantially downregulated in these cells (**Figure 1B**). Overall, out of 35 genes of the T_{RM} core signature, 20 were significantly up- or downregulated in the $CD69^+$ $CD8^+$ T cell compartment compared to the $CD69^-$ $CD8^+$ T cell pool from the lung. Importantly, the transcription factor Hobit (encoded by *Zfp683*), which we have recently identified as a key regulator of tissue-residency (20), was also significantly upregulated in lung $CD8^+$ T_{RM} cells, as compared to the circulating memory $CD8^+$ T cell population in the lung (**Figures 1B,C**). In contrast, expression levels of the related transcription factor Blimp-1 (encoded by *Prdm1*) were not significantly different between the two memory subsets (**Figure 1C**). Members of the common γ-chain cytokines, in particular IL-2, IL-7, and IL-15, play an important role in the maintenance of memory $CD8^+$ T cells. In the lung, both circulating and resident memory $CD8^+$ T cells expressed the individual components of the IL-7 (*Il7r*, *Il2rg*) and IL-15 receptor (*l2rb*, *Il2rg*) (**Figure 1D**). In contrast, the alpha chain of the IL-2 receptor was upregulated in lung $CD8^+$ T_{RM} cells compared to $CD69^-$ memory $CD8^+$ T cells in the lung. Furthermore, in comparison to their circulating counterparts in the lung, the $CD69^+$ lung T_{RM} cells expressed significantly higher levels of pro-inflammatory cytokines, chemokines and cytotoxic mediators, including colony-stimulating factor-1

(*Csf1*), lymphotactin (*Xcl1*) granzyme B (*Gzmb*) and TNF-related apoptosis-inducing ligand (TRAIL, *Tnfsf10*), indicative of a poised effector state (**Figures 1E–G**). Taken together, influenza virus infection induced a distinct population of $CD69^+$ $CD8^+$ T cells in the lungs, which were identified as bona fide T_{RM} cells by transcriptional analysis. Importantly, these lung-resident $CD8^+$ T cells exhibited elevated transcript levels of effector molecules and were characterized by high expression of the T_{RM}-associated transcription factor Hobit.

$CD8^+$ T_{RM} Cell Formation in the Lung Requires Hobit and/or Blimp-1

Given its selective expression in lung $CD8^+$ T_{RM} cells, we hypothesized that Hobit may contribute to the development of these cells. In other tissues, including the skin, liver, kidney, and small intestine, Hobit regulates the generation and/or maintenance of $CD8^+$ T_{RM} cells together with its homolog Blimp-1 (20). In order to investigate the role of these two transcription factors in the development of lung $CD8^+$ T_{RM} cells, mixed bone marrow (BM) chimeric mice were generated, containing a WT compartment and a compartment lacking functional Hobit and Blimp-1 (double knock-out, DKO) (**Figure 2A**). An approach with mixed BM chimeric mice was chosen to minimize indirect effects on CD8 T cell differentiation through differences in viral clearance. Mice were infected intranasally with HKx31 virus, and the virus-specific (D^b $NP366^+$) $CD8^+$ T cell response was analyzed over time. Previous studies have demonstrated a critical role for Blimp-1 in terminal effector cell (TEC) differentiation (24, 25). In line with these findings, analysis of virus-specific D^b $NP366^+$ $CD8^+$ T cells in the blood at the peak of the anti-viral effector $CD8^+$ T cell response (day 10 p.i.) revealed a substantial decrease in $KLRG1^+$ $CD127^-$ TECs in the DKO compared to the WT compartment (**Figures 2B–D**). Concomitantly, D^b $NP366^+$ cells deficient for both Hobit and Blimp-1 exhibited a sharp increase in $CD127^+$ $KLRG1^-$ memory precursor effector cells (MPECs) compared to their WT counterparts (**Figures 2C,D**). In lung tissue, a distinct $CD69^+$ population was already observed at the effector stage, while CD103 expression was minimal (**Figure 2F**). Both the WT and the DKO compartment gave rise to similar frequencies of $CD69^+$ $CD103^-$ and $CD69^+$ $CD103^+$ cells at this stage, suggesting little impact of Hobit and Blimp-1 deficiency on the formation of these cells (**Figures 2E–G**). In contrast, D^b $NP366^+$ DKO cells generated less T_{RM} cells in the lung at the memory phase than their WT counterparts (**Figures 2H,I**). This defect was most pronounced for $CD69^+$ $CD103^+$ cells, which were decreased in both frequencies and absolute numbers in the DKO compartment compared to the WT compartment (**Figures 2I,J**). Interestingly, DKO cells formed $CD69^+$ $CD103^-$ T_{RM} cells at near similar frequencies as WT cells, indicating little effect of combined Hobit and Blimp-1 deficiency on the generation of this population (**Figures 2I,K**). Apart from CD69 and CD103, $CD8^+$ T_{RM} cells across tissues express additional tissue-residency markers, including the chemokine receptor CXCR6 and the integrin CD49a (26–29). Influenza-virus-specific WT $CD8^+$ T cells in the lungs co-expressed CXCR6 and CD49a at similar frequencies as the residency marker CD69, suggesting that both molecules also identify $CD8^+$ T_{RM} cells in this tissue (**Figures 2L,M**). Interestingly, combined deficiency for Hobit and Blimp-1 impaired the formation of $CXCR6^+$ $CD49a^{high}$ cells, which were decreased in both frequencies and absolute numbers in the DKO compartment compared to the WT compartment (**Figures 2L,M**). In all, these results show that the combined genetic ablation of Hobit and Blimp-1 results in reduced TEC and enhanced MPEC formation during the effector $CD8^+$ T cell response, and impairs the generation of $CD103^+$ lung T_{RM} cells in the memory $CD8^+$ T cell response.

Hobit and Blimp-1 Impair Formation of $CD8^+$ T_{CM} Cells

Apart from $CD8^+$ T_{RM} cells in the lung, influenza virus infection also induces the formation of circulating effector memory (T_{EM}) and central memory (T_{CM}) $CD8^+$ T cells (30, 31). To assess the impact of co-deficiency of Hobit and Blimp-1 on these circulating memory subsets, we analyzed virus-specific (D^b $NP366^+$) WT and DKO cells isolated from secondary lymphoid organs of mixed BM chimeric mice after HKx31 infection. In both spleen and lung-draining lymph nodes (mediastinal lymph nodes, mLN), virus-specific $CD44^{high}$ $CD62L^+$ $CD8^+$ T_{CM} cells were present at elevated levels in the DKO compartment compared to the WT compartment (**Figures 3A,B**), both in frequencies and in absolute numbers. In contrast, no effect of Blimp-1 and Hobit deficiency was observed in the $CD44^{high}$ $CD62L^-$ $CD8^+$ T_{EM} subset, as these cells were present in both the WT and the DKO compartment in similar numbers (**Figure 3C**). Taken together, these data suggest that Hobit and/or Blimp-1 not only drive the formation of $CD8^+$ T_{RM} cells, but also suppress the development of $CD8^+$ T_{CM} cells after influenza virus infection.

Generation of Lung $CD8^+$ T_{RM} Cells Depends on Blimp-1, but Not Hobit

We next investigated whether Hobit and Blimp-1 collaborated in the development of lung $CD8^+$ T_{RM} cells, as observed in other tissues (20), or whether either one was the dominant transcription factor driving $CD8^+$ T_{RM} formation in the lungs. To this end, three groups of mixed BM chimeric mice were generated, consisting of one control group, containing two WT compartments (CD45.1 and CD45.2) and two experimental groups, containing a WT (CD45.1) compartment next to either a Hobit KO (CD45.2) compartment or a Blimp-1 KO (CD45.2) compartment. The mixed BM chimeric mice were infected intranasally with HKx31 virus, and the virus-specific (D^b $NP366^+$) $CD8^+$ T cell response was analyzed over time. As expected, similar to cells lacking both functional Hobit and Blimp-1 (**Figures 2C,D**), D^b $NP366^+$ Blimp-1 KO cells contained elevated frequencies of $CD127^+$ $KLRG1^-$ MPECs at the peak of the anti-viral $CD8^+$ T cell effector response (**Figures 4A,B**). Moreover, D^b $NP366^+$ Blimp-1 KO cells nearly lacked $KLRG1^+$ $CD127^-$ TECs, which was also observed for cells with combined deficiency for Hobit and Blimp-1 (**Figures 2C,D**). Neither of these phenotypes was observed for D^b $NP366^+$ Hobit-deficient cells, which contained TECs and MPECs at similar

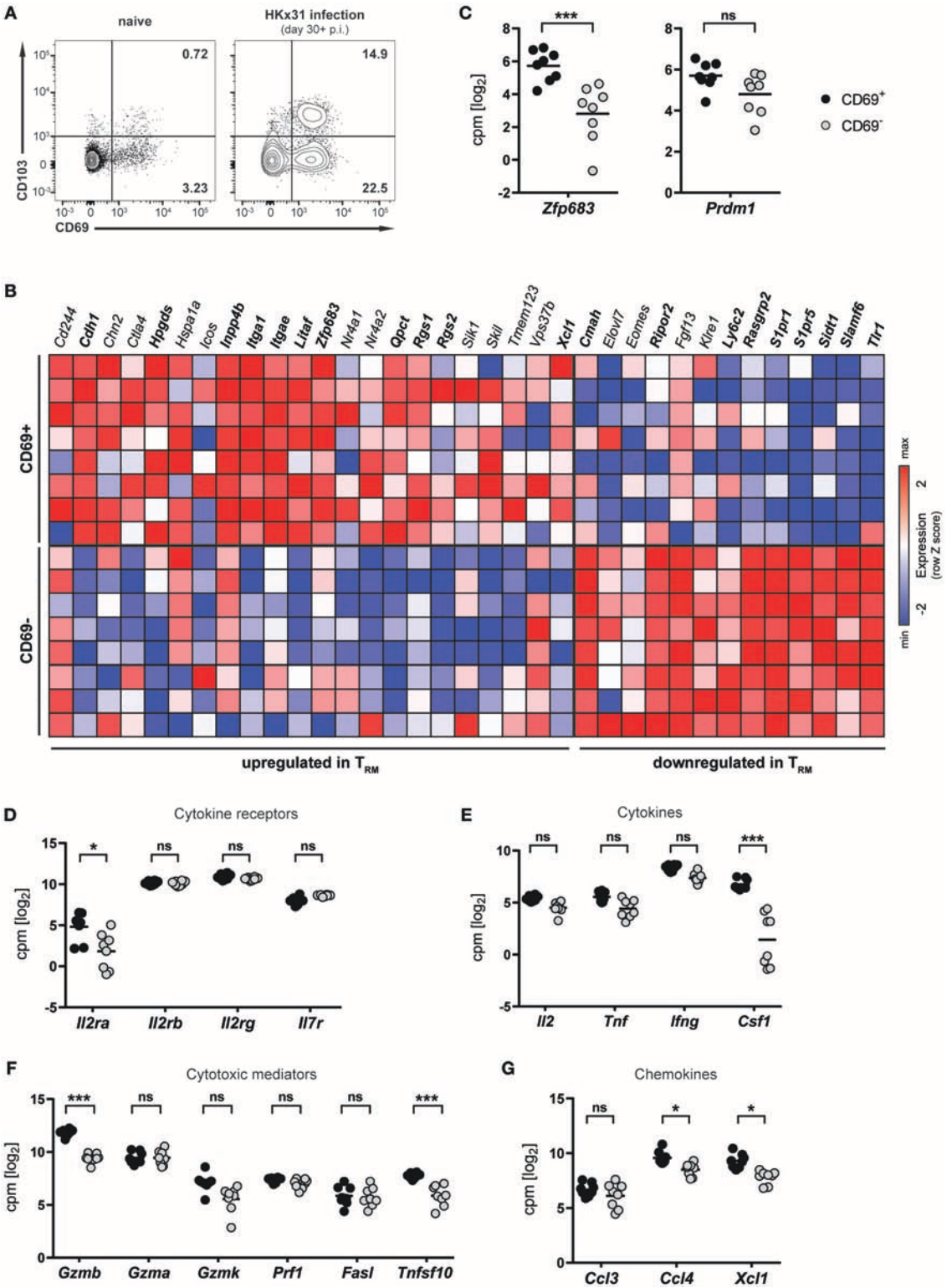

FIGURE 1 | Transcriptional profile of lung $CD8^+$ T_{RM} cells arising after influenza virus infection. **(A)** Representative flow cytometry plots are shown of CD69 and CD103 expression on memory ($CD44^{high}$ $CD62L^{lo}$) $CD8^+$ T cells isolated from lungs of naïve mice or lungs of mice at day 30+ (memory phase) after intranasal HKx31 influenza virus infection. **(B)** Expression (column Z-score) of mRNA from genes belonging to the T_{RM} core signature (12) in $CD69^+$ and $CD69^-$ memory ($CD44^{high}$ $CD62L^{lo}$) $CD8^+$ T cells isolated from murine lungs at day 30+ after HKx31 infection. Genes in bold are differentially expressed in $CD69^+$ vs. $CD69^-$ cells. Expression

(Continued)

FIGURE 1 | of gene *Usp33*, part of the T_{RM} core signature, was not detected by our analysis. **(C–G)** Expression (log2 read counts per million, voom normalized expression) of **(C)** Hobit (*Zfp683*) and Blimp-1 (*Prdm1*), genes encoding for **(D)** common γ-chain cytokine receptors, **(E)** cytokines, **(F)** cytotoxic mediators, and **(G)** selected chemokines is shown in CD69$^+$ and CD69$^-$ memory (CD44^{high} CD62L^{lo}) CD8$^+$ T cells isolated from murine lungs at day 30+ after HKx31 infection. Symbols represent individual mice and line indicates the mean. Data from one experiment ($n = 8$), taken from Hombrink et al. (23). *FDR adjusted $P < 0.05$; *** FDR adjusted $P < 0.001$; ns: not significant.

