IL-17-producing γδ T cells switch migratory patterns between resting and activated states

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Interleukin 17-producing γδ T (γδT17) cells have unconventional trafficking characteristics, residing in mucocutaneous tissues but also homing into inflamed tissues via circulation. Despite being fundamental to γδT17-driven early protective immunity and exacerbation of autoimmunity and cancer, migratory cues controlling γδT17 cell positioning in barrier tissues and recruitment to inflamatory sites are still unclear. Here we show that γδT17 cells constitutively express chemokine receptors CCR6 and CCR2. While CCR6 recruits resting γδT17 cells to the dermis, CCR2 drives rapid γδT17 cell recruitment to inflamed tissues during autoimmunity, cancer and infection. Downregulation of CCR6 by IRF4 and BATF upon γδT17 activation is required for optimal recruitment of γδT17 cells to inflamed tissue by preventing their sequestration into uninflamed dermis. These findings establish a lymphocyte trafficking model whereby a hierarchy of homing signals is prioritized by dynamic receptor expression to drive both tissue surveillance and rapid recruitment of γδT17 cells to inflammatory lesions.
Interleukin-17-producing γδ T cells (γδT17 cells) are innate-like lymphocytes crucial for early defence against extracellular bacterial and fungal pathogens. γδT17 effector function is programmed in Vγ4– and Vγ6– cells during thymic development, resulting in their homeostatic localization to barrier tissues and ability to be rapidly activated by innate-derived cytokines. Production of interleukin 17A (IL-17A) and other inflammatory cytokines by γδT17 cells within hours of pathogen encounter orchestrates early neutrophil responses critical for mucocutaneous defence. However, dysregulated γδT17 cells responses contribute to pathogenesis associated with several models of autoimmunity and can enhance tumour growth and metastasis.

How γδT17 cells populate homeostatic barrier tissues and then infiltrate inflamed tissues from circulation is unclear. γδT17 cells seed dermis and mucosal tissues during perinatal life. Although parabiosis experiments demonstrate that the majority of Vγ4– γδT17 cells in skin-draining lymph nodes (sLN)s are permanently resident, studies using photolabelling, adoptive transfers and recepto-antagonist suggest that γδT17 cells constitutively circulate between dermis, sLN and blood. Nevertheless, sLN γδT17 cells expand during autoimmune inflammation and infiltrate target tissues via circulation. Furthermore, dermal Vγ4– γδT17 cells home from skin to sLN, proliferate, and repopulate inflamed and distal unaffected skin during psoriasis. Thus despite a largely tissue-restricted distribution, γδT17 cells are motile and move between lymphoid and barrier tissues under homeostasis and experimental inflammatory conditions.

Chemokine receptor CCR6, involved in both homeostatic and inflammatory trafficking of leukocytes in barrier tissues, is expressed by both T helper 17 (Th17) and γδT17 cells. We reported a largely redundant function for CCR6 in recruitment of granulocyte–macrophage colony stimulating factor-producing encephalitogetic Th17 cells to the central nervous system (CNS) during experimental autoimmune encephalomyelitis (EAE). Instead, these cells display a CCR6–CCR2– phenotype and infiltrate the CNS via CCR2, which is critical for T-cell–mediated injury. However, Vγ4– γδT17 cells downregulated CCR6 expression, whereas Th17 cells co-express CCR6 and CCR2 during development, but lose CCR6 expression upon activation.

Results

γδT17 cells downregulate CCR6 upon activation. We recently reported that Th17 cell development during EAE is coupled with a dynamic, temporally regulated switch from CCR6 to CCR2 expression as Th17 cells propagate their differentiation. Expression patterns of CCR6 and CCR2 define distinct effector phenotypes of Th17 cells, with a CCR6–CCR2+ phenotype marking the encephalitogetic granulocyte–macrophage colony-stimulating factor/interferon-γ-producing population. Unlike Th17 cells, γδT17 cell effector function is programmed during thymic development and these cells populate barrier tissues prior to inflammation. Thus, we initially examined CCR6 and CCR2 expression in sLN and dermis in uninimmunized Il17a–/– x Rosa26*YFP mice, where Il17a expression drives persistent marking of cells with eYFP. γδT17 cells in these compartments constitutively co-expressed CCR2 and CCR6 (Fig. 1a and Supplementary Fig. 1a). Expression of CCR6 and CCR2 was restricted to γδ T cells bearing a CD27+CD44hi phenotype, characteristic of γδT17 cells (Supplementary Fig. 2a). CCR6/CCR2 co-expression was similar between Vγ4+ and Vγ6+ γδT17 cell subsets as distinguished by both Vγ4 expression and CD3/T-cell receptor (TCR) expression level, as previously reported (CD3bright staining) (Supplementary Fig. 1b,c), and both receptors were functional as determined by ex vivo chemotaxis (Fig. 1b). However, examination of γδT17 cells from diverse tissues revealed a heterogeneous pattern of CCR6 expression. While thymic and most lymphoid γδT17 cells uniformly expressed both CCR6 and CCR2, populations of γδT17 cells lacking CCR6 expression (CCR6–CCR2+) were prominent in lung and gut-associated tissues (Fig. 1c). As the gut is tonically immunologically active due to interactions with commensal microbiota, we hypothesized that γδT17 cells downregulate CCR6 expression during inflammation.

In support of this idea, activation of sLN and spleen γδT17 cells in vivo during EAE resulted in downregulation of CCR6 expression compared to uninimmunized mice (Fig. 1d). CNS-infiltrating γδT17 cells were also largely CCR6+. BrdU incorporation revealed that CCR6 expression was downregulated in proliferated γδT17 cells, while BrdU– cells remained CCR6+ (Fig. 1e). Other γδ T-cell subsets did not express CCR2 or CCR6 at rest, and did not gain expression of these receptors over the course of EAE (Supplementary Fig. 2a). Unlike Th17 cells, γδT17 cells are predominantly activated by TCR-independent signals including IL-23 and IL-1β. In vitro stimulation of lymphocytes with a range of known stimuli including IL-23/IL-1β, IL-23/IL-18 (ref. 26), IL-7 (ref. 27) and γδ TCR signalling uniformly repressed CCR6 surface expression in γδT17 cells (Fig. 1f and Supplementary Fig. 2b). IL-12 did not impact CCR6 expression, consistent with a reported absence of IL-12R expression by γδT17 cells (Supplementary Fig. 2b). Activation-induced CCR6 downregulation correlated with induction of activation markers CD69 and CD25 and increased CD44 expression, and occurred in both Vγ4+ and Vγ6+ γδT17 cells (Supplementary Fig. 2c,d). In all in vivo and in vitro systems, γδT17 cells maintained high levels of CCR2 following activation, and virtually all γδT17 cells were CCR2+ (Fig. 1a,c,d,f). Therefore, γδT17 cells are programmed to co-express CCR6 and CCR2 during development, but lose CCR6 expression upon activation.

CCR2 drives γδT17 cell recruitment to inflamed tissues. Tissue-infiltrating γδT17 cells are best understood in the context of cancer and autoimmunity. γδT17 cells infiltrate B16 melanomas and promote tumour growth, and infiltrate the CNS at disease onset and exacerbate disease pathogenesis during EAE. How γδT17 cells infiltrate these inflammatory lesions is unknown. We thus used these models to investigate CCR6 and CCR2 function in control of γδT17 cell migration during inflammation. Consistent with the observation that activation induces downmodulation of CCR6 expression, CCR6-deficiency did not affect γδT17 cell infiltration of B16 melanomas (Fig. 2a,b), nor recruitment to the CNS during EAE onset.
Figure 1 | γδT17 cells downregulate CCR6 upon activation. (a) Representative flow cytometry of CCR6 and CCR2 expression in skin-draining lymph nodes (sLN) and dermal CD3+ TCR-γδ+ IL-17A-YFP+ γδT17 cells from Il17aCre+ Rosa26eYFP mice (n = 3). (b) Ex vivo transwell chemotaxis of Il17aCre+ Rosa26eYFP+ splenic IL-17A+ /− γδ T cells to CCL20 and CCL2 (n = 3). (c) Representative flow cytometry of CD45+ γδT17 cells from organs of naïve Il17aCre+ Rosa26eYFP+ mice (n = 3). mLN, mesenteric lymph node; PP, Peyer’s patches; siLPL, small intestinal lamina propria lymphocytes. (d) Representative flow cytometry and quantitation of CCR6 and CCR2 expression by γδT17 cells from organs of Il17aCre+ Rosa26eYFP+ mice either naïve (n = 6) or at experimental autoimmune encephalomyelitis (EAE) onset (n = 7) or peak (n = 5). CNS, central nervous system; iLN, inguinal lymph node; ND, not detected. (e) Representative flow cytometry and quantitation of CCR6 expression by γδT17 cells from wild type (WT) mice given BrdU at d3 post-immunization for EAE, and analysed at d8 (n = 4). (f) Representative flow cytometry and frequency of CCR6 and CCR2 expression by γδT17 cells from Il17aCre+ Rosa26eYFP+ lymphocytes cultured with indicated stimuli for 72 h (n = 5). See also Supplementary Figs 1 and 2. Mean ± s.e.m. (a–c) Representative of two experiments. (d–f) Pooled from two experiments. (e) Paired two-tailed Student’s t-test, (f) one-way paired ANOVA with Dunnett’s multiple comparisons test relative to unstimulated control. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
Figure 2 | CCR2 recruits γδT17 cells to inflammatory sites. (a) CD45<sup>+</sup>CD3<sup>+</sup>TCRγδ<sup>+</sup>IL-17A<sup>+</sup> γδT17 cell numbers in tumour-infiltrating lymphocytes (TIL) following B16 melanoma challenge (n = 5/time point). (b) γδT17 cell numbers in TIL at d7 post-challenge with B16 melanoma in wild type (WT) (n = 12) and Ccr6<sup>−/−</sup> mice (n = 13). (c) γδT17 cell numbers in central nervous system (CNS) at experimental autoimmune encephalomyelitis (EAE) onset in WT (n = 7) and Ccr6<sup>−/−</sup> mice (n = 6). (d) γδT17 cell numbers in TIL and inguinal lymph nodes (iLN) d7 post-challenge with B16 melanoma in WT (n = 15 (TIL), 9 (iLN)), Ccr2<sup>−/−</sup> (n = 13 (TIL), 10 (iLN)) and Ccr2<sup>−/−</sup>Ccr6<sup>−/−</sup> mice (n = 9 (TIL), 5 (iLN)). (e) ELISA for CCL2 in tumour supernatant from WT mice challenged with B16 melanoma (n = 5/time point). (f) γδT17 cell numbers in CNS and iLN at EAE onset in WT (n = 14), Ccr2<sup>−/−</sup> (n = 13) and Ccr2<sup>−/−</sup>Ccr6<sup>−/−</sup> mice (n = 12). (g) γδT17 cell numbers in CNS at peak disease in WT (n = 6), Ccr2<sup>−/−</sup> (n = 5) and Ccr2<sup>−/−</sup>Ccr6<sup>−/−</sup> mice (n = 6). (h) ELISA for CCL2 in CNS of WT mice with EAE (n = 4/time point). (i) Ly5.1 mice (n = 4) at d5 post-challenge with B16 melanoma were transferred i.v. with in vitro-expanded γδT17 cells from Ccr2<sup>−/−</sup> (CD45.2<sup>+</sup>) and F<sub>1</sub> (CD45.1<sup>+</sup>CD45.2<sup>+</sup>) mice. Ccr2<sup>−/−</sup>:F<sub>1</sub> total, Vy4 and Vy6 γδT17 cell ratios in spleen and tumours were normalized to input ratio. Vy4 and Vy6 γδT17 cells were determined by CD3<sup>bright</sup> gating. Representative flow cytometry of CD45.2<sup>+</sup> γδT17 cells at d7 or input. (j) Ly5.1 mice (n = 7) at EAE onset were transferred with F<sub>1</sub> and Ccr2<sup>−/−</sup> γδT17 cells as in (i). Twenty-four hours later, ratios of Ccr2<sup>−/−</sup>:F<sub>1</sub> γδT17 cells in spleen, blood and CNS were normalized to input. Representative flow cytometry of CD45.2<sup>+</sup> γδT17 cells 24 h later or input. See also Supplementary Figs 3 and 4. Mean ± s.e.m. (a,c,e,i) Representative of two experiments. (b,d,f,j) Pooled from two experiments. (b,e) Unpaired two-tailed Student’s t-test, (f,g,j) one-way ANOVA with Bonferroni’s multiple comparisons test (paired in j), (i) paired two-tailed Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
(Fig. 2c). Thus, γδT17 cell trafficking to inflamed tissues in these settings occurs independently of CCR6.

In contrast, deficiency of Ccr2 abrogated γδT17 cell infiltration of B16 melanomas but did not affect their expansion in draining lymph nodes (LNs) (Fig. 2d). CCR2-driven infiltration of γδT17 cells was consistent with upregulation of its major ligand CCL2 in tumours (Fig. 2e). Similar results were found in EAE, where Ccr2-deficiency inhibited γδT17 cell recruitment to the CNS at both onset and peak disease (Fig. 2f,g), time points at which CCL2 was induced in the CNS as reported33 (Fig. 2h). CCR2 appeared to operate independently of CCR6 in regulation of γδT17 cell trafficking, as compound deficiency of Ccr6 and Ccr2 (Ccr6−/−Ccr2−/−) did not further affect γδT17 cell infiltration in either model (Fig. 2d,f,g).

Ccr2−/−Ccr6−/− mice exhibited enhanced tumour growth, while Ccr2−/− and Ccr2−/−Ccr6−/− mice had decreased EAE severity (Supplementary Fig. 3). Therefore, to examine the cell-intrinsic requirements of CCR2 for γδT17 cell migration, we developed a novel in vitro expansion protocol to generate large numbers of purified activated γδT17 cells, which contained both Vγ4+ and Vγ6+ subsets and maintained functional CCR2 expression (Supplementary Fig. 4). Equal ratios of in vitro-expanded genetically marked wild type (WT) and Ccr2−/− γδT17 cells were co-transferred into B16 melanoma-bearing recipients. While donor γδT17 cells recovered from spleen retained the input ratio, Ccr2−/− γδT17 cells did not migrate efficiently to tumours. This observation was true for both Vγ4+ and Vγ6+ subsets (Fig. 2i). Similar experiments using the EAE model revealed equivalent WT-Ccr2−/− γδT17 cell ratios in spleen and blood but reduced Ccr2−/− γδT17 cell recruitment to the CNS (Fig. 2j). Thus, CCR2, but not CCR6, drives activated γδT17 cell migration to inflammatory sites during B16 melanoma and EAE.

**CCR2 is essential for protective γδT17 cell responses.** The above models involve γδT17 cell infiltration from circulation, as the CNS and tumours lack resident γδT17 cell populations. However, many inflammatory scenarios implicate tissue-resident γδT17 cells, which survey and rapidly defend against infection at barrier surfaces. The extent to which γδT17 cell migration contributes to host defence during ongoing inflammation is unknown. To investigate whether CCR2 also directs tissue-infiltrating γδT17 cells during infection, we used experimental Streptococcus pneumoniae infection, immunity to which requires γδT17 cells4. Accordingly, Tcd−/− mice had higher bacterial burden and reduced neutrophils in the nasal wash (NW) than WT at 72 h post-infection (Fig. 3a,b). *S. pneumoniae* infection induced γδT17 cell expansion in draining LNs and nasal-associated lymphoid tissue (Fig. 3c). CCL2 was induced in the nasal passages (NPs) upon infection (Fig. 3d), and co-transfer of *in vitro*-expanded WT and Ccr2−/− γδT17 cells into infected mice revealed an intrinsic requirement of CCR2 for γδT17 cell accumulation in NP (Fig. 3e). Thus, CCR2 drives circulating γδT17 cell infiltration of mucosal tissue during *S. pneumoniae* infection.

To elucidate the ability of recruited γδT17 cells to control infection, we transferred purified *in vitro*-expanded γδT17 cells into Tcd−/− hosts prior to *S. pneumoniae* infection. In this model, tissue-infiltrating γδT17 cells provide the only source of γδ T-cell-driven protection. Transfer of WT γδT17 cells reduced nasopharyngeal bacterial burden by ~10-fold, whereas Ccr2−/− γδT17 cells completely failed to control infection (Fig. 3f). Hence, CCR2 drives recruitment of protective γδT17 cells to the nasal mucosa during *S. pneumoniae* infection. Collectively, we conclude that γδT17 cell trafficking to diverse inflamed tissues is critically dependent on CCR2 signalling.

**CCR6 regulates homeostatic positioning of γδT17 cells.** Expression of CCR6 during γδT17 cell thymic development followed by rapid downregulation upon activation suggests that CCR6 plays a more prominent function in regulation of γδT17 cell homeostasis. While CCL20 is induced during inflammation, it is constitutively expressed in barrier tissues including skin, Peyers’ patches and large intestine34-37. Both Ccr6−/− and Ccr2−/−Ccr6−/− mice had markedly reduced number and frequency of γδ T cells expressing intermediate amounts of CD3/TCR in the dermis (γδT18), a population previously reported to produce IL-17 and distinct from TCRγδ dendritic epidermal T cells22 (Fig. 4a). We confirmed that γδT10 cells were entirely marked by eYFP in Ili17a−/− x Rosα267−/− mice, despite negligible IL-17A production following *ex vivo* restimulation (Supplementary Fig. 5a). In contrast to a previous report10, Ccr6-deficiency reduced the number of both Vγ4+ and Vγ4− (Vγ6−) γδT18 cells, although the ratio was skewed slightly towards Vγ6− cells (Fig. 4b). Examination of other organs revealed that deficiency in Ccr2 had no effect on γδT17 cell homeostasis, while Ccr6-deficiency increased γδT17 cells in the peritoneal cavity (Supplementary Fig. 5b). We conclude that CCR6 regulates dermal γδT17 cell residence.

To determine whether CCR6 drives recruitment of circulating γδT17 cells into dermis, we transferred unstimulated WT or Ccr6−/− lymphocytes into naïve mice and tracked their accumulation in dermis. Transferred WT γδT17 cells were substantially enriched in dermis, demonstrating that γδT17 cells can constitutively populate the skin from circulation. In contrast, Ccr6−/− γδT17 cells were defective in infiltration of dermis and pooled in the blood (Fig. 4c). In support of earlier results, both Vγ4+ and Vγ4− γδT17 cells were recruited to the dermis, the ratio of which was unaltered by Ccr6 deficiency, suggesting both populations are dependent on CCR6 for circulation-to-dermis trafficking (Fig. 4c). While constitutive expression of CCL20 in epidermis was reported, whether it is expressed in uninflamed dermis is unclear34,38. We found that Cd20 was constitutively expressed in the dermis by an uncharacterized CD31− CD90− CD140α− stromal population (Fig. 4d). Thus, CCR6 directs homeostatic recruitