

The developmental pathway for CD103⁺CD8⁺ tissue-resident memory T cells of skin

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Tissue-resident memory T cells (T_{RM} cells) provide superior protection against infection in extralymphoid tissues. Here we found that CD103⁺CD8⁺ T_{RM} cells developed in the skin from epithelium-infiltrating precursor cells that lacked expression of the effector-cell marker KLRG1. A combination of entry into the epithelium plus local signaling by interleukin 15 (IL-15) and transforming growth factor- β (TGF- β) was required for the formation of these long-lived memory cells. Notably, differentiation into T_{RM} cells resulted in the progressive acquisition of a unique transcriptional profile that differed from that of circulating memory cells and other types of T cells that permanently reside in skin epithelium. We provide a comprehensive molecular framework for the local differentiation of a distinct peripheral population of memory cells that forms a first-line immunological defense system in barrier tissues.

Infection primes a population of effector T cells that migrate to all manner of peripheral tissues. The entry of those cells into the tissues is controlled by a 'migration-imprinting' program that depends on the origin of infection and by the induction of local chemokines that recruit T cells into sites of active inflammation¹. As the infection resolves and local inflammation subsides, the infiltration of T cells into nonlymphoid organs gradually decreases^{2,3}, partly as a consequence of the loss of cells that express the homing receptors that permit trafficking into organs such as gut and skin^{4–6}. Nonetheless, long-term T cell immunity can be maintained in regional tissues because of the action of a subset of cells that lodge during the early phase of peripheral infection and thereafter never return to the circulation^{7,8}. Those are called 'tissue-resident memory T cells' (T_{RM} cells); the best characterized are CD8⁺ memory T cells that bear the α -chain (CD103) of the integrin $\alpha_E\beta_7$ together with the activation marker CD69 (ref. 9). Such CD103⁺CD8⁺ T_{RM} cells have been identified in various tissues^{9–11}, where they can dominate immunity to localized infections^{5,9,12–15}.

Memory T cells in the blood and secondary lymphoid organs have been defined as belonging to the subsets of lymphoid tissue-recirculating central memory T cells (T_{CM} cells) and extralymphoid tissue-infiltrating effector memory T cells (T_{EM} cells)¹⁶. While some peripheral T cells probably equilibrate with the blood, some do not¹⁷, and the latter belong to the T_{RM} cell category^{4,9,13}. At present, it remains unclear how the subsets of T_{EM} cells, T_{CM} cells and T_{RM} cells are related. Considerable information is available about the

developmental processes that give rise to the distinct populations of T_{EM} cells and T_{CM} cells^{18,19}, although how T_{RM} cells fit into those lineages remains unclear. It is known that T_{RM} cells lodge in tissues during the effector phase of the immune response^{4,9}, at a time when memory precursor cells are present in the circulation. Such precursor cells can be defined by differences in their expression of the receptor KLRG1 and interleukin 7 (IL-7) receptor α -chain (CD127). Long-lived circulating memory cells are progressively derived from KLRG1[–]CD127^{hi} and KLRG1[–]CD127^{hi} precursor cells, while KLRG1⁺CD127[–] cells give rise to short-lived effector or effector-like memory populations^{18–20}. Since tissue T cells are known to have enhanced effector abilities²¹, it might be expected that T_{RM} cells would originate from the KLRG1⁺ precursor cells. However, unlike circulating effector cells, CD103⁺CD8⁺ T_{RM} cells are particularly long lived, which means that their origins are more likely to be the KLRG1[–] precursor cells that give rise to persisting memory cells, such as the T_{CM} cells found in the circulation.

Clear definition of the development of T_{RM} cells requires understanding of what controls the entry of precursor cells into nonlymphoid compartments. CD103⁺CD8⁺ T_{RM} cells often reside in the epithelium in a range of different tissues, including the gut, skin and female reproductive tract^{9,22,23}. Such restricted localization of T_{RM} cells may be linked to the production of transforming growth factor- β (TGF- β) in those environments^{24,25}, as this cytokine is a major driver of CD103 expression^{26–28}. As a consequence, it might be expected that migration into epithelium would be a critical step in the maturation of CD103⁺CD8⁺ T_{RM} cells. Here we found that this prediction held true

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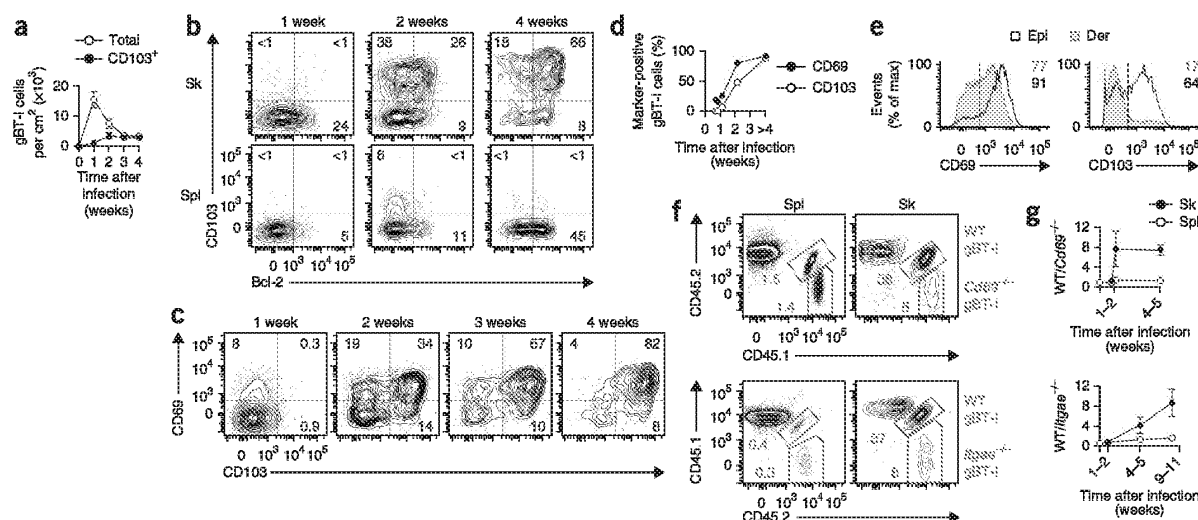


Figure 1 CD103 and CD69 regulate the lodgement and persistence of T_{RM} cells. (a) Quantification of total and CD103⁺ gBT-I cells in skin from mice given naive gBT-I cells and then infected with HSV; gBT-I cells were tracked by their expression of $V_{\alpha}2$, CD45.2 and CD45.1. (b) Expression of CD103 and Bcl-2 by gBT-I cells in the skin (Sk) and Spleen (Spl) of mice as in a at 1, 2 or 4 weeks after infection (above plots), assessed by flow cytometry with gating on gBT-I cells. Numbers in quadrants indicate percent events in each throughout. (c, d) Expression of CD69 and CD103 by gBT-I cells in skin from mice as in a at 1–4 weeks after infection (above plots; c) and frequency of CD69⁺ and CD103⁺ gBT-I cells in the skin of those mice (d). (e) Expression of CD69 and CD103 by gBT-I cells in the epidermis (Epi) and dermis (Der) of mice 11 d after infection as in a. Numbers in plots indicate percent CD69⁺ cells (left) or CD103⁺ cells (right); colors match key. (f) Expression of CD45.1 and CD45.2 in gBT-I cells from the spleen (gated on CD8⁺ cells) and skin (gated on $V_{\alpha}2$ ⁺ cells) of host mice given transfer of wild-type (WT) and *Cd69*^{−/−} gBT-I cells (top row) or wild-type and *Itgae*^{−/−} gBT-I cells (bottom row), with distinct patterns of expression of CD45.1 and CD45.2, followed by infection of the host with HSV and analysis >30 d after infection. Numbers adjacent to outlined areas indicate percent CD45.1⁺CD45.2⁺ (wild-type) cells (both rows, top), CD45.1⁺CD45.2[−] (*Cd69*^{−/−}) cells (top row, bottom) or CD45.1[−]CD45.2⁺ (*Itgae*^{−/−}) cells (bottom row, bottom). (g) Ratio of wild-type cells to *Cd69*^{−/−} cells (top) or *Itgae*^{−/−} cells (bottom) at various times after infection (horizontal axes) of mice as in f. Data are pooled from three experiments with 8–12 mice per time point (a), 4–12 mice per time point (d) or 9–12 mice per time point (g; error bars (a,d,g), s.e.m.), are from one experiment with 4 mice (e) or are representative of three experiments (b,c,f).

for the skin, with entry into the epidermis being a pivotal transition point for the successful maturation of T_{RM} cells. We exploited that requirement for entry into the epidermis to characterize the development of T_{RM} cells and to distinguish those memory cells from their T_{CM} and T_{EM} counterparts in lymphoid tissues.

RESULTS

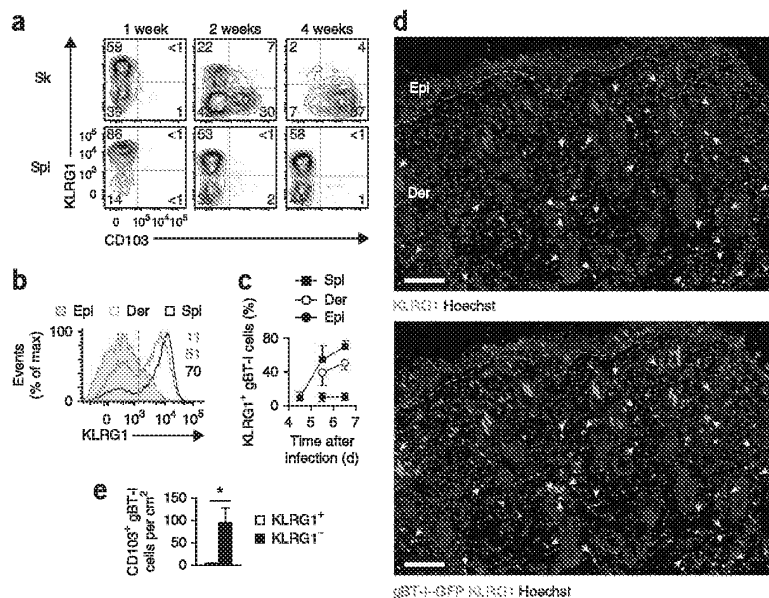
Roles of CD69 and CD103 in the development of T_{RM} cells

To analyze the formation of CD103⁺CD8⁺ T_{RM} cells in skin, we used a combination of infection with herpes simplex virus (HSV) along with adoptive transfer of gBT-I cells, which have transgenic expression of a T cell antigen receptor (TCR) specific for that pathogen. Tracking gBT-I cells by their expression of the TCR α -chain variable region 2 ($V_{\alpha}2$) and of the markers CD45.1 and/or CD45.2 revealed their rapid accumulation in infected skin, with numbers peaking around day 7 and then rapidly falling thereafter (Fig. 1a). In contrast, the number of CD103⁺ gBT-I cells increased at a more modest rate to reach a stable plateau around 2 weeks after infection (Fig. 1a). Consistent with the lack of contraction of the CD103⁺ subset, the T cells upregulated expression of the prosurvival molecule Bcl-2 (Fig. 1b). Skin-infiltrating gBT-I cells also upregulated expression of the activation marker CD69 (Fig. 1c), which preceded the expression of integrin α_E chain (CD103) (Fig. 1d). We also found analogous patterns of progressive upregulation of the expression of Bcl-2, CD69 and CD103 in endogenous nontransgenic T cells (Supplementary Fig. 1a,b). Separating the skin into epidermis and dermis at day 11 after infection showed that virus-specific T cells had heterogeneous CD69 expression in the dermis but were nearly all CD69⁺ in the epidermis (Fig. 1e). At the

same time, a large portion of epidermal gBT-I cells expressed CD103, while their counterparts in the dermis were predominantly CD103[−] (Fig. 1e). It should be noted that dermal preparations contain hair follicles with residual epithelial cells, including intraepithelial T cells (Supplementary Fig. 1c).

Both CD69 and CD103 were necessary for the optimal formation and/or survival of CD8⁺ T_{RM} cells in the skin, as deletion of these molecules resulted in a reduction in T_{RM} cell numbers after adoptive cotransfer of equal numbers of wild-type and CD69-deficient (*Cd69*^{−/−}) or CD103-deficient (*Itgae*^{−/−}) gBT-I cells before infection (Fig. 1f,g). CD69 was also necessary for the persistence of T cells in the sensory ganglia (Supplementary Fig. 1d). Given the sequential nature of the induction of expression of CD69 and CD103 (Fig. 1d), we anticipated that the absence of the individual molecules would affect the development of T_{RM} cells in a progressive way, with loss of CD69 having an early effect and deletion of CD103 having a downstream effect on T cell numbers. Indeed, *Cd69*^{−/−} gBT-I cells were lost at a much faster rate than were *Itgae*^{−/−} gBT-I cells (Fig. 1g). In each case, the starting ratio of wild-type gBT-I cells to mutant gBT-I cells in skin and spleen was roughly 1, with the latter remaining close to 1 at all times examined (Fig. 1g). Histology showed that CD103 was dispensable for the entry of T cells into the epidermis, since both wild-type and *Itgae*^{−/−} gBT-I cells were able to reach that compartment (Supplementary Fig. 1e). These combined results suggested that deficiency in CD69 had an effect shortly after T cells entered the skin, while deletion of CD103 affected the persistence of T_{RM} cells long after the cells had reached the epidermis.

Figure 2 T_{RM} cells develop from KLRG1⁺ precursor cells that selectively infiltrate the epidermis during acute infection. (a) Expression of KLRG1 and CD103 by gBT-I cells in the skin and spleen of mice ($n = 12$ per time point) given naive CD45.2⁺CD45.1⁺ gBT-I cells, followed by infection with HSV and analysis during acute infection (1 week), after resolution of infection (2 weeks) and during early memory (4 weeks); plots are gated on $V_{\alpha}2^{+}$ CD45.2⁺CD45.1⁺ events. (b,c) KLRG1 expression by gBT-I cells isolated from the epidermis, dermis and spleen of mice ($n = 8-9$ per time point) as in a, assessed at 6.5 d (b) or 4.5–6.5 d (c) after infection. Numbers adjacent to plot (b) indicate percent KLRG1⁺ gBT-I cells (colors match key). (d) Microscopy of skin from mice given GFP⁺ gBT-I cells and infected as in a (6 d after infection), to detect KLRG1 by staining with fluorescence-labeled antibody to KLRG1 (anti-KLRG1; top) and identify gBT-I cells by GFP expression (gBT-I-GFP; bottom); arrows indicate examples of KLRG1⁺ cells (yellow arrows) and KLRG1⁺ gBT-I cells (red arrows); dashed lines indicate the epidermal-dermal junction. Scale bars, 100 μ m. (e) Quantification of CD103⁺ gBT-I cells in skin from recipient mice ($n = 12$ per group) infected with HSV and, 4 d later, given transfer of effector gBT-I cells (sorted into KLRG1⁺ and KLRG1⁺ subsets from spleens of donor mice 6 d after infection with HSV), assessed 3 weeks after infection of recipients. * $P < 0.05$ (two-tailed Mann-Whitney test). Data are representative of three experiments (a) or two experiments (b,d) or are pooled from two experiments (c) or three experiments (e; error bars, s.d. (c) or s.e.m. (e)).



T_{RM} cells derive from KLRG1⁺ precursor cells

After skin infection with HSV, there was a gradual shift of gBT-I cells in the spleen from CD62L⁺CD127⁺KLRG1⁺ effector cells to a predominantly CD62L⁺CD127⁺KLRG1⁺ T_{CM} cell population at late times (1 year) after infection, with heterogeneous populations of T_{CM} cells and T_{EM} cells (the latter being CD62L⁺CD127⁺KLRG1⁺ or CD62L⁺CD127⁺KLRG1⁺) at intermediate times (Supplementary Fig. 2a). We also noted changes in skin T cells when we examined expression of KLRG1 and CD103 (Fig. 2a). Early after infection (week 1), skin T cells were predominantly KLRG1⁺CD103⁺ effector cells, while at memory times (week 4), the surviving memory cells were almost exclusively CD103⁺ T_{RM} cells with little or no KLRG1 expression (Fig. 2a), as has been described for T_{RM} cells in other organs^{11,22}. Endogenous CD103⁺ T cells in the skin also largely lacked expression of KLRG1 (Supplementary Fig. 2b). While over 50% of splenic gBT-I cells were KLRG1⁺ 4 weeks after infection, less than 10% of the corresponding skin T cells expressed that marker (Fig. 2a). That difference was consistent with published studies showing disequilibrium between T_{RM} cells in the skin and circulating memory cells and, in particular, cells of the T_{EM} phenotype (which largely express KLRG1) that survive through to the time of memory development^{5,13}.

Those data supported the hypothesis that the skin CD103⁺ T_{RM} cell population developed from KLRG1⁺ precursor cells. Consistent with that, separation of the skin into dermis and epidermis at early times after infection showed that the latter contained almost exclusively KLRG1⁺ gBT-I cells, whereas the former contained a mixture of KLRG1⁺ and KLRG1⁺ cells, similar to what was found in the spleen (Fig. 2b,c). To better visualize the localization of the different populations, we examined, by immunofluorescence microscopy, KLRG1 expression by skin acutely infected with HSV (Fig. 2d). In those sections, the KLRG1⁺ gBT-I cells exhibited a weaker but clearly discernable fluorescence signal (Fig. 2d), in contrast to an

undefined population with strong fluorescence. In confirmation of the data obtained by flow cytometry, almost all gBT-I cells in the epidermis and in the upper regions of the dermis lacked expression of KLRG1 (Fig. 2d). Finally, re-transfer of gBT-I effector cells isolated from spleen 6 d after infection with HSV and sorted into KLRG1⁺ and KLRG1⁺ subsets showed that only the latter generated CD103⁺ T_{RM} cells in the skin of HSV-infected recipient mice (Fig. 2e). These data formally demonstrated that KLRG1⁺ precursor cells gave rise to intraepithelial T_{RM} cells in skin. As an extension of those results, we found that KLRG1⁺ cells also 'preferentially' generated T_{RM} cells in the sensory ganglia (Supplementary Fig. 2c).

The formation of T_{RM} cells requires epithelial infiltration

Our data indicated that infiltration of the epithelium and the formation of T_{RM} cells by virus-specific CD8⁺ T cells were functionally linked; however, our results fell short of demonstrating that entry into the epithelium was indeed required for the maturation of T_{RM} cells. To examine the migration of T cells from the dermis to the epithelium independently of their initial priming or recruitment from the blood, we activated gBT-I cells *in vitro* by culturing them with HSV glycoprotein B peptide-pulsed splenocytes and then directly injected the gBT-I cells into the dermis to determine if those cells could develop into epithelium-lodged CD103⁺ T_{RM} cells. The population of injected cells gradually increased their expression of CD69 and CD103 (Supplementary Fig. 3a) and, concomitant with those phenotypic changes, eventually localized to the epidermis and hair-follicle epithelium (Supplementary Fig. 3b). To show that entry into the epithelium was necessary for the formation of T_{RM} cells, we treated the *in vitro*-activated T cells with pertussis toxin before injecting them intradermally into the flank skin of recipient mice. Pertussis toxin inactivates G protein-coupled receptors and thereby blocks chemokine-driven migration²⁹. While untreated cells gave



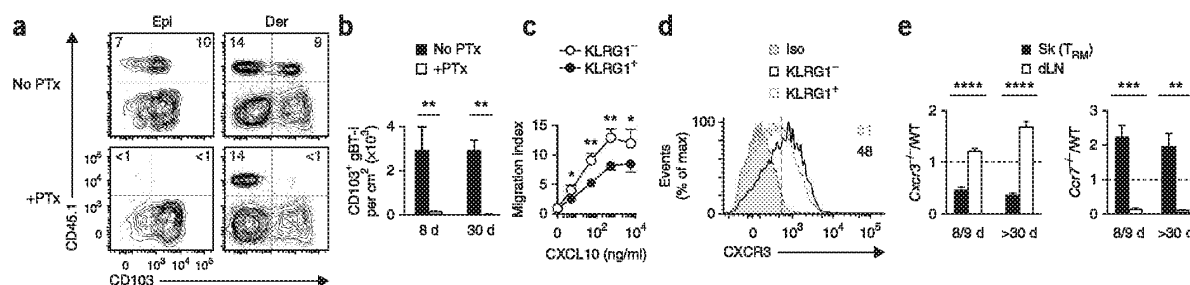


Figure 3 Opposing chemokines regulate the entry into the epidermis and differentiation of T_{RM} cells versus egress from tissues. (a) Expression of CD45.1 and CD103 by cells isolated from the epidermis and dermis of CD45.2⁺ recipient mice given intradermal injection of *in vitro*-activated CD45.2⁺CD45.1⁺ gBT-I cells left untreated (No PTx) or treated with pertussis toxin (+PTx), assessed 8 d after cell transfer, with gating on CD45.2⁺ events; gBT-I cells were identified as V α 2⁺CD45.1⁺ events (top quadrants). (b) Quantification of CD103⁺ gBT-I cells in total skin preparations from mice as in a ($n = 6$ per group), at 8 or 30 d after transfer. (c) Migration of KLRG1⁺ and KLRG1⁺ effector gBT-I cells toward CXCL10 gradients *ex vivo* after enrichment from spleens of mice 7 d after infection with HSV. Numbers adjacent to plot indicate percent CXCR3⁺ gBT-I cells (colors match key). Iso, isotype-matched control antibody. (d) CXCR3 expression by KLRG1⁺ and KLRG1⁺ effector gBT-I cells isolated from the spleen 6.5 d after infection with HSV. Numbers adjacent to plot indicate percent CXCR3⁺ gBT-I cells (colors match key). (e) Ratio of *Ccr3*^{+/+} (left) or *Ccr7*^{+/+} (right) CD8⁺ effector T cells to wild-type effector cells in draining lymph nodes (dLN) and skin (CD103⁺; Sk (T_{RM})) from recipient mice given intradermal cotransfer of *in vitro*-activated cells, assessed at various times (horizontal axes) after transfer. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ (two-tailed Mann-Whitney test (b) or two-tailed paired *t*-test (c,e)). Data are pooled from two experiments with 5 mice per group (b), five experiments with cells pooled from 5 mice in each (c) or two to three experiments with 7–12 mice per group (e; error bars (b,c,e), s.e.m.) or are representative of two experiments (a,d).

rise to a population of CD69⁺CD103⁺ T_{RM} cells after intradermal injection, treatment with pertussis toxin blocked both infiltration of the epidermis and *in vivo* CD103 expression (Fig. 3a,b and Supplementary Fig. 3c). Pertussis toxin-treated gBT-I cells were still able to upregulate CD103 expression when exposed to TGF- β *in vitro* (Supplementary Fig. 3d), which indicated that the lack of upregulation *in vivo* resulted from a migration defect rather than from an intrinsic block in CD103 expression itself. Combined, these data indicated that migration to the epidermis was necessary for the development of CD103⁺CD8⁺ T_{RM} cells in the skin.

Chemokines regulate epidermal entry of T_{RM} cell precursors

We next sought to identify the chemokines that promoted the entry of cells into the epidermis and thus the formation of T_{RM} cells. The

chemokines CXCL9 and CXCL10 have been shown to facilitate entry into the epithelium during infection of mucosal surfaces with HSV type 2 (HSV-2)¹⁵, and we found that mRNAs encoding those chemokines were synthesized by keratinocytes during infection of the skin with HSV (Supplementary Fig. 4a). Notably, KLRG1⁺ precursors of T_{RM} cells showed 'preferential' migration toward those chemokines in Transwell assays *ex vivo* (Fig. 3c and Supplementary Fig. 4b), consistent with higher expression of CXCR3 (the receptor for CXCL9 and CXCL10) on this subset than on KLRG1⁺ effector cells (Fig. 3d). Intradermal injection of *in vitro*-activated CD8⁺ T cells lacking expression of CXCR3 (*Ccr3*^{-/-}) resulted in the generation of fewer CD103⁺ T_{RM} cells than did injection of wild-type CD8⁺ T cells (Fig. 3e). That decrease was not a consequence of poor survival of the *Ccr3*^{-/-} T cells, as they were present in higher frequency in

Figure 4 Signaling via the TGF- β receptor and IL-15 is required for the development of CD103⁺ T_{RM} cells. (a) CD103 expression by wild-type (WT) and *Tgfb2*^{fl/dLck-Cre} OT-I cells in the skin of recipient mice after *in vitro* activation and intradermal transfer (Act transfer) or transfer of naive cells and infection of host mice with ovalbumin-expressing HSV (Naive transfer + HSV.OVA), assessed 4–30 d (above plots) after cell transfer (top) or infection (bottom); plots are gated on OT-I cells. (b) Ratio of wild-type OT-I cells to *Tgfb2*^{fl/dLck-Cre} OT-I cells in the skin and spleen of mice ($n = 10$ –11 per group) treated as in a, bottom row. (c,d) Quantification of CD103⁺ (Sk) or total (Spi) gBT-I cells in wild-type and *IL15*^{-/-} host mice ($n = 11$ –12 per group; c) or bone marrow chimeras (described below; $n = 8$ –10 per group; d) infected with HSV and, 4 d later, given transfer of effector gBT-I cells enriched from the spleens of wild-type mice 6 d after infection with HSV, analyzed 2 and >4 weeks (c) or 3 and 6 weeks (d) after infection of recipients. Chimeras (d): wild-type mice given transfer of wild-type (WT→WT) or *IL15*^{-/-} (*IL15*^{-/-}→WT) bone marrow, and *IL15*^{-/-} mice given transfer of wild-type (WT→*IL15*^{-/-}) or *IL15*^{-/-} (*IL15*^{-/-}→*IL15*^{-/-}) bone marrow. Each symbol (c) represents an individual mouse; small horizontal lines indicate the mean. NS, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ (two-tailed Mann-Whitney test (c) or Kruskal-Wallis test followed by Dunn's multiple-comparison post-test (d)). Data are representative of two experiments (a) or are pooled from two experiments (b,d) or three to four experiments (c; error bars (b,d), s.e.m.).

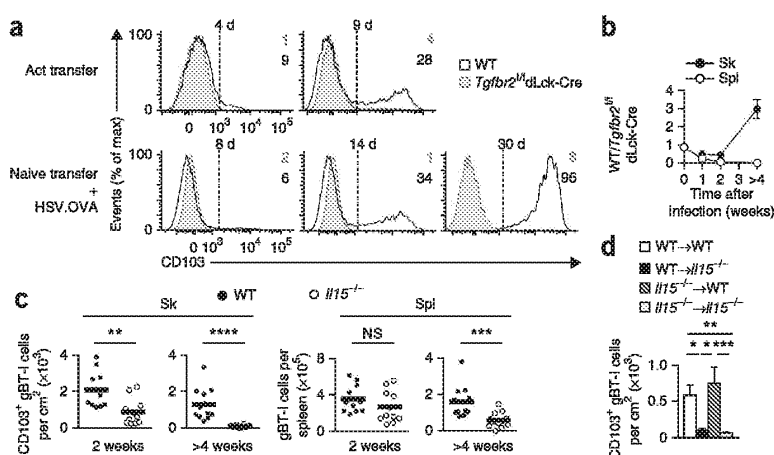


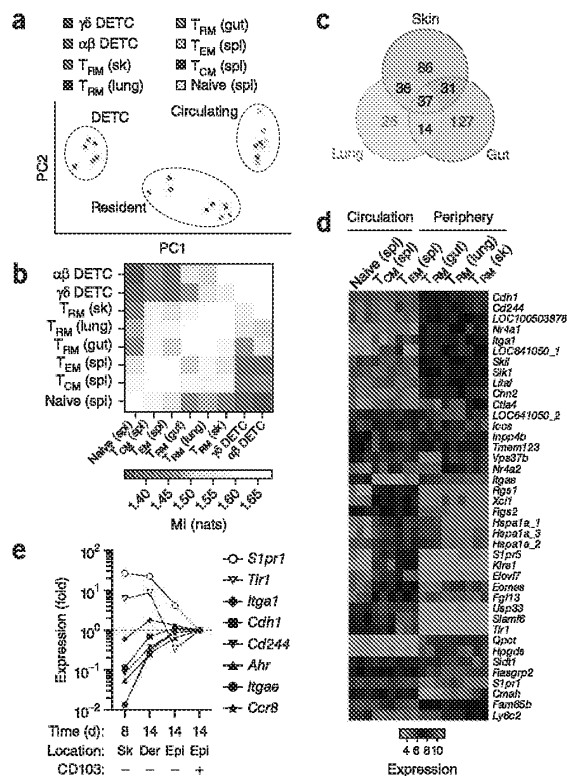
Figure 5 Definition of a core transcriptional profile of T_{RM} cells that is progressively engaged during differentiation. (a) PCA of gene-expression data for various subtypes of lymphoid and peripheral $CD8^+$ T cells and DETCs (key); circles indicate three distinct clusters of T cells. (b) MI analysis of the genome-wide gene-expression distributions for each pair of cell types (presented as information entropy of natural logarithms ('nats')). (c) Gene expression of T_{RM} cells from the skin, lung and gut relative to that of T_{CM} cells and T_{EM} cells (pairwise comparison); numbers in overlapping regions indicate gene transcripts shared by the overlapping T_{RM} cell subsets; numbers in non-overlapping regions indicate gene transcripts unique to each T_{RM} cell subset. (d) Heat map of transcripts with different expression in the lymphoid group (Circulation) than in the T_{RM} cell group (Periphery) presented as $\log_2$ -normalized expression (key). (e) Quantitative PCR analysis of the expression of various genes (key) in gBT-I cells sorted from total skin, dermis or epidermis of host mice that received naive gBT-I cells followed by infection of the recipients with HSV, assessed 8 or 14 d after infection, during T_{RM} differentiation; results are presented as median values relative to expression by T_{RM} cells. Data are pooled from two to five experiments.

lymphoid organs (Fig. 3e), which suggested more efficient return to the circulation. Given that last result, we sought to determine what would happen if we blocked egress from the skin. It is known that the chemokine receptor CCR7 is required for exit from tissues via the lymphatics^{30,31}, so we reasoned that CCR7-deficient cells might give rise to greater numbers of $CD103^+$ T_{RM} cells. Indeed, $Ccr7^{-/-}$ $CD8^+$ T cells displayed greater formation of T_{RM} cells than did their wild-type counterparts, with a concomitant decrease in their return to the lymph nodes (Fig. 3e). Thus, it seemed that facilitating the entry of T cells into the epidermis or inhibiting their egress from the skin promoted the formation of $CD103^+$ T_{RM} cells.

Development of T_{RM} cells in skin requires TGF- β and IL-15

TGF- β , which is expressed in the skin epithelium²⁴, is a known driver of the upregulation of $CD103$ expression and has been shown to be important in promoting the maturation of T_{RM} cells in other tissues²⁸. However, its contribution to the formation of T_{RM} cells in the skin has remained unknown. Thus, we sought to determine what effect signaling via the TGF- β receptor had on the formation of T_{RM} cells in the skin. Our initial experiments involved direct intradermal injection of wild-type OT-I cells (which have transgenic expression of an ovalbumin-specific TCR) and OT-I cells deficient in the type II TGF- β receptor ($Tgfb\beta 2^{fl/dLck-Cre}$ OT-I cells) activated by splenocytes coated with the cognate ovalbumin peptide before transfer. After intradermal injection, only the wild-type OT-I cells upregulated $CD103$ expression (Fig. 4a, top). In addition to their lack of $CD103$ expression, the TGF- β receptor-deficient cells were also selectively lost from the skin but not from the spleen (Supplementary Fig. 5a). We verified that the TGF- β receptor was also required for the upregulation of $CD103$ expression after infection. For this, we transferred a mixture of wild-type and $Tgfb\beta 2^{fl/dLck-Cre}$ OT-I cells into C57BL/6 mice and then infected the recipient mice with recombinant ovalbumin-expressing HSV. We noted upregulation of $CD103$ expression only on skin-infiltrating wild-type OT-I cells, whereas $Tgfb\beta 2^{fl/dLck-Cre}$ OT-I cells lacked $CD103$ expression and gradually disappeared from the skin (Fig. 4a,b).

The formation and/or survival of $CD103^+$ T_{RM} cells was also dependent on IL-15, as its absence resulted in fewer T_{RM} cells than in wild-type mice (Fig. 4c). In these experiments, we primed effector gBT-I cells in wild-type donor mice and re-transferred them into infected wild-type or IL-15-deficient ($Il15^{-/-}$) recipient mice to minimize the potential effect of initial T cell priming in the absence of IL-15. Reduced formation of T_{RM} cells in $Il15^{-/-}$ mice was evident by 2 weeks after infection of the secondary recipients



with HSV, with very low numbers of T_{RM} cells present in the skin of $Il15^{-/-}$ mice by week 4 and, similarly, also in their dorsal root ganglia (Supplementary Fig. 5b). That reduction correlated with muted induction of Bcl-2 expression in $CD103^+$ skin T cells in the absence of IL-15 (Supplementary Fig. 5c). We also noted such IL-15 dependence for splenic memory cells, although to a lesser extent than for the skin T_{RM} cells (Fig. 4c). The generation of T_{RM} cells was completely restored in bone marrow chimeras in which IL-15 production was restricted to radioresistant cells (Fig. 4d). Thus, IL-15 from radioresistant cells such as skin keratinocytes and Langerhans cells was sufficient to support optimal development and survival of T_{RM} cells.

A unique transcriptional profile of T_{RM} cells

Although transcriptional analyses of T_{EM} cells and T_{CM} cells have been published³², no comparison between those transcriptomes and that of the T_{RM} cell subset has been made. Thus, we analyzed by microarray the expression patterns of $CD103^+CD8^+$ T_{RM} cells from the skin, gut or lungs after infection with HSV, lymphocytic choriomeningitis virus (LCMV) or influenza virus, respectively, and compared those with the expression patterns of T_{CM} cells and T_{EM} cells isolated from HSV-infected mice. To facilitate the identification and isolation of subsets of memory cells, we used T cells with transgenic expression of TCRs of the desired specificity and transferred them into C57BL/6 mice before infection. For infection with HSV or LCMV, we used mice given adoptive transfer of T cells specific for the natural viral determinants (from gBT-I mice and P14 mice (which have transgenic expression of a TCR specific for an epitope of LCMV glycoprotein), respectively), while for infection with influenza virus, we used a recombinant virus (flu.gB) that expresses HSV glycoprotein



Table 1 Transcripts that define the core signature of T_{RM} cells

Gene	Product	Entrez ID	Probe ID	Reg
<i>Cd244</i>	CD244 natural killer cell receptor 2B4	18106	10351644	↑
<i>Cdh1</i>	Cadherin 1	12550	10575052	↑
<i>Chn2</i>	Chimerin 2	69993	10538356	↑
<i>Ctla4</i>	Cytotoxic T lymphocyte-associated protein 4	12477	10346790	↑
<i>Hpgds</i>	Hematopoietic prostaglandin D synthase	54486	10545101	↑
<i>Hspa1a</i>	Heat-shock protein 1A	193740	10450369	↑
			10444589	↑
			10450367	↑
<i>Icos</i>	Inducible T cell costimulator	54167	10346799	↑
<i>Inpp4b</i>	Inositol polyphosphate-4-phosphatase, type II	234515	10573082	↑
<i>Itga1</i>	Integrin α_1	109700	10412298	↑
<i>Itgae</i>	Integrin α_E , epithelium associated	16407	10378286	↑
<i>Ltfa</i>	Lipopolysaccharide-induced tumor-necrosis factor	56722	10437687	↑
LOC100503878	Zinc-finger protein 683-like	100503878	10508909	↑
LOC641050	Hypothetical protein LOC641050	641050	10504761	↑
			10538892	↑
<i>Nr4a1</i>	Nuclear receptor subfamily 4, group A, member 1	15370	10427035	↑
<i>Nr4a2</i>	Nuclear receptor subfamily 4, group A, member 2	18227	10482772	↑
<i>Qpct</i>	Glutaminy-peptide cyclotransferase (glutaminy cyclase)	70536	10447056	↑
<i>Rgs1</i>	Regulator of G protein signaling 1	50778	10358408	↑
<i>Rgs2</i>	Regulator of G protein signaling 2	19735	10358389	↑
<i>Sik1</i>	Salt-inducible kinase 1	17691	10449741	↑
<i>Skil</i>	SKI-like	20482	10491300	↑
<i>Tmem123</i>	Transmembrane protein 123	71929	10583145	↑
<i>Vps37b</i>	Vacuolar protein sorting 37B (yeast)	330192	10533729	↑
<i>Xcl1</i>	Chemokine (C motif) ligand 1	16963	10359697	↑
<i>Cmah</i>	Cytidine monophospho-N-acetylneuraminic acid hydroxylase	12763	10404132	↓
<i>Elovl7</i>	ELOVL family member 7, elongation of long chain fatty acids (yeast)	74559	10407072	↓
<i>Eomes</i>	Eomesodermin homolog (<i>Xenopus laevis</i>)	13813	10589994	↓
<i>Fam65b</i>	Family with sequence similarity 65, member B	193385	10404152	↓
<i>Fgf13</i>	Fibroblast growth factor 13	14168	10604751	↓
<i>Klre1</i>	Killer cell lectin-like receptor family E member 1	243655	10542205	↓
<i>Ly6c2</i>	Lymphocyte antigen 6 complex, locus C2	100041546	10429573	↓
<i>Rasgrp2</i>	RAS, guanyl releasing protein 2	19395	10460968	↓
<i>S1pr1</i>	Sphingosine 1-phosphate receptor 1	13609	10501586	↓
<i>S1pr5</i>	Sphingosine 1-phosphate receptor 5	94226	10591494	↓
<i>Sid1</i>	SID1 transmembrane family, member 1	320007	10439583	↓
<i>Slamf6</i>	SLAM family member 6	30925	10351691	↓
<i>Tlr1</i>	Toll-like receptor 1	21897	10530145	↓
<i>Usp33</i>	Ubiquitin specific peptidase 33	170822	10496919	↓

Transcripts commonly regulated in T_{RM} cells from the skin, gut and lung, upregulated (↑) or downregulated (↓) in T_{RM} cells relative to their expression in T_{CM} cells and T_{EM} cells from the spleen (overlapping region, Fig. 5c; heat map, Fig. 5d). Entrez ID, accession code for the gene database of the National Center for Biotechnology Information; Probe ID, Affymetrix probe set identifier.

B in combination with gBT-1 cells. As our study had focused on skin T_{RM} cells, we also included two other skin-resident intraepithelial T cell subsets in these comparisons: $\gamma\delta$ TCR-expressing dendritic epidermal T cells ($\gamma\delta$ DETCs) and a population of $\alpha\beta$ TCR-bearing cells ($\alpha\beta$ DETCs) that populate the epidermal niche in mice that lack $\gamma\delta$ DETCs (*Tcrd*^{-/-} mice).

We subjected the transcriptional data to principal-component analysis (PCA) (Fig. 5a) to delineate variations in the data, and we calculated the similarities and distances between T cell subsets by a mutual information (MI) approach (Fig. 5b). The PC1 and PC2 axes of variation in the PCA distinguished the circulating T cell subsets from those that were resident or DETC like (Fig. 5a). That pattern was consistent with the pairwise relationships defined by the MI analysis (Fig. 5b), which showed that T_{RM} cells from different tissues were transcriptionally related. The PCA showed that the transcriptomes of the T_{EM} cell and T_{CM} cell subsets were more similar to the transcriptome of naive T cells than that of T_{RM} cells, which were themselves distinct from the $\gamma\delta$ and $\alpha\beta$ DETCs. Thus, both the PCA- and MI-based metrics demonstrated that the various T_{RM} cell populations

formed a transcriptomically distinct subset that as a group was less similar to the T_{EM} cell and T_{CM} cell subsets than the T_{EM} cell and T_{CM} cell subsets were to each other or to circulating naive T cells.

We made a detailed comparison of genes regulated differently in T_{RM} cells than in subsets of circulating memory cells (Fig. 5c,d). For this comparison, we used combined thresholds of a change in gene expression of 1.5-log₂ fold and P values of less than 0.05 to identify genes with different expression in the circulating and resident populations of memory cells. Each T_{RM} cell subset had unique elements, with between 25 and 127 transcripts specific for each of the individual T_{RM} cell populations from the different tissues (Fig. 5c), such as transcripts encoding the chemokine receptors CCR9 (expressed only in gut T_{RM} cells) and CCR8 (expressed only in skin T_{RM} cells). We also found transcript expression common to T_{RM} cell populations, with 51–73 transcripts shared by any given pair. Those included transcripts, such as that encoding the aryl hydrocarbon receptor (*Ahr*), that were selectively upregulated in gut and skin T_{RM} cells but not the corresponding population in the lungs. Notably, our comparison identified 37 transcripts commonly up- or downregulated by all three T_{RM} cell subsets (Fig. 5c,d and Table 1). About 60% of the transcripts with a difference in expression were upregulated on T_{RM} cells relative to their expression on circulating memory T cells, although genes encoding some key molecules were downregulated, including that encoding the sphingosine 1-phosphate receptor S1P₁. Overall, those 37 transcripts and their products distinguished T_{RM} cells from their counterparts in the circulation and thus represented the core transcriptional signature of this population.

Progressive engagement of the T_{RM} cell transcriptome

Finally, we tracked the changes in the expression of selected genes as T cells progressed along the T_{RM} cell maturation pathway in the skin. From the results presented above, we predicted that T_{RM} cells would mature from the time they entered the dermis at the peak of the effector response, through to their recruitment into the epidermis and, finally, their acquisition of surface expression of CD103 (Supplementary Fig. 6). We focused on KLRG1⁺ T cells in this analysis, as neither skin T_{RM} cells nor their precursors expressed KLRG1. We isolated T cells from the dermis and/or epidermis at either the height of the effector response (day 8) after infection with HSV or during memory conversion (day 14). Quantitative PCR analysis of genes commonly regulated in all subsets of T_{RM} cells (*Cdh1*, *S1pr1*, *Tlr1*, *Itga1*, *Itgae* and *Cd244*) or selected subsets of T_{RM} cells (*Ccr8* and *Ahr*) showed appropriate up- or downregulation as the cells progressed along this putative maturation pathway in skin (Fig. 5e). Overall, this analysis showed the transcriptional development of T_{RM} cells from early KLRG1⁺ effector-type precursor cells to fully mature CD103⁺ T_{RM} cells lodged in the epidermis.

DISCUSSION

Our study has provided a detailed description of the development and extended phenotype of CD103⁺CD8⁺ T_{RM} cells. It has defined a maturation pathway for the development of skin T_{RM} cells from KLRG1⁺ precursor cells that enter the tissues during the early effector response, with an important checkpoint being their migration into the epidermis in a chemokine-dependent manner. We found that IL-15 and TGF- β were involved in the formation and/or survival of skin T_{RM} cells, with the latter probably driving the upregulation of CD103 expression, consistent with what has been noted for a variety of other tissues^{27,28,33}. The precursors of T_{RM} cells were not end-stage differentiated effector cells or mature T_{EM} cells. Both of those populations have KLRG1 expression^{19,20}, whereas the precursors of CD8⁺ T_{RM} cells that reached the epithelium and the mature skin-resident memory cells did not. Once formed, the T_{RM} cells do not seem to undergo any contraction but instead remain numerically stable over a considerable time^{5,14,21}. As for that longevity, CD103⁺CD8⁺ T_{RM} cells seem to be more closely related to the circulating T_{CM} cells than to the T_{EM} cells that are ultimately lost from the circulation^{34,35}. That matches their derivation from KLRG1⁺ precursor cells, which are also the origin of the long-lived T_{CM} cell population^{20,36,37}. In addition, T_{RM} cells seem able to proliferate *in situ* after restimulation^{38,39} and, although this is not universal¹¹, in some cases they do so after *in vivo* transfer²², attributes typically associated with the T_{CM} cell subset³⁵.

Entry into the epidermis involved the action of CXCR3 ligands, although additional molecules probably also participate in this event, as the absence of CXCR3 did not completely abrogate the formation of T_{RM} cells. Nonetheless, the proposal for CXCR3-mediated recruitment to the epithelium is consistent with the published finding that exogenous application of CXCR3 ligands can promote the formation of T_{RM} cells in the epithelium of the lower female reproductive tract¹⁵. The formation of T_{RM} cells was enhanced in the absence of CCR7 expression on T cells introduced directly into skin, probably as a consequence of reduced return of precursor cells to the circulation, as CCR7 promotes the egress of T cells from the skin and other tissues^{30,40}. Such a scenario would suggest that the precursor cells are not fully committed to the T_{RM} cell fate when they first enter the skin.

While transcriptional analysis of brain T_{RM} cells has been reported⁴¹, the broader comparison we used here uniquely identified just under 40 transcripts that defined a core set of genes expressed differently in this population. One of the transcripts with a difference in expression encodes the receptor S1P₁, which controls the entry of T cells into the lymphatics⁴² and must be downregulated for the retention of T_{RM} cells in peripheral tissues⁴³. Complementing that, CD69 expression is rapidly upregulated once the T cells reach the skin, which provides additional impetus for retention in the tissues, as this molecule is known to functionally inhibit S1P₁ (ref. 44). We found that CD69 independently affected the number of T_{RM} cells, similar to what has been shown for CD103⁺CD8⁺ T cells in the lungs²⁷. However, while previously it was thought that loss of CD69 inhibits the ongoing recruitment of T cells²⁷, our data here suggested that it affected early retention of precursors of T_{RM} cells. Our microarray analysis also showed that expression of a second member of the family of S1P receptors, S1P₅, was downregulated. In contrast to S1P₁, S1P₅ is not inhibited by CD69 (ref. 45). Additional gene products associated with tissue retention are RGS-1 and RGS-2, both known G protein-coupled inhibitors⁴⁶. Separate from that, T_{RM} cells upregulate their expression of CD49a, E-cadherin and integrin α E β 7, all associated with cell tethering or adhesion⁴⁷. Overall, our results showed that downregulation of egress from the tissues seemed to be a critical feature of the maturation of CD103⁺CD8⁺ T_{RM} cells.

While retention in the tissues is a central feature of the T_{RM} cell phenotype, the array data indicated that these were not simply circulating memory cells trapped within the extralymphoid environs. Instead, we found that T_{RM} cells were a phenotypically distinct population. For example, genes encoding molecules associated with the modulation of T cell signaling were also present in the core transcriptional gene set and absent from that of the circulating memory cell subsets. The expression of genes encoding the immunological modifiers CTLA-4, ICOS, KLRE1 and various members of the SLAM family, along with transcription factors, such as members of the Nr4a family and Litaf, was regulated differently in T_{RM} cells. Combined, these results indicated a distinct phenotype for the resident subset of cells. That proposal was reinforced by the PCA and pairwise MI analyses, which showed that the T_{RM} cell transcriptomes differed from those of the subsets of circulating memory cells. Notably, the bioinformatics analysis also showed that the skin T_{RM} cells differed from the main intraepithelial T cells naturally found in mouse skin, notably the $\gamma\delta$ DETCs and the $\alpha\beta$ TCR-expressing cells that fill the void in the absence of the former. To some extent, this makes sense, as DETCs are involved in innate processes such as skin homeostasis and wound healing^{48,49} rather than contributing to adaptive immunity and immune memory, as do CD103⁺CD8⁺ T_{RM} cells⁸.

Overall, our gene-expression and maturational tracking studies showed that T_{RM} cells were derived from the same precursor cells that gave rise to the long-lived memory cells found in the circulation. The memory precursor cells underwent an *in situ* developmental program that resulted in their differentiation into specialized memory cells adapted to the extralymphoid compartment. The development of T_{RM} cells is not a 'default' program but instead is dependent on location, requiring the action of tissue-specific cytokines and, in some cases, local antigen recognition^{11,14}. As a consequence, T_{RM} cells are focused on regional immunity, consistent with the emerging view that they provide superior control of peripheral infection^{9,13,14}. By identifying the precursors of T_{RM} cells and describing mechanisms associated with their retention and maturation, we have provided a foundation for studies designed to manipulate the generation and lodgment of T_{RM} cells for the purpose of enhanced immunological protection.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. GEO: microarray data, GSE47045.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

L.K.M., F.R.C. and T.G. designed the experiments; L.K.M., A.R., J.Z.M., N.C., A.T.S., M.-L.H. and T.G. did the experiments; L.K.M., A.R., J.Z.M., A.T.S., W.R.H., M.L., F.R.C. and T.G. analyzed the data; J.V.-R., P.L., S.N.M., T.S. and D.C.T. contributed reagents; F.R.C. led the research program and, with the help of L.K.M. and T.G. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.



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