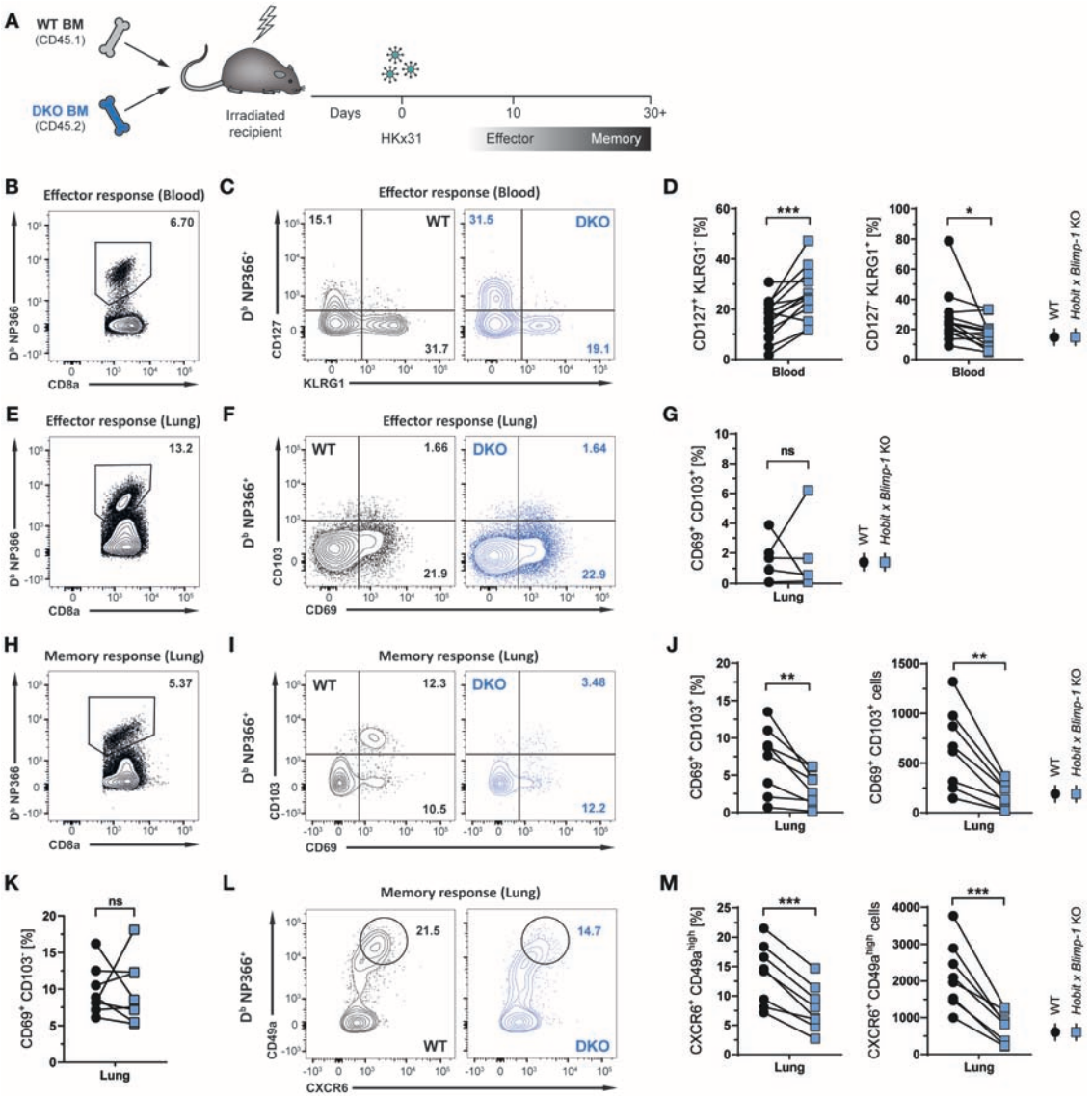

FIGURE 2 | Formation of lung CD8$^+$ T_{RM} cells depends on Hobit and/or Blimp-1. **(A)** Experimental scheme shows the generation of mixed bone marrow (BM) chimeras from WT and Hobit and Blimp-1 KO (DKO) mice (1:1 ratio) and HKx31 influenza virus infection of these chimeric mice. **(B–G)** Analysis at the effector time point is shown. **(B**,**E)** Representative flow cytometry plot shows frequency of D^b NP366$^+$ cells within CD8$^+$ T cell population in **(B)** blood and **(E)** lung at day 10 post infection. **(C**,**F)** Representative flow cytometry plots show **(C)** expression of CD127 and KLRG1 and **(F)** expression of CD69 and CD103 on D^b NP366$^+$ donor CD8$^+$ T cells of the WT and DKO compartment from **(C)** blood and **(F)** lungs at day 10 post infection. **(D**,**G)** The frequencies of **(D)** CD127$^+$KLRG1$^-$ and KLRG1$^+$CD127$^-$ and **(G)** CD69$^+$ CD103$^+$ D^b NP366$^+$ donor CD8$^+$ T cells of the WT and DKO compartment from **(D)** blood and **(G)** lungs at day 10 post infection were quantified. **(H–M)** Analysis at the memory time point is shown. **(H)** Representative flow cytometry plot shows frequency of D^b NP366$^+$ cells within CD8$^+$ T cell population in lung at day 30+ post infection. **(I**,**L)** Representative flow cytometry plots show **(I)** expression of CD69 and CD103 and **(L)** expression of CXCR6 and CD49a on D^b NP366$^+$ donor CD8$^+$ T cells of the WT and DKO compartment from lungs at day 30+ post infection. **(J**,**K**,**M)** The frequencies and absolute numbers of **(J)** CD69$^+$CD103$^+$, **(K)** CD69$^+$CD103$^-$, and **(M)** CXCR6$^+$CD49a^{high} D^b NP366$^+$ donor CD8$^+$ T cells of the WT and DKO compartment from lungs at day 30+ post infection were quantified. Data from **(G)** one experiment ($n = 5$) or combined data from **(C**,**J**,**K**,**M)** two independent experiments ($n = 8$). Symbols represent individual mice, lines connect paired samples. Paired *t*-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, ns: not significant.

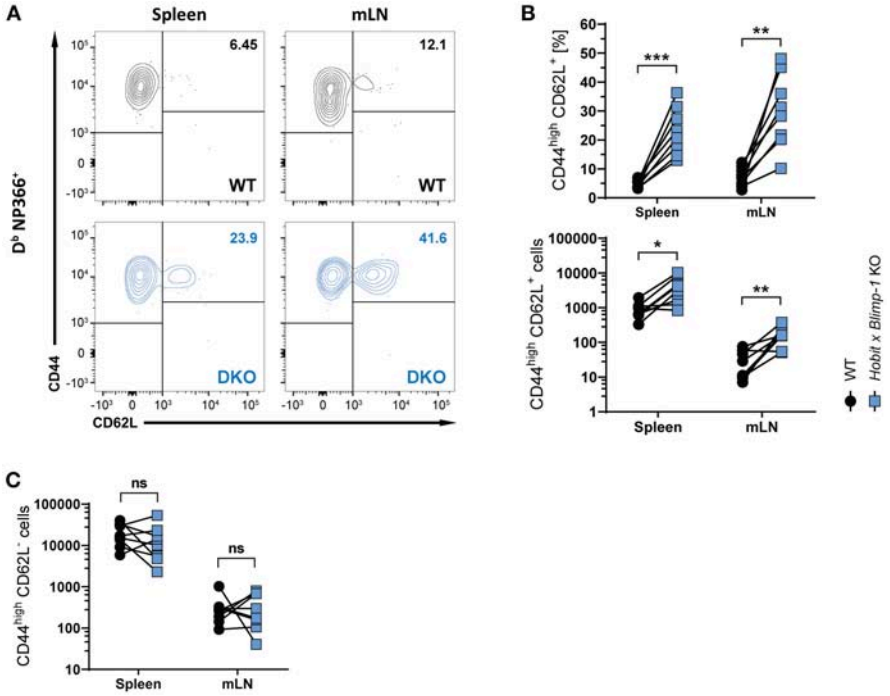

FIGURE 3 | Increased $CD8^+$ T_{CM} formation upon combined loss of Hobit and Blimp-1. **(A–C)** WT: Hobit × Blimp-1 KO (DKO) mixed bone marrow chimeras were infected intranasally with HKx31 influenza virus. The phenotype of virus-specific (D^b $NP366^+$) WT and DKO donor $CD8^+$ T cells was analyzed at day 30+ post infection (memory phase). **(A)** Representative flow cytometry plots show expression of CD44 and CD62L on D^b $NP366^+$ donor $CD8^+$ T cells of the WT and DKO compartment from spleen and mediastinal lymph nodes (mLN). **(B,C)** The frequencies and absolute numbers of **(B)** $CD44^{high}CD62L^+$ D^b $NP366^+$ donor $CD8^+$ T cells and **(C)** absolute numbers of $CD44^{high}CD62L^-$ D^b $NP366^+$ donor $CD8^+$ T cells of the WT and DKO compartment in spleen and mLN were quantified. Combined data from two independent experiments ($n = 8$). Symbols represent individual mice, lines connect paired samples. Paired *t*-test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, ns: not significant.

frequencies as their WT counterparts (**Figures 4A–C**). These data indicate a dominant role of Blimp-1 over Hobit in regulating the balance between terminal effector and memory precursor cell differentiation during the effector response. After clearance of the infection, the D^b $NP366^+$ Hobit KO compartment exhibited no defects in the formation of $CD8^+$ T_{RM} cells in the lung (**Figures 4D,E**). In contrast, the Blimp-1 deficient compartment of virus-specific cells was severely impaired in the formation of $CD69^+$ $CD103^+$ $CD8^+$ T_{RM} cells (**Figures 4D,E**). As previously observed for cells with combined deficiency for Hobit and Blimp-1, D^b $NP366^+$ Blimp-1 KO cells did not exhibit a substantial defect in generating $CD69^+$ $CD103^-$ cells in the lung (**Figure 4F**). Thus, Blimp-1, but not Hobit, appears to be essential for the formation of terminal effector cells and $CD69^+$ $CD103^+$ $CD8^+$ T_{RM} cells in the lung.

Blimp-1 Suppresses $CD8^+$ T_{CM} Formation

We next considered the role of Blimp-1 and its homolog Hobit in the formation of other memory $CD8^+$ T cell subsets. Consistent with previous research demonstrating the repressive role of Blimp-1 in the formation of $CD8^+$ T_{CM} cells (24, 25), we observed elevated frequencies of $CD44^{high}$ $CD62L^+$ D^b $NP366^+$ cells in the Blimp-1 KO compartment of mixed BM chimeras already during the effector $CD8^+$ T cell response (**Figures 5A,B**). Importantly, Blimp-1 deficiency resulted in a strong increase in the frequency of D^b $NP366^+$ $CD8^+$ T_{CM} cells in the circulation, lung and secondary lymphoid organs during the memory phase (**Figures 5C–E**). This effect was not observed for cells lacking functional Hobit, neither during the effector nor the memory response (**Figures 5B,D**). Consequently, these data suggest that Blimp-1 suppresses $CD8^+$ T_{CM} development as early as in the effector response, while Hobit does not appear to have an essential role in this process.

Blimp-1 Suppresses TCF-1 Expression in Lung $CD8^+$ T_{RM} Cells

The transcription factors Hobit and Blimp-1 regulate the development of $CD8^+$ T_{RM} cells in part by suppressing genes, which are incompatible with the establishment of tissue-residency (20). A direct target of both Hobit and Blimp-1 is T-cell factor 1 (TCF-1). TCF-1 is a nuclear effector of the canonical Wingless/Integration 1 (Wnt) signaling pathway and constitutes an essential transcription factor for the development of $CD8^+$ T_{CM} cells (32, 33). Given the enhanced formation of $CD8^+$ T_{CM} cells upon genetic ablation of Blimp-1, we analyzed TCF-1 expression in different memory $CD8^+$ T cells subsets arising after influenza infection. As expected, WT D^b $NP366^+$ $CD8^+$ T_{CM} cells exhibited high uniform expression of

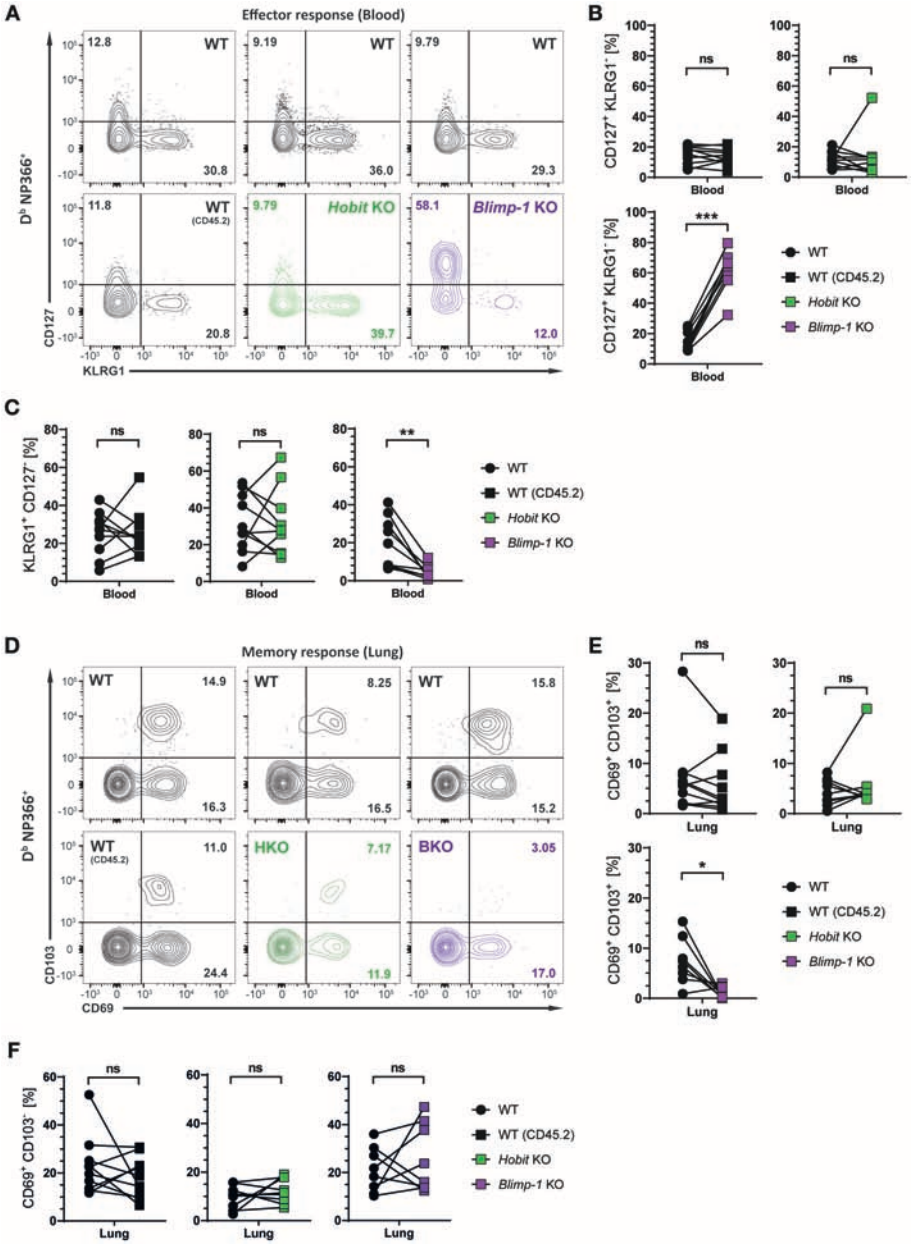

FIGURE 4 | Formation of lung CD8$^+$ T_{RM} cells depends on Blimp-1, but not Hobit. Mixed bone marrow chimeras [WT:WT; WT:Hobit KO (HKO); WT:Blimp-1 KO (BKO)] were infected intranasally with HKx31 influenza virus. The phenotype of virus-specific (D^b NP366$^+$) WT (CD45.1$^+$), WT (CD45.2$^+$), HKO (CD45.2$^+$), and BKO (CD45.2$^+$) donor CD8$^+$ T cells was analyzed. **(A)** Representative flow cytometry plots show expression of CD127 and KLRG1 by D^b NP366$^+$ donor CD8$^+$ T cells from the blood of mixed bone marrow chimeras at day 10 post infection. **(B,C)** Frequencies of **(B)** CD127$^+$KLRG1$^-$ and **(C)** KLRG1$^+$CD127$^-$ D^b NP366$^+$ donor CD8$^+$ T cells from the blood of mixed bone marrow chimeras at day 10 post infection were quantified. **(D)** Representative flow cytometry plots show expression of CD69 and CD103 by D^b NP366$^+$ donor CD8$^+$ T cells from the lungs at day 30+ post infection. **(E,F)** Frequencies of **(E)** CD69$^+$CD103$^+$ and **(F)** CD69$^+$CD103$^-$ D^b NP366$^+$ donor CD8$^+$ T cells from the lungs at day 30+ post infection were quantified. Combined data from two independent experiments (n = 9–10). Symbols represent individual mice, lines connect paired samples. Paired t-test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, ns: not significant.

TCF-1 at the protein level, while virus-specific WT CD8$^+$ T_{RM} cells in the lung showed markedly lower expression of TCF-1 (**Figures 6A,B**). Hobit deficiency did not significantly impact TCF-1 expression in both CD8$^+$ T_{CM} and T_{RM} populations. Loss of Blimp-1 had no effect on the high levels of TCF-1 expression in CD8$^+$ T_{CM} cells. However, Blimp-1 deficiency resulted in strongly increased TCF-1 protein expression in the remaining virus-specific CD8$^+$ T_{RM} cells present in the lung (**Figures 6A,B**). These results indicate that Blimp-1 mediates suppression of TCF-1 expression in lung CD8$^+$ T_{RM} cells, which may contribute to the instruction of CD8$^+$ T_{RM} development in the lungs.

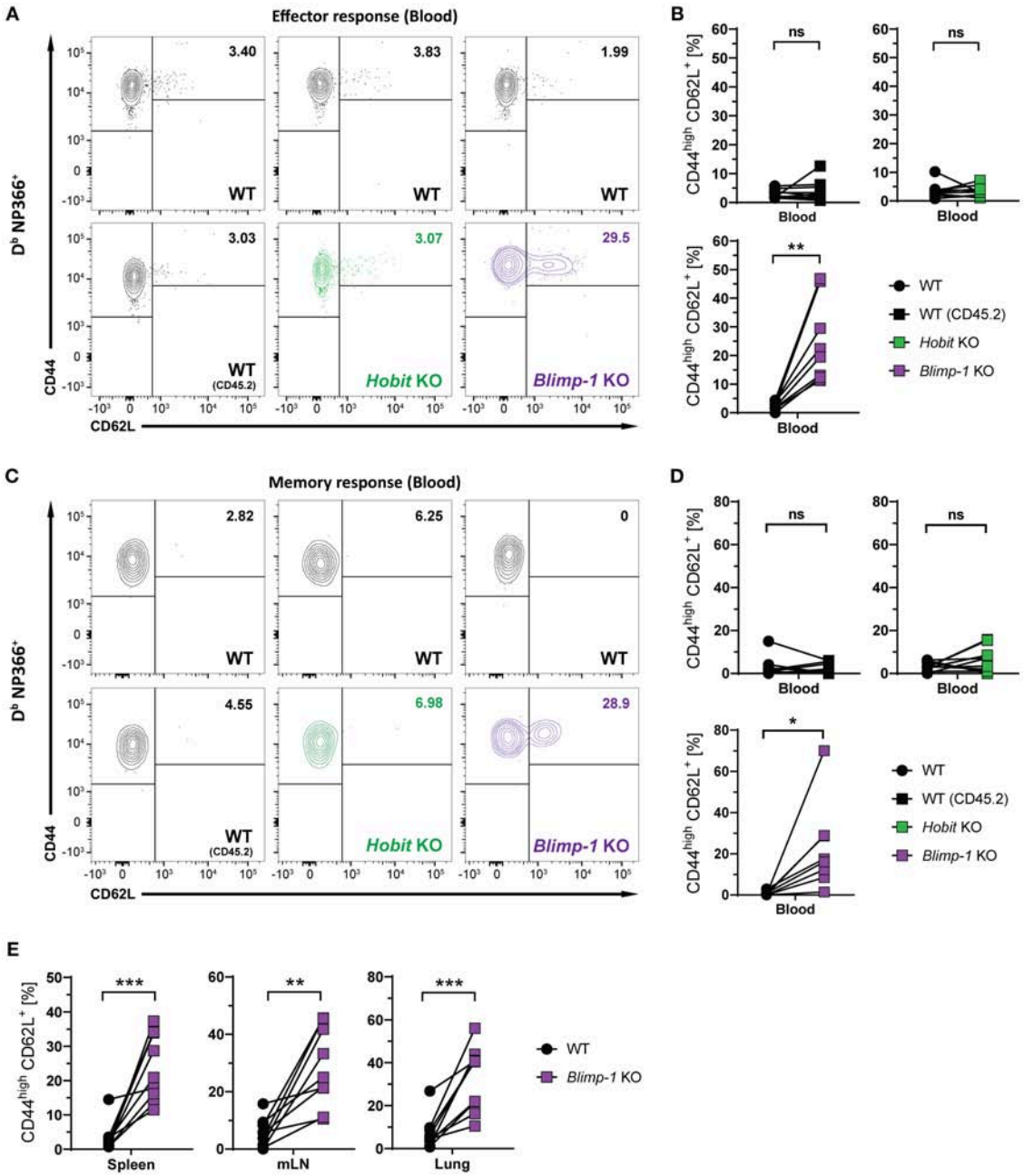

FIGURE 5 | $CD8^+$ T_{CM} formation is suppressed by Blimp-1. Mixed bone marrow chimeras [WT:WT; WT:Hobit KO (HKO); WT:Blimp-1 KO (BKO)] were infected intranasally with HKx31 influenza virus. The phenotype of virus-specific (D^b $NP366^+$) WT ($CD45.1^+$), WT ($CD45.2^+$), HKO ($CD45.2^+$) and BKO ($CD45.2^+$) donor $CD8^+$ T cells was analyzed in blood **(A–D)** and in the indicated tissues **(E)**. **(A,C)** Representative flow cytometry plots show expression of CD44 and CD62L on D^b $NP366^+$ donor $CD8^+$ T cells from the blood at **(A)** day 10 and **(C)** day 30+ post infection. **(B,D,E)** Frequencies of $CD44^{high}CD62L^+$ cells D^b $NP366^+$ donor $CD8^+$ T cells from **(B)** blood at day 10 post infection, and from **(D)** blood as well as from **(E)** spleen, mediastinal lymph nodes (mLN) and lung at day 30+ post infection were quantified. Combined data from two independent experiments ($n = 9$–10). Symbols represent individual mice, lines connect paired samples. Paired *t*-test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, ns: not significant.

DISCUSSION

The maintenance of lung $CD8^+$ T_{RM} cells is distinct from that of $CD8^+$ T_{RM} cells at other sites. In contrast to $CD8^+$ T_{RM} cells in skin and small intestine, lung-resident $CD8^+$ T cells are not maintained as stable populations, but slowly decline over time. Here, we show that $CD8^+$ T_{RM} cells in the lungs diverge from T_{RM} cells in the liver, intestine and skin in terms of their transcriptional regulation. Unlike their counterparts in other tissues, differentiation and/or maintenance of lung $CD8^+$

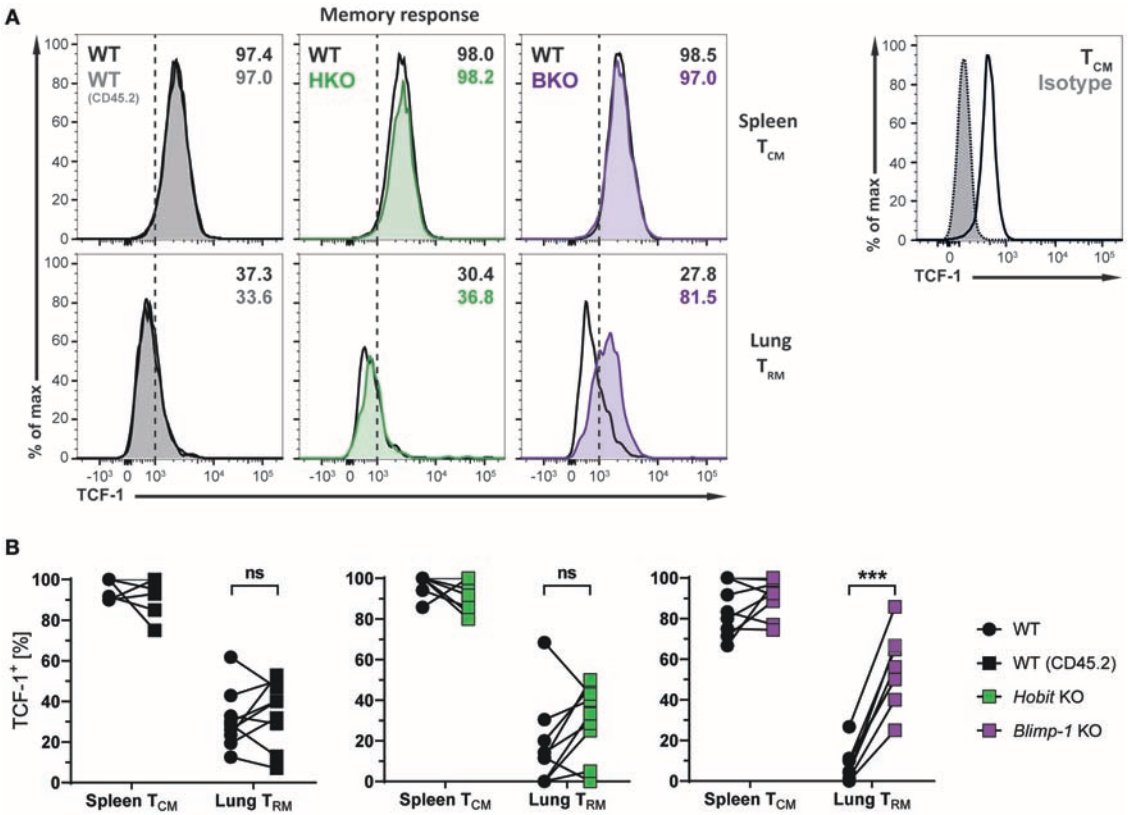

FIGURE 6 | TCF-1 expression in lung $CD8^+$ T_{RM} cells is suppressed by Blimp-1. **(A,B)** Mixed bone marrow chimeras [WT:WT; WT:Hobit KO (HKO); WT:Blimp-1 KO (BKO)] were infected intranasally with HKx31 influenza virus. At day 30+ post infection (memory phase), the expression of TCF-1 by virus-specific (D^b NP366$^+$) WT (CD45.1$^+$), WT (CD45.2$^+$), HKO (CD45.2$^+$), and BKO (CD45.2$^+$) donor $CD8^+$ T cells was analyzed. Expression of TCF-1 was analyzed in donor $CD8^+$ T_{CM} cells ($CD44^{high}CD62L^+$) from spleen and donor $CD8^+$ T_{RM} cells ($CD69^+CD103^+$) from lungs. TCF-1 staining was validated using isotype control staining on $CD8^+$ T_{CM} cells ($CD44^{high}CD62L^+$) from spleen. **(A)** Representative histograms show expression of TCF-1, and **(B)** graphs display frequencies of $TCF\text{-}1^+$ cells. Combined data from two independent experiments (n = 9–10). Symbols represent individual mice, lines connect paired samples. Paired t-test. $^{***}P < 0.001$, ns: not significant.

T_{RM} cells after influenza virus infection was entirely independent of Hobit. Instead, we found that $CD103^+$ lung $CD8^+$ T_{RM} cells exclusively depended on Blimp-1. Blimp-1 repressed the formation of virus-specific $CD8^+$ T_{CM} cells and the expression of the transcription factor TCF-1 in lung $CD8^+$ T_{RM} cells. These findings may suggest that Blimp-1 controls the lineage choice between $CD8^+$ T_{CM} and T_{RM} cells after influenza infection and highlight a unique aspect of the transcriptional regulation of $CD8^+$ T_{RM} cells in the lung.

In the skin, liver, kidney, and small intestine, $CD8^+$ T_{RM} cell formation and/or maintenance is co-regulated by Hobit and Blimp-1 (20). Our data demonstrate that $CD103^+$ $CD8^+$ T_{RM} cells in the lungs do not require Hobit for their formation, but entirely rely on Blimp-1. These findings may reflect differences in signals from the local microenvironment modulating Hobit and Blimp-1 expression. While influenza virus causes an acute infection of the airways, residual viral antigen can persist in the lung and the draining lymph nodes for extended periods of time after clearance of the infection (34, 35). Presence of remaining viral antigen can induce T cell responses long after the infection and modulates the migration and localization of virus-specific memory $CD8^+$ T cells (34–36). Persistent antigen may thus impact on the local $CD8^+$ T_{RM} cell pool in the airways. Previous research has demonstrated the requirement of local antigen encounter for the establishment of $CD8^+$ T_{RM} cells in the lungs (37). However, it remains unclear whether residual antigen also influences the maintenance of lung $CD8^+$ T_{RM} cells. TCR signaling can induce *Blimp-1* expression in T cells (38). Persistent viral antigen in the lungs may thus modulate Blimp-1 expression in $CD8^+$ T_{RM} cells at this site and in turn bias the dependence of lung $CD8^+$ T_{RM} cells toward Blimp-1. In addition, *Blimp-1* expression is upregulated by pro-inflammatory cytokines, including IL-2 and IL-12 (39). After influenza virus infection, $CD8^+$ T_{RM} cells localize to sites of tissue regeneration in the lungs (18), which may be characterized by residual inflammation, thus favoring *Blimp-1* expression. Our transcriptional analysis revealed selective upregulation of the IL-2 receptor alpha chain (*Il2ra*) by $CD69^+$ $CD8^+$ T_{RM} cells arising in the lungs after influenza infection, potentially indicating increased responsiveness to IL-2. Furthermore, IL-2 signaling is required for residency of $CD4^+$ T_{RM} cells in the lungs (40). Consequently, IL-2 and other pro-inflammatory cytokines

may also act on lung $CD8^+$ T_{RM} cells and enhance Blimp-1 expression, resulting in Blimp-1 dependent maintenance of these memory T cells.

In contrast to Blimp-1, Hobit does not appear to play an essential role in the differentiation and/or maintenance of lung $CD8^+$ T_{RM} cells. Nevertheless, Hobit is specifically expressed by $CD69^+$ $CD8^+$ T cells in the lungs after influenza virus infection, indicating the presence of local cues driving Hobit mRNA expression. The cytokine interleukin-15 (IL-15) mediates *Hobit* expression in a T-bet-dependent manner (20). Local IL-15 and potential other signals may thus promote Hobit mRNA expression in lung $CD8^+$ T_{RM} cells. Epithelial cells in the lung airways constitutively express IL-15 (41), and the cytokine mediates the recruitment of effector $CD8^+$ T cells into the inflamed lung after influenza virus infection (42). Furthermore, IL-15 is required for persistence of $CD103^+$ $CD8^+$ T_{RM} cells in the lungs (43), as well as for $CD8^+$ T_{RM} maintenance in skin, salivary glands, and kidney (43, 44). Taken together, these findings suggest a prominent role for IL-15 in modulating $CD8^+$ T cell responses in the lungs. Whether IL-15 facilitates Hobit expression in lung $CD8^+$ T_{RM} cells in a similar fashion as in $CD8^+$ T_{RM} cells at other sites, remains to be determined. Despite being differentially expressed, Hobit does not appear to have an essential role in the formation of lung $CD8^+$ T_{RM} cells. Using transcriptional profiling, we have detected Hobit and Blimp-1 expression at the mRNA level. However, this does not necessarily reflect the expression of Hobit and Blimp-1 at the protein level, which may be subject to posttranscriptional regulation. Currently, there are no tools available to study Hobit protein expression in mice. Regarding Blimp-1, we have recently demonstrated a discrepancy between the expression of Blimp-1 at the transcriptional and protein level in memory $CD8^+$ T cells (9), suggesting regulation at the level of translation or degradation. In line with this, Blimp-1 is similarly expressed by both $CD69^+$ and $CD69^-$ memory $CD8^+$ T cells in the lungs at the transcript level, while Blimp-1 deficiency only perturbs the formation of lung $CD8^+$ T_{RM} cells. Therefore, differences at the level of posttranscriptional regulation of Hobit and Blimp-1 expression may shape the unique dependency of lung $CD8^+$ T_{RM} cells on Blimp-1.

Our data demonstrate that lung $CD8^+$ T_{RM} cells do not require Hobit for their development, and this may have implications for their effector functions. In contrast to circulating memory $CD8^+$ T cells, $CD8^+$ T_{RM} cells in many tissues maintain high levels of the cytotoxic mediator granzyme B (8, 26, 45). On a transcriptional level, we could show that lung $CD8^+$ T_{RM} cells express elevated levels of granzyme B mRNA. However, lung $CD8^+$ T_{RM} cells do not retain granzyme B protein expression and exhibit little cytotoxicity (46, 47). We recently showed that Hobit is essential for maintenance of granzyme B expression in liver and intestinal $CD8^+$ T_{RM} cells (9). This may indicate a functional link between the poor cytolytic potential of lung $CD8^+$ T_{RM} cells and their independence from Hobit-mediated regulation. Whether Blimp-1 and/or Hobit regulate effector responses of lung $CD8^+$ T_{RM} cells upon reinfection, remains to be determined.

Immunosurveillance of peripheral tissues for reinfection is primarily performed by $CD8^+$ T_{RM} cells (48). These resident populations are characterized by shared phenotypic, transcriptional and functional features, but also exhibit distinct differences across tissues (20, 28). In addition, growing evidence suggests further heterogeneity of $CD8^+$ T_{RM} cells within one tissue. Expression of CD103 and CD49a delineates $CD8^+$ T_{RM} cell populations with discrete functional capacities in the intestine and skin, respectively (29, 49). In the lung, both $CD69^+$ $CD103^-$ and $CD69^+$ $CD103^+$ $CD8^+$ T_{RM} cells arise after influenzas virus infection. While genetic ablation of Blimp-1 greatly impaired the formation of $CD69^+$ $CD103^+$ $CD8^+$ T_{RM} cells, the $CD69^+$ $CD103^-$ population was less affected, suggesting distinct transcriptional regulation of the two subsets. Moreover, $CD103^-$ $CD8^+$ T_{RM} cells may occupy different sites, as they are unable to undergo interaction with E-cadherin expressing epithelial cells, a process requiring CD103 expression (50, 51). In line with this, $CD103^-$ $CD8^+$ T_{RM} cells in the intestine display localization and functional properties separate from their $CD103^+$ counterparts (49). Hence, lung tissue may harbor distinct $CD8^+$ T_{RM} cell populations, delineated by CD103 expression and dependency on Blimp-1.

The transcriptional programs governing the differentiation of naïve $CD8^+$ T cells into different subsets of memory $CD8^+$ T cells, namely T_{CM}, T_{EM} and T_{RM} cells, are gradually being uncovered. However, it is still incompletely understood how transcription factors control the fate choice between the memory $CD8^+$ T cell subsets. Previous research has highlighted the importance of the transcription factors Runx3 and Notch in the formation and/or maintenance of $CD8^+$ T_{RM} cells in the lungs. While Runx3 is required for the formation of lung $CD8^+$ T_{RM} cells, Notch regulates the maintenance of these cells (23, 52). Here, we show that the transcription factor Blimp-1 promotes formation of lung $CD8^+$ T_{RM} cells, while suppressing the development of $CD8^+$ T_{CM} cells. These phenotypes were confined to the Blimp-1 deficient, but not the WT compartment of mixed BM chimeras, and are thus likely due to cell-intrinsic effects rather than indirect effects involving competition for shared resources. Interestingly, Blimp-1 had no impact on the formation of $CD44^{high}$ $CD62L^-$ $CD8^+$ T_{EM} cells, indicating that this transcription factor primarily regulates the lineage choice between $CD8^+$ T_{RM} and T_{CM} cells. We have previously demonstrated that Blimp-1 suppresses the expression of TCF-1 via direct binding to the TCF-1 encoding *Tcf7* locus. Consistently, *Tcf7* downregulation is a common feature of resident lymphocyte populations (20). We also observed low expression of TCF-1 protein in lung $CD8^+$ T_{RM} cells, which was strongly increased upon genetic ablation of Blimp-1. TCF-1 is an essential transcription factor driving the development of circulating memory $CD8^+$ T cells, in particular of $CD8^+$ T_{CM} cells (32, 33, 53). The suppression of TCF-1 by Blimp-1 may suggest that Blimp-1, in concert with TCF-1, controls the fate choice between $CD8^+$ T_{RM} and T_{CM} cells. It will be interesting to determine if and how these two transcription factors drive this lineage choice during $CD8^+$ T cell differentiation. This is of particular interest in the context of therapeutic approaches aiming to modulate the balance between different memory $CD8^+$ T cell subsets.

Similar to mice, crossprotection against influenza virus in humans is strongly correlated with the presence of $CD8^+$ T cells specific to conserved viral epitopes (54, 55). Moreover, $CD8^+$ T cells recognizing respiratory viruses, including influenza virus, are enriched in the human lung (56, 57). A substantial fraction of these virus-specific $CD8^+$ T cells express markers of tissue-residency, including CD69 and CD103 (58, 59), indicating the presence of bona fide influenza-specific $CD8^+$ T_{RM} cells within the human lung. Transcriptional profiling of human lung $CD8^+$ T_{RM} cells has demonstrated a strong overlap with gene signatures of $CD8^+$ T_{RM} cells from different murine tissues, including the selective upregulation of tissue retention molecules (e.g., CD49a, CD103, CD69), and the downregulation of tissue egress receptors (e.g., S1PR1, S1PR5) (23, 28). In line with our findings in mice, $CD8^+$ T_{RM} cells in the human lung exhibit no differential expression of Blimp-1 on the transcriptional level in comparison to circulating $CD8^+$ T_{EM} cells (23). In contrast to their murine counterparts, Hobit mRNA is not differentially expressed in human lung $CD8^+$ T_{RM} cells, relative to the expression in $CD8^+$ T_{EM} cells (23, 28), partly because the latter population also expresses Hobit in humans (23, 60). This may suggest that Hobit is not specifically involved in the differentiation of human lung $CD8^+$ T_{RM} cells. It remains to be determined if Blimp-1, on the other hand, promotes $CD8^+$ T_{RM} differentiation in humans.

We show that $CD103^+$ $CD8^+$ T_{RM} cell formation in the lungs after influenza virus infection is dependent on the transcription factor Blimp-1. Blimp-1 driven suppression of TCF-1 expression may instruct the lineage choice between $CD8^+$ T_{CM} and T_{RM} cells during memory $CD8^+$ T cell formation. As there is growing recognition of the clinical importance of tissue-resident T cell memory, we believe that insights into the transcriptional mechanisms governing local $CD8^+$ T_{RM} cell differentiation are an important prerequisite for the development of novel vaccination approaches in the lungs.

MATERIALS AND METHODS

Mice

C57BL/6JRj ($CD45.2^+$ WT) mice were purchased from Janvier and B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ ($CD45.1^+$ WT) mice from the Jackson Laboratory. Both lines were crossed to generate B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ x C57BL/6JRj mice. *Zfp683*$^{-/-}$ (*Hobit* KO) (61), *Prdm1*$^{flox/flox}$ × Lck Cre (*Blimp-1* KO) mice (24) and *Zfp683*$^{-/-}$ × *Prdm1*$^{flox/flox}$ × Lck Cre mice (20) were maintained on a C57BL/6JRj background. For the generation of mixed bone marrow (BM) chimeras, CD45.1 × CD45.2 (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ × C57BL/6JRj) recipient mice were irradiated (2x 5 Gy) and reconstituted with i.v. transfer of $10*10^6$ BM cells per genotype. Mixed BM chimeras were used 12–16 weeks after reconstitution and chimerism of lymphocytes was confirmed prior to usage. All mice were maintained under SPF conditions in the animal facility of the Netherlands Cancer Institute (NKI). Animal experiments were conducted according to institutional and national guidelines.

Influenza Virus Infection

Mice were infected intranasally with 100x $TCID_{50}$ (median tissue culture infectious dose) of HKx31 influenza A virus in a volume of 50 µl after anesthetization by inhalation of isoflurane. HKx31 virus was kindly provided by Dr. Guus Rimmelzwaan (University of Veterinary Medicine Hannover). At the indicated time points after infection, mice were sacrificed, and tissues were harvested for analysis of $CD8^+$ T cell responses.

Tissue Preparation

Single cell suspensions from spleen and lymph nodes were prepared by mechanical disruption via passing of the tissues over a 70 µm cell strainer. Lung tissue was cut into pieces of 1 mm^2 and enzymatically digested for 30 min at 37°C with 750 U ml^{-1} Collagenase Type I (Worthington) and 0.31 mg ml^{-1} DNase I (Roche, from bovine pancreas, grade II) in RPMI 1,640 supplemented with 10% (v/v) fetal calf serum (FCS). Single cell suspensions were generated by filtering over a 70 µm cell strainer and the isolated lymphocytes were purified by density centrifugation on a 66/44% Percoll gradient (GE Healthcare). Lung lymphocytes were extracted from the interphase of the Percoll gradient. Contaminating erythrocytes were removed using red blood cell lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA).

Flow Cytometry

Cells were incubated with antibodies and tetramers for 25 min at 4°C and washed with PBS supplemented with 0.5% (v/v) FCS. The following anti-mouse monoclonal antibodies were used: anti-CD3 (17A2), anti-CD4 (RM4-5), anti-CD8a (53–6.7), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD49a (Ha31/8), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD103 (M290), anti-CD127 (A7R34), anti-CXCR6 (SA051D1), anti-KLRG1 (2F1), anti-TCF-1 (S33-966), mouse IgG1, κ isotype control (P3.6.2.8.1). Antibodies were purchased from BioLegend, eBiosciences, BD Biosciences, or BD Pharmingen. Influenza-virus-specific $CD8^+$ T cells were detected using H-2 D^b ASNENMETM ($NP_{366-374}$) tetramers (D^b NP366), which were a kind gift of Dr. Anja ten Brinke (Sanquin Research). Exclusion of dead cells was performed with live/dead fixable near-IR dead cell stain kit (Thermo Fisher Scientific). For staining of intracellular molecules, the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) was used according to the manufacturer's specifications. Samples were acquired on an LSR Fortessa flow cytometer (BD) and data was analyzed using FlowJo V10 (Tree star) software.

RNA-Seq Analysis

A subset of previously published RNA-seq (CEL-seq) data (geo accession number GSE79774) (23) was reanalysed. DMSO-treated $CD69^+$ and $CD69^-$ memory ($CD44^{high}$ $CD62L^{lo}$) $CD8^+$ T cells isolated from murine lungs at day 30+ after HKx31 infection were analyzed to determine the tissue-resident phenotype of these populations. Reads were first aligned to mm10 genome with STAR, followed by read count quantification with featureCounts using Ensembl's v92 annotation with modification of *Zfp683*. Since CEL-seq was used, only the 3′ end of the genes

is sequenced, and inspecting the alignments manually revealed that the reads that mapped to *Zfp683* were outside Ensembl's defined region of the gene. We therefore extended the 3′ end of *Zfp683* with 1,057 nucleotides to include all reads of this gene. To find differentially expressed (DE) genes, first low expressed genes were removed (retained genes had more than 1 cpm (counts per million) in at least four samples), followed by limma voom and quantile normalization. A linear model was fitted to each gene using a design that also included mouse (paired) and library (batch) effect, and eBayes moderated t-statistics were used to find DE genes between $CD69^+$ and $CD69^-$ memory $CD8^+$ T cells. Benjamini-Hochberg procedure was used for FDR correction, and adjusted $P < 0.05$ were considered significant.

Statistical Analysis

For pairwise comparisons, a standard two-sided Student's *t*-test (paired), was applied with GraphPad Prism 6 software. $P < 0.05$ were considered statistically significant ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). The Morpheus software (https://software.broadinstitute.org/morpheus), developed by the Broad Institute, was used to generate heat maps. Values were converted to heat map colors using the mean and maximum values for each row.

ETHICS STATEMENT

This study was carried out in accordance with the national guidelines of the Central Commission for Animal Experiments (Centrale Commissie Dierproeven). The experimental protocols were approved by the Animal Welfare Body of the Netherlands Cancer Institute (NKI).

AUTHOR CONTRIBUTIONS

FB and KvG conceived and designed studies. FB, NK, TW, and RS performed animal experiments and FB analyzed data. BN analyzed RNAseq data provided by PH and DA. FB and KvG wrote the manuscript. All authors provided critical feedback and approved the manuscript.

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REVIEW
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Effector Functions of CD4+ T Cells at the Site of Local Autoimmune Inflammation—Lessons From Rheumatoid Arthritis

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Infiltration of memory CD4+ T cells in synovial joints of Rheumatoid Arthritis (RA) patients has been reported since decades. Moreover, several genome wide association studies (GWAS) pinpointing a key genetic association between the HLA-DR locus and RA have led to the generally agreed hypothesis that CD4+ T cells are directly implicated in the disease. Still, RA is a heterogeneous disease and much effort has been made to understand its different facets. T cell differentiation is driven by mechanisms including antigen stimulation, co-stimulatory signals and cytokine milieu, all of which are abundant in the rheumatic joint, implying that any T cells migrating into the joint may be further affected locally. In parallel to the characterization and classification of T-cell subsets, the contribution of different effector T cells to RA has been investigated in numerous studies though sometimes with contradictory results. In particular, the frequency of Th1 and Th17 cells has been assessed in the synovial joints with various results that could, at least partly, be explained by the stage of the disease. For regulatory T cells, it is largely accepted that they accumulate in RA synovial fluid and that the equilibrium between regulatory T cells and effector cells is a key factor in controlling inflammation processes involved in RA. Recent phenotypic studies describe the possible implication of a novel subset of peripheral T helper cells (Tph) important for T-B cell cross talk and plasma cell differentiation in the RA joint of ACPA+ (autoantibodies against citrullinated proteins) RA patients. Finally, cytotoxic CD4+ T cells, historically described as increased in the peripheral blood of RA patients have attracted new attention in the last years. In view of the recently identified peripheral T-cell subsets, we will integrate immunological data as well as information on genetic variants and therapeutic strategy outcomes into our current understanding of the width of effector T cells. We will also integrate tissue-resident memory T cell aspects, and discuss similarities and differences with inflammatory conditions in skin (psoriasis) and mucosal organs (Crohn's disease).

Keywords: rheumatoid arthritis, CD4+ T cells, effector function, T-cell subsets, helper, cytotoxic, tissue-resident

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INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic inflammatory disease targeting peripheral joints leading to bone erosion, impairment of mobility, and decreased quality of life. It is affecting 0.5–1% of the population worldwide and is more prevalent in women than in men (1). The pathogenesis of RA is mainly localized in the synovial joint where immune cells composed of T cells, B cells, macrophages, and dendritic cells infiltrate the synovium. Moreover, fibroblast-like synoviocytes present in the sublining layer of the synovium proliferate and contribute to cartilage damage (2). Memory CD4+ T cells are enriched in affected joints of RA patients (3) and highly expanded CD4+ T cell clones are found in synovial tissue of early disease (4) suggesting that T cell expansion could be due to local antigen-induced proliferation. The efficiency of co-stimulation blockade targeting CD80/CD86-CD28 interaction further illustrates the importance of T cells in the pathogenesis of RA (5).

A central function of CD4+ T cells in RA has also been deducted from genetic studies. An early report by Stastny (6) identified an association between RA and HLA-DRB1 that was further confirmed by genome-wide association studies (GWAS) (7). This association led to the "shared epitope hypothesis" whereby a five-amino acid sequence found in certain HLA-DRB1 alleles was associated with increased susceptibility to RA (8). In about 2/3 of RA patients, serum antibodies to citrullinated protein antigens (ACPAs) are present and these are associated with the HLA-DRB1 risk alleles (1). Altogether, these findings have led to the hypothesis that citrullinated peptides might be preferentially presented by HLA-DRB1 risk alleles (9). Such peptide presentation has indeed been demonstrated both functionally (10) and by peptide-HLA crystal structure determination (11). Several citrullinated candidate peptides can be presented by HLA-DRB1*04:01 and other shared epitope alleles such as *04:04 and *10:01 (10, 12, 13) and the search for immunodominant T cell epitopes is still an important area of investigation in the field of RA. The relevance of antigen specificities has already been discussed elsewhere (13) and will not be detailed in this review but instead will be discussed in the context of effector T cell functions.

Infiltration of CD4+ T cells at the site of inflammation is a characteristic feature of several autoimmune syndromes. In the scope of this review, we present and discuss up-to-date understanding of effector functions of CD4+ T cells (**Figure 1**) present in the joint of RA patients. Examples of CD4+ T cell effector functions from other chronic inflammatory conditions (psoriasis and Crohn's disease) are selected to contrast and discuss our current knowledge in the field of RA. In particular, many common therapeutic strategies have been evaluated in RA, psoriasis and Crohn's disease with different outcomes that shed light on the different pathways implicated in the pathogenesis of these inflammatory disorders. Due to lack of space, this review will be mainly dedicated to findings in human inflammatory conditions.

TH1 CELLS AND ASSOCIATED EFFECTOR FUNCTIONS

In 1986, Mosmann and Coffman proposed that mouse CD4+ helper T (Th) cells could be subdivided in Th1 or Th2 subsets based on their differential capacity to secrete IFNγ, IL-2, and TNF or IL-4, and IL-5, respectively (14). Subsequently, several reports identified human T cell clones separating into Th1 and Th2 categories (15). Th1 CD4+ T cells are crucial in the defense against intracellular pathogens such as mycobacteria (16) whereas Th2 CD4+ T cells mediate the immune defense against parasites such as helminths (17).

Th1 Cells in Circulation and at Site of Inflammation

CD4+ T cells prone to secrete IFNγ (18, 19) were identified in synovial fluids from RA patients while IL-4 production (18) and IL-4+ T cell clones (19) were not increased in synovial fluid compared to peripheral blood. RA was subsequently defined as a Th1-driven disease while Th2 immunity was proposed to have a therapeutic potential in RA (20). CXCR3 was identified as a surface marker for Th1 cells (21) and T-bet as a master transcription factor (22). CXCR3 binds the two IFNγ-induced chemokines CXCL9 and CXCL10 (23). CXCR3 expression on CD4+ T cells (24) as well as CXCL9 and CXCL10 are enriched in synovial fluids (25). Although the vast majority of CD4+ T cells present in synovial joints are of memory phenotype (CD45RO+) (3) and hence antigen-experienced, our insight into their antigen specificity is scarce. Non-specific CD4+ T cells infiltrating the inflamed joint are likely to bias the analysis of the phenotype of relevant CD4+ T cells.

In that context, *ex vivo* peptide-HLA-DR-tetramer analysis provides a more relevant picture of antigen-specific i.e., citrulline-reactive T cells. Hereby, around 40% of citrulline-reactive CD4+ T cells were found to be CXCR3+ in the blood of RA patients (26) pointing again toward a Th1 signature of autoreactive T cells in RA. Presence of IL-12, IL-18, IFNγ, drivers of Th1 differentiation has also been reported in the synovial tissues of RA patients but not in osteoarthritis patients (**Figure 1**) (27, 28). However, there is still a lack of information concerning the phenotype of antigen-specific CD4+ T cells at the site of inflammation. Finally, immunodominant T cells epitopes have yet to be discovered in RA that will facilitate the more common use of peptide-HLA-DR-tetramer.

Downstream Effects of Th1 Activity

Th1 cells classically induce macrophage activation (29) characterized in the context of the synovial joint by an increased capacity to produce pro-inflammatory cytokines such as TNF (30). Long-lived resident macrophages are present in synovial tissues from healthy donors (31) while inflammatory macrophages are mainly derived from blood monocytes in active RA (32). The interplay between Th1 cells and these two different subsets of macrophages in the context of the synovial joint is unknown. It will be particularly important to understand if Th1 cells can modify the properties of resident macrophages which could then contribute to perpetuation of the disease (33). Th1

Possible triggering factors in RA	CD4+ T cell subsets		Possible effector function in RA	Evidence for involvement in RA
IL-12 (27), IL-18 (28) IFNγ (18, 19)	Th1 T-bet	CXCR3	• Macrophage activation • B-cell isotype switching	• Th1 cells in SF (18) and in synovial tissue (19) • IgG1 and IgG3-class switched B-cells in RA patients (34, 35) • Activated macrophages in SF (32)
TGF-β, IL-6, IL-1β IL-21, IL-23 (66-69)	Th17 RORγT	CCR6 CD161	• Induction of proinflammatory cytokines • Neutrophil recruitment • Ostoclastogenesis	• IL-17 in SF (60) and Th17 cells in synovial tissue (61) • Neutrophils and NETs in synovial fluids (71) • Collagen–induced arthritis is decreased in IL-17 knock-out mice (72)
IL-2 (141), TGFβ (142)	Treg FOXP3	CD25	• Regulatory function dampened by inflammatory milieu	• Tregs in SF (131, 132) and in synovial tissue (133)
IL-6 (111), ICOSL (112)	Tfh Bcl6	CXCR5 ICOS PD-1	• B-cell activation and isotype switching	• Autoantibodies in RA (94) • Tfh in synovial tissue (113, 114) • Germinal centers in synovial tissues of some RA patients (95, 96)
unknown	Thelper-like Blimp	PD-1high MHC Class II	• B-cell activation and isotype switching	• Autoantibodies in RA (94) • Thelper-like in synovial tissue (113) • Germinal centers in synovial tissues of some RA patients (95, 96)
IL-2 (141), IL-15 (175) 4-1BB (176)	Cytotoxic EOMES Hobit	Prf1 GZMB	• Cell death • Citrullination	• Cytotoxic CD4+ T cells in SF (165, 166) and in synovial tissue (164)

FIGURE 1 | Important CD4+ T-cell subsets in Rheumatoid Arthritis (SF, Synovial Fluid; NETs, Neutrophil Extracellular Traps).

cells have been proposed to influence class switching toward IgG1 and IgG3 in humans (20). In RA, polyclonal antibodies against type II collagen are predominantly of IgG1 and IgG3 subclasses (34) and autoantibodies against citrullinated fibrin are mainly IgG1 (35) suggesting previous interaction with IFNγ-producing cells. Nevertheless, Ig class switching is probably influenced by a multitude of other factors during the course of inflammation and should not be oversimplified by a link to a specific CD4+ T-cell subset. T helper cells also provide help to CD8+ T cells as demonstrated in the context of cancer immunology (36). Despite a reported presence of CD8+ T cells in synovial joints (37), the influence of CD4+ T cells on their activation is currently unknown.

Th1 Targeted Therapy

Evidences of pathogenic function of Th1 cells in RA were contradicted by the lack of efficiency of therapeutic strategy targeting IFNγ (Fontolizumab) initiated in a phase II clinical trial in active RA. This clinical trial was terminated because the first phase did not reach the goals of primary endpoint (38). In the same line, in IFNγ receptor knock-out mice, collagen-induced arthritis was accelerated (39). In this particular mouse model, it has been proposed that IFNγ suppresses inflammation through inhibition of Th17 responses (40). It is however currently unknown if this hypothesis holds true in a human setting. It should be mentioned that biologic therapies targeting TNF, a Th1 cytokine are successful treatments in RA (41). Hence, Th1 cells could act on at least two opposing levels by directly contributing to tissue damage through TNF production or by suppressing Th17 responses.

Since Th1 cells were one of the first T helper cell subsets described, their contribution to the pathogenesis of autoimmune diseases has been investigated in numerous studies. This is also the case both for psoriasis (42, 43) and Crohn's disease (44) that were both initially suggested to be Th1-driven diseases. IFNγ-producing cells were indeed identified at the site of inflammation in these two diseases (42, 45). However, in a phase II clinical trial, Fontolizumab did not induce a robust beneficial clinical effect in Crohn's disease (46). Similarly, in a small study, therapeutic targeting of IFNγ with a humanized anti-IFNγ (HuZAF) showed no significant efficacy in psoriasis patients (47). In these three diseases, despite the clear presence of Th1 cells at the site of inflammation, therapeutic targeting of IFNγ did not lead to beneficial results. IFNγ might be important in the very early phases of the disease through, for instance, the induction of TNF in macrophages (48). It has also been shown that IFNγ induces the expression of vascular cellular adhesion molecule-1 (VCAM-1) on endothelial cells (49) which facilitates lymphocyte migration to the tissue. Finally, through reciprocal regulation, Th1 cells might also suppress the generation of pathogenic T cells such as Th17 cells that contribute to tissue damage.

TH17 CELLS AND ASSOCIATED EFFECTOR FUNCTIONS

The Th1/Th2 hypothesis was revisited in 1995, when a third T-cell subset named Th17 cells based on the production of the newly identified cytokine IL-17 (50, 51) was discovered. The IL-17 family comprises 6 members with IL-17A (historically referred as IL-17) and IL-17F being the most closely related, in addition to IL-17B, IL-17C, IL-17D, and IL-17E (52). Th17 cells were initially described as co-expressing the chemokine receptors CCR6 and CCR4 (53) and expressing the master transcription factor RORγT (**Figure 1**) (54). In addition, CD161 was recently described as a marker of all IL-17 producing cells (55). In epithelial, endothelial and fibroblastic cells, IL-17A stimulates the production of pro-inflammatory cytokines such as IL-6, IL-8, and GM-CSF (56) and promotes neutrophil recruitment (57). Th17 cells are particularly important in protective immunity against fungal and extracellular bacterial infections (*Staphylococcus aureus*) (58).

Classic Th17 Responses and RA

Early on, production of IL-17 was demonstrated in synovial tissues (59) and synovial fluid (60) of RA patients but not in that of osteoarthritis patients. In addition, IL-17-producing CD4+ T cells from synovial tissues from RA patients could readily be identified (61). The reported frequency of Th17 cells in peripheral blood of RA patients varies according to different studies where either an increase (62, 63) or a status quo in their frequency (64, 65) has been observed. Moreover, only few citrulline-specific CD4+ T cells were CCR6+ positive in peripheral blood of RA when analyzed by *ex vivo* peptide-HLA-DR-tetramer analysis (26). Th17-inducing cytokines (IL-6 IL1-β, IL-21, TGF-β, and IL-23) (66–69) are present in the synovial joint (**Figure 1**). Further, synovial IL-17 from RA patients was shown to induce bone resorption (70). Finally, IL-17 contributes to neutrophil recruitment, a hallmark of RA synovial fluid (71). In IL-17-deficient mice, collagen-induced arthritis was decreased supporting the notion that Th17 cells play a pathogenic role in the development of the arthritis (72). It was therefore unexpected that therapeutic targeting of IL-17A (Secukinumab) or the IL-17 receptor (brodalumab) in phase II studies was less successful in RA than in other inflammatory conditions such as psoriasis (73, 74). It was recently proposed that Th17 cells might migrate to the synovium in CCP+ (anti-cyclic citrullinated peptide) early RA patients (75). Hence, in RA, IL-17-producing T cells might contribute during early stages of the disease or be more prominent in a subtype of RA patients.

The Different Facets of IL-17- Anti vs. Pro-inflammatory Features

Another level of complexity arises from evidence that Th17 cells are implicated in different immune responses depending on co-expressed cytokines (76). Indeed, T cells co-expressing IL-17 and IL-10 are thought to be important in mucosal defense but not pathogenic as T cells co-expressing IL-17, IFNγ, or GM-CSF are (66). After anti-TNF treatment, Th17 cells were shown to acquire IL-10 production in RA (77) implying that Th17 cells could also be protective and participate in dampening inflammation in RA. While GM-CSF appears to be a critical component of Th17 pathogenicity in the experimental autoimmune encephalomyelitis (EAE) mouse model (78), it is associated with the Th1 axis in multiple sclerosis (79). Likewise, in synovial joints of RA patients, GM-CSF production is enriched in Th1 cells and not in Th17 cells (80). Several clinical trials targeting GM-CSF are ongoing in RA (81) and will shed light on the pathogenic function of GM-CSF in the context of RA. It also implies that additional markers for Th17 pathogenic subsets are needed to predict which patients are more likely to respond to such therapies.

Different Responses to Anti-IL-17 Blockade in Inflammatory Diseases

While the importance of Th17 cells seems to vary according to the stage and subsets of RA, psoriasis vulgaris can currently be defined as an IL-17-mediated inflammatory skin disease (82). IL-17-secreting cells are found in psoriatic lesions and include CD4+ (83), CD8+ (84), and γδ T cells (85). Phase III trials with a human anti-IL-17A monoclonal antibody (Secukinumab) were successful in psoriasis with response rates of 72–82% at week 12 (86). Th17 cells are also present in the gut of patients with Crohn's disease (87) but IL-17 blockade with Secukinumab was not effective and adverse cases of fungal infections and worsening of the disease were observed (88). In this case, IL-10-producing Th17 cells having a regulatory function (89, 90) might have been targeted contributing to the exacerbation of the disease.

Although early studies suggested that Th17 cells are crucial in most of human inflammatory conditions, different responses to IL-17 blockade contradict this hypothesis. Th17 cells are present at the site of inflammation in RA, Crohn's disease and psoriasis but the difference in the response to anti-IL-17 therapies strongly suggests that their direct implications in the pathogenesis of these diseases differ and reflect different effector functions in tissues. In psoriasis, blocking of IL-17 will alleviate the direct effects of IL-17 on keratinocyte proliferation. In the gut, Th17 cells play an important role in mucosal host defense (58, 91), which is reflected by the secondary effects observed in Crohn's patients under anti-IL-17A therapies. Th17 cells also produce IL-22 which is involved in intestinal epithelial barrier integrity (92). In active RA, targeting IL-17 might not be sufficient to dampen the ongoing cytokine cascade and resorb migration of neutrophils already sequestered in the synovial joint. Targeting of IL-17 at earlier time points or in conjunction with other anti-cytokine blockade might be more effective. Indeed, the combination of IL-17 and TNF synergize to induce the expression of P and E-selectins on endothelial cells that induce an influx of neutrophils (93).

T CELLS PROVIDING B CELL HELP–TFH and TPH

Local CD4+ T-cell help to B cells is likely to be a prominent driver of humoral immunity in RA patients seropositive for ACPA (anti-citrullinated protein antibodies) and/or RF

(Rheumatoid Factor). About 60–70% of RA patients present with ACPAs and 50-80% of patients are seropositive for RF (94). Already in 1992, synovial T cells from RA patients were shown to induce B cell Ig production *in vitro* (3). Moreover, ectopic germinal centers are observed in synovial tissues of some RA patients (95, 96). In 2000, a subset of follicular CD4+ T cells (Tfh) expressing CXCR5 and specialized in stimulating antibody responses was described in germinal centers in secondary lymphoid organs (97). They typically express the master transcription factor Bcl-6 as well as IL-21, PD-1, and ICOS. IL-6 and ICOS triggering regulate their differentiation (98) (**Figure 1**).

B Cell Helpers in Circulation

Circulating Tfh can be analyzed in peripheral blood where their characteristics slightly differ from the follicular ones with a lack of expression of Bcl-6 (98–100). Blood Tfh can be further subdivided into Th1, Th2, Th17 cell subsets with Tfh2 and Tfh17 being the only subsets capable of sustaining the B-cell Ig switch (101). Several studies have described an increased frequency of CXCR5+ICOS+CD4+ Tfh cells in peripheral blood of RA patients which correlates with serological anti-CCP titers and disease severity (102–105). This is accompanied by increased concentrations of IL-21 in the serum of RA patients when compared to healthy donors (102–106). Few reports have investigated the frequency and function of CXCR5+ follicular T cells in psoriasis and Crohn's disease probably due to the lower prevalence of humoral immunity in these patients as compared to RA patients (107, 108). One study reported an increase of Tfh17 CD4+ T cells in peripheral blood of psoriasis patients that correlated with disease activity (109). An increased frequency of Tfh1 and Tfh17 CD4+ T cells has been observed in peripheral blood of patients with Crohn's disease (110).

B Cell Helpers in Tissue

IL-6 (111) and ICOS expression on CD4+ T cells (112), inducers of Tfh differentiation, have been reported in the rheumatic joint. In synovial tissues, few CD4+ T cells express CXCR5 (113, 114) which is surprising given the reported expression of CXCL13, the ligand for CXCR5, in synovial fluids and tissues of RA patients (115, 116). It has recently been proposed that another subset of memory T cells, the peripheral T helper cell (T_{ph}) subset expressing MHC class II and high levels of the co-inhibitory receptor PD-1 provide B-cell help in the synovial joint (**Figure 1**) (113). These cells lack Bcl-6 but express other typical markers for B-cell help such as IL-21, CXCL13, ICOS, and MAF. It is currently unknown if this subset shares the same ontogeny as Tfh. This study supports earlier reports showing the importance of PD-L1 (program death ligand-1)/PD-1 interaction in RA. Indeed, most of C57BL/6-Pdcd1-/- mice develop arthritis (117) and CD4+ T cells were shown to express PD-1 in synovial joints of RA patients (118). CD4+PD-1+ infiltrating T cells have also been described in the context of breast cancer (119) where they display comparable features with T_{ph} such as ICOS and CXCL13 expression (120). Importantly, these cells also express IFNγ in both RA synovial fluid (113) and breast cancer (119) showing that these T cells have the capacity to convey multiple effector functions contradicting the original Th1/Th2 hypothesis. Recently, the occurrence of case reports of RA following PD-1 or PD-L1 blockade in the context of cancer therapies have also highlighted the role of this checkpoint regulation in the balance between cancer and autoimmunity (121). Cases of psoriasis have also been reported (122) suggesting the importance of PD-1 in the pathogenesis of this disease as well. Although this subset of pathogenic T cells has not been extensively studied in psoriasis, PD-1 expression on Th17 cells has been reported in psoriatic skin of patients (123). So far, the subset of pathogenic T_{ph} cells driving B-cell responses has only been described in the synovial joint of RA patients but is probably relevant to other antibody-positive autoimmune diseases.

REGULATORY T-CELL SUBSETS

The concept of regulatory T cells was revisited in 1995 when the group of Sakaguchi described a population of CD4+ CD25+ T cells capable of preventing the development of several autoimmune diseases in mice (124). Shortly after, the key function of the transcription factor FOXP3 (Forkhead box P3) in the development and function of regulatory T cells was demonstrated in mice (125) and humans (126). Regulatory T cells (Tregs) also express CTLA-4 (cytotoxic T lymphocyte-associated antigen-4) and other markers e.g., GITR (glucocorticoid-induced TNFR family related gene) and CD39 whose expression might vary depending on the context (127). In peripheral blood, Tregs can be divided into naïve and activated regulatory T cells based on the expression of CD45RA (128). Importantly, in humans, FOXP3 expression is not strictly restricted to regulatory T cells as it is transiently up-regulated also in effector T cells (129). Therefore, in humans, regulatory T cells cannot only be defined by the phenotypic expression of FOXP3 and CD25 but need to be supplemented by assessment of their *in vitro* suppressive capacity and/or the demethylation of the *FOXP3* TSDR (Treg cell-specific demethylated region) (130).

Tregs at Site of Inflammation

In RA patients, FOXP3+CD25+CD4+ T cells accumulate in synovial fluid (131, 132) and in synovial tissue (**Figure 1**) (133). *In vitro* suppressive capacity and demethylation of the *FOXP3* TSDR showed that these regulatory T cells are functional (133, 134). However, effector T-cell proliferation and pro-inflammatory cytokines have been demonstrated to reduce FOXP3 regulatory T-cell function *in vitro* (134), which is likely to happen also *in situ*. Indeed, adding TNF during co-culture experiments was shown to inhibit regulatory T-cell functions (135, 136). Whether this effect is mediated through a direct action on effector T cells or regulatory T cells is still a matter of debate (137) since TNF can also induce conventional T-cell proliferation (138). The ontogeny of FOXP3+CD4+ T cells in synovial tissues is still unknown. FOXP3+ Tregs can originate from the thymus (thymus Tregs or tTregs) or be induced *in situ* from conventional T cells during infections or other inflammatory processes (induced Tregs or iTregs) (139).

Regulatory T cells from peripheral blood and inflamed joints of juvenile arthritis patients were shown to harbor a different T

cell Receptor Vβ usage than conventional T cells suggesting that Tregs would be generated independently of conventional T cells (140). Whether this is also the case in RA is currently unknown but IL-2 (141) and TGFβ (142), important for induced regulatory T-cell generation are present in synovial fluids of RA.

In plaques of psoriasis patients, the frequency of FOXP3+ CD4+ T cells is increased when compared to healthy skins (143) where only few Tregs are found in the dermis and epidermis (143). FOXP3+CD4+ T cells present in the psoriatic lesions also co-expressed IL-17 (143, 144). Hence, as for RA, it has been suggested that the inflammatory milieu through for instance IL-6 (145) may affect regulatory T-cell function.

Therapeutic Interventions Affecting Treg Function and/or Frequencies

Current therapies given in RA that alleviate inflammation are therefore likely to restore or increase regulatory T-cell function. In RA patients treated with anti-TNF therapy, an increased frequency of CD4+CD25 high T cells was observed in responders when compared to non-responders (146). Similarly, an expansion of CD4+CD25+FOXP3+ T cells has been observed after anti-IL-6R blockade and was accompanied by a decrease in Th17 frequency (147). Treg and Th17 cells have opposite functions but their differentiation both rely on the presence of TGFβ. In the absence of pro-inflammatory cytokines such as IL-6, Treg differentiation is enhanced whereas in the presence of IL-6, Th17 differentiation is promoted. Hence, targeting IL6R contributes to increase the ratio between Treg and Th17 cells in RA patients (147). Recently, a therapeutic strategy based on the use of low-dose of IL-2 has been developed to directly induce the expansion of Tregs *in vivo* in autoimmune patients (148). This concept relies on the fact that Treg development and expansion is dependent on low levels of IL2R signaling (149). Clinical trials investigating such treatments in RA are currently ongoing.

IL-10 Producing Tr1 Cells

Another subset of T regulatory cells named T regulatory type 1 cells (Tr1) is defined by their suppressive function combined with their capacity to produce IL-10 (150). So far, no unique cell surface marker specific for Tr1 cells has been identified but the expression of several markers such as ICOS, PD-1, CD49b, TIM-3, and LAG3 is increased on this subset (151). Tr1 cells have been extensively studied in the context of intestinal mucosal immunity and the prevention of colitis (152, 153). The importance of IL-10 in intestinal immunity is also illustrated by the identification of mutations in *IL-10*, *IL-10RA,* and *IL-10RB* genes in children suffering from inflammatory bowel disease (IBD) (153). The frequency of Tr1 cells (defined by production of IL-10 and low production of IL-2 and IL-4) was found to be decreased in peripheral blood and synovial fluid of RA patients when compared to osteoarthritis patients and healthy donors (154). However, IL-10 does not only have an anti-suppressive effect but is also involved in B-cell activation and antibody production (155) and is secreted by follicular helper T cells (156). Clearly, IL-10 alone is not sufficient to define Tr1 cells and additional markers are needed to understand their possible function in synovial tissues. As of today, Tr1 cells have been clearly implicated in intestinal mucosal immunity but their contribution to the synovial joint homeostasis is less clear.

CYTOTOXIC CD4+ T CELLS

Although not part of the general text book, cytotoxic features of CD4+ T cells have been observed already more than 20 years ago but were initially described in T cell clones (157) raising the concern that their generation might be an artifact due to repeated *in vitro* stimulation. However, the presence of CD4+ T cells with cytotoxic activities (CD4+ CTLs) has been confirmed *ex vivo* in human diseases driven by a variety of viruses like CMV (158) or dengue (159) as reviewed in Juno et al. (160). In healthy individuals, the frequency of peripheral CD4+ CTLs is usually very low (161).

Cytotoxic T Cells in RA

In peripheral blood of a subset of RA patients, several groups have reported an increased frequency of a population of CD4+CD28null cells expressing perforin, granzymes, and other cytotoxic features (162–164) (**Figure 1**). Although CD4+ CD28null T cells are not enriched in synovial fluid, the presence of perforin+CD4+ T cells has been repeatedly reported in synovial fluids and tissues (164–166). No unique marker is associated with CD4+CD28null T cells but they express proteins related to their cytotoxic functions which are more commonly found in CD8+ CTLs and Natural Killer (NK) cells including granzyme B, granzyme A, and perforin-1. NK cell activating receptors such as NKG2D are also found on CD4+CD28null cells (167). Further investigation of CD4+CD28null cells or an updated approach of studying such cells in RA is warranted in the light of recent characterization of CD4+ CTLs at the single cell level (168, 169). In particular, the recently described transcription factor Hobit was identified in CD4+ CTLs where its precise function remains to be determined (170). We recently demonstrated that the transcription factor EOMES, implicated in terminal T-cell differentiation and the transcription of perforin-1 (171), is increased in CD4+ T cells from synovial fluids of RA patients (166). Using single cell transcriptomics, expanded T cell clones present in the synovium of RA patients were also shown to express EOMES and granzyme B when compared to circulating expanded clones (172). Antigen-specificity of CD4+ CTLs in RA is still debated although an increase in their frequency is more prominent in CMV-seropositive patients suggesting a link between CMV infections and the generation of this T-cell subset in RA patients (173). Nevertheless, repeated antigen stimulation, a classical feature of chronic inflammation, seems to be a constant feature in CD4+ CTL generation (160). Although CD4+ CTLs were initially suggested to derive from Th1 cells, it has also been proposed that they represent an independent lineage with CRTAM (class I-restricted T cell-associated molecule) as a possible marker for precursors of CD4+ CTLs (174). IL-2 and IL-15 as well as 4-1BB triggering are thought to favor their generation (160). Importantly, IL-2 (141) and IL-15 (175) are present in synovial fluids of RA patients while soluble forms of 4-1BB and 4-1BB ligand are increased in peripheral blood of RA patients (176). The functional implications of

CD4+ CTLs interactions with HLA class-II expressing cells in synovial joints such as macrophages, dendritic cells, neutrophils (177), chondrocytes (178), or endothelial cells remains largely unknown. They might directly contribute to joint damage as it has been shown that CD4+ CTLs can directly lyse EBV-infected B cells (179). Another possibility is that they participate in the hypercitrullination of NETs (Neutrophil Extracellular Traps) through a perforin-dependent mechanism (180). We indeed observed that the level of ACPAs correlated with the frequency of perforin+CD4+ T cells in synovial joint of RA patients (166). It was recently shown that CIA was attenuated in granzyme A-/- mice (181) that presented reduced osteoclastogenesis. The source of granzyme A was not identified in this study but we have shown that CD4+ T cells producing granzyme A are present in synovial joints of RA patients (166). Granzyme A also stimulates monocytes to produce IL-6, IL-8, and TNF (182) which can contribute to increased inflammation in the RA joint. The presence of CD4+ CTL at the site of inflammation has been reported in several autoimmune diseases (183–185). Cytotoxic CD4+ T cells expressing NKG2D were identified in the lamina propria of patients with Crohn's disease (186). Likewise, perforin+ CD4+ T cells have been observed in skin lesions from patients with psoriasis (187).

Therapeutic Strategies Affecting Cytotoxic CD4+ T Cells

TNF has been shown to repress the expression of the *CD28* gene (188). In an early study, the expression of CD28 was indeed increased on CD4+ T cells in RA patients undergoing anti-TNF therapy but markers of cytotoxicity were not investigated (189). Direct approaches to target cytotoxic CD4+ T cells can be achieved by targeting specific molecules expressed on these cells. For instance, an antibody targeting NKG2D induced a reduction in disease activity in some Crohn's disease patients in a phase II clinical trial (190). Cytotoxic CD4+ T cells also express CX3CR1, the receptor for fractalkine, a chemokine expressed on synoviocytes and endothelial cells of synovial tissues from RA patients (191). A phase II clinical trial is currently investigating the effect of fractalkine blockade in RA patients refractory to TNF inhibitors or methotrexate therapy. Clearly, the results of these new therapeutic blockades will bring new insights into the contribution of cytotoxic CD4+ T cells to RA.

TISSUE-RESIDENT MEMORY T CELLS

Tissue-resident memory T cells (Trm) are memory T cells that remain in a given tissue during a long period of time. They are well-described in mucosal tissues where they contribute to the first line of adaptive defense after re-exposure to a specific pathogen. For instance, influenza-specific resident memory CD8+ T cells have been described in the lung (192). The transcriptional signature of Trm cells differs from circulating T cells and includes genes important for their migration and retention in a given tissue (193). While the markers defining CD4+ Trm T cells are likely to slightly differ depending on the tissue, receptors such as CD69, CD49a, PD-1, and CXCR6 are commonly expressed (193). Persistence of memory T cells in tissues is beneficial in the rapid intervention against pathogenic infections infections but is also proposed to participate in the maintenance of pathogenicity in autoimmune inflammatory conditions.

Trm T Cells in Disease

Psoriasis is the best example of a clear implication of resident memory T cells in the pathogenesis and resurgence of the disease. Indeed, Th17 Trm cells are present in recurrent psoriatic skin lesions and persist in resolved skin even after effective treatment (194). These data highlight the implication of Trm T cells in the reappearance of psoriatic lesions in a site-specific manner (194). The presence of T cells in perivascular areas of healthy synovial joints has been reported but is largely inferior to the number of T cells observed in mucosal tissues at steady state (195). Persistence of inflammation in synovial joints is observed in RA patients even in clinical remission (196) and might be indicative of Trm involvement in RA as described in psoriasis. Recently, CD8+ T cells with features of Trm cells such as CD69, PD-1, and CD103 have been identified in synovial fluids of juvenile arthritis patients (197). A fraction of CD4+ T cells express PD-1 (118) and CD69 (198) in synovial fluid of RA patients but whether these cells are bonafide Trm cells is so far unknown. Importantly, the peripheral T helper cell subset recently described in synovial joints (113) also expresses PD-1 and CD69 suggesting at least some overlap with resident memory T-cell markers. In synovial fluids, T cell clones with identical TCR sequences persist over time indicative of retention mechanisms in the joint (199). However, clonal T-cell expansions have not been studied in the context of Trm markers. In particular, the maintenance of Trm T cells in synovial tissues during the course of the disease and during relapses has not been assessed in RA. This set of experiments would provide information about the nature of effector T-cell functions implicated in tissue damage as exemplified by the persistence of Th17 Trm cells in psoriasis.

Given the recent discovery of Trm T cells, no specific therapeutic strategy is currently targeting this population. The persistence of Trm T cells in the plaques of psoriasis patients show that they probably resist current therapies. Hence, future therapies targeting the maintenance of resident T cells in tissues represent an attractive perspective.

GENETIC RISK VARIANTS AND T-CELL SUBSETS

The study of genetic risk variants can allow a better understanding of the pathogenesis of the disease and the cell subsets involved and also helps to validate therapeutic targets (200). The first genetic contribution to RA is located in the HLA-DRB1 locus (8). Genome-wide association studies have also identified 100 additional loci associated to RA (**Table 1** and **Supplementary Table 1**) (201–203) that are predicted to target immune pathways. This set of gene loci does not

TABLE 1 | Shared genetic variants associated to Rheumatoid Arthritis (RA), Psoriasis and Crohn's disease referenced at Immunobase corresponding to GO biological process (enrichr), p = adjusted p-value.

	Psoriasis	RA	Crohn's disease	GO biological process
Shared among the three diseases	*REL, TAGAP, TNFRSF9 TYK2, UBE2L3*	*REL, TAGAP, TNFRSF9 TYK2, UBE2L3*	*REL, TAGAP, TNFRSF9 TYK2, UBE2L3*	Interleukin-23-mediated signaling pathway (p = 0.04802) Negative regulation of interferon-beta production (p = 0.04802) Interleukin-27-mediated signaling pathway (p = 0.04802)
Shared between Psoriasis and RA	*ELMO1, ETS1 IRF4, TNFAIP3*	*ELMO1, ETS1 IRF4, TNFAIP3*		Regulation of toll-like receptor 3 signaling pathway (p = 0.01682) T-helper 17 cell lineage commitment (p = 0.01682) T-helper cell lineage commitment (p = 0.01682)
Shared between RA and Crohns' disease		*IFNGR2, IKZF3, IL2 IL6ST, IRF8, PTPN2 PTPN22, RASGRP1 SPRED2, STAT4, YDJC CD40, IL2RA, IL21 CXCR5, BACH2*	*IFNGR2, IKZF3, IL2 IL6ST, IRF8, PTPN2 PTPN22, RASGRP1 SPRED2, STAT4, YDJC CD40, IL2RA, IL21 CXCR5, BACH2*	regulation of tyrosine phosphorylation of STAT protein (p = 3.079e-7) positive regulation of interferon-gamma secretion (p = 0.0003406) interleukin-21-mediated signaling pathway (p = 0.0003774)
Shared between Psoriasis and Crohns' disease	*ERAP1, HLA-C, IL12B IL23R, NOS2, SOCS1 STAT3, STAT5A, STAT5B TNIP1, ZMIZ1*		*ERAP1, HLA-C, IL12B IL23R, NOS2, SOCS1 STAT3, STAT5A, STAT5B TNIP1, ZMIZ1*	Interleukin-23-mediated signaling pathway (p = 8.300e-7) Cellular response to interleukin-7 (p = 3.344e-8) Regulation of T-helper 17 cell lineage (p = 0.00007020)

correspond to a unique T-cell subset signature. However, epigenetic chromatin modifications (trimethylation of histone H3 at lysine 4) of RA-associated risk alleles are enriched in primary CD4+ regulatory T cells (201) suggesting that the function of this subset might be implicated in RA. Psoriasis represents a clear example where part of the 35 genetic loci can be assigned to the IL-23/Th17 pathway (204). Some of the genetic variants shared between psoriasis and Crohn's disease correspond to the IL-23 pathway and the T-helper 17 cell lineage (**Table 1**) based on gene ontology biological process analysis [Enrichr (205)]. Shared genetic variants between RA and Crohn's disease highlight a positive regulation of IFNγ secretion that might reflect part of the Th1 component of these diseases (**Table 1**). Many of the genetic variants associated with RA are not common to psoriasis or Crohn's disease emphasizing the importance of distinct mechanisms in the pathogenesis of the disease (**Supplementary Table 1**). For instance, genetic variants in the *IL-10* and *IL-10R* loci are only found associated with Crohn's disease, which is striking given the importance of IL-10 regulatory function in the intestinal barrier as well as in inducing IgA class switch (206). Similarly, a risk locus encompassing PADI4 (peptidylarginine deiminase type 4) is found only in RA (**Supplementary Table 1**). PADI4 controls citrullination processes that are highly relevant in RA where anti-citrullinated peptides antibodies (ACPAs) are commonly found. Still, a clear correlation between genetic risk variants in RA and a specific T-cell subset is lacking. Nevertheless, this can be explained by several factors. First, although some of the locus variants directly have an effect on the expression of the assigned gene (201) (eQTL (expression quantitative trait loci effect)), in most cases the functional consequences of the genetic variants have not been elucidated. We recently demonstrated that the *PTPN22* risk allele (rs2476601) favors the development of EOMES+ CD4+ T cells with cytotoxic features in RA (166). This finding and the fact that *EOMES* risk variants are associated with RA (**Supplementary Table 1**) suggest that cytotoxic T cells probably contribute to the disease. Second, RA is a complex disease that might encompass several sub-phenotypes with distinct stages and genetic signatures that are not uncovered in current GWAS. Finally, our understanding of genetic variants is evolving together with our knowledge on T-cell differentiation mechanisms and will be revisited in the light of emerging data on new T-cell subsets.

CONCLUSIONS AND FUTURE DIRECTIONS

Rheumatoid Arthritis is a complex disease where several T-cell subsets have been proposed to be involved. During the last 10 years, new therapeutic trials as well as extended GWAS have provided new data to reinvestigate the contribution of T-cell subsets in RA. Based on the therapeutic intervention and the genetic data, RA cannot be classified as a Th17-driven disease such as for example psoriasis. Moreover, it has become clear that in human inflammatory contexts, CD4+ T cells harbor multiple

effector function profiles that do not follow the classical dogma of T helper classification. IFNγ and IL-17 are present in synovial joints of RA patients but their blockade does not necessarily improve disease suggesting that their effector function is not rate-limiting for the downstream processes. These cytokines initiate a cascade of proinflammatory cytokines that may no longer be reversed by blocking IFNγ or IL-17 alone. Earlier targeting of these cytokines or combined therapeutic targeting of more downstream cytokines such as GM-CSF and TNF might be more effective. Th1 cells might also have already differentiated into cytotoxic CD4+ T cells capable of inducing cytotoxic damage and a cascade of proinflammatory cytokines. In that context, granzyme A represents a good candidate target since it induces osteoclastogenesis (181) as well as proinflammatory cytokines (182). In ACPA+ RA patients, recent identification of pathogenic T_{ph} cells driving B-cell responses show that, in addition to IL-10, these cells also produce IFNγ and perforin-1 (113). How these multiple effector functions are integrated during T cell/APC interactions is currently not known. Still, the elevated expression of PD-1 on this subset confirms that this co-inhibitory signaling pathway is important in RA and represents a possible target. The emergence of the concept of resident memory T cells capable of perpetuating the disease represent a breakthrough in the understanding of the mechanisms behind disease chronicity and might also favor the development of new therapeutics. Based on the comparison between these three inflammatory conditions, it is clear that some pathogenic pathways are common to these diseases while some others are very distinct and are probably a reflection of different tissue-mediated immunity components. These data should also encourage us to stratify RA patients in subgroups who might be more likely to respond to certain therapies based on the stage of the disease as well as the genetic variants associated. Moreover, a more common use of single cell technologies will allow the dissection of functional properties of rare CD4+ T cells present in inflammatory tissues. However, caution should be taken when analyzing T-cell subsets present in inflammatory tissues since bystander T cells can bias our view of pathogenic T cells. The presence of specific T cell types in inflammatory tissues does not imply that they are necessarily involved in the pathogenesis of the disease. Hence, the analysis of antigen-specific T cells might give a more accurate picture of important effector T-cell functions in the aforementioned inflammatory conditions. Finally, instead of targeting a distinct T-cell subset or effector function, an alternative approach would be to perform antigen-specific targeting and hence to target pathogenic T cells irrespective of their phenotype. We hope that, with this review, we provide a better understanding of current knowledge of CD4+ T-cell functions in RA and highlight the possible ways to identify pathogenic T cells that could be therapeutically targeted.

AUTHOR CONTRIBUTIONS

KC, CG, and VM discussed the content of the review. KC wrote the manuscript. CG and VM edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00353/full#supplementary-material

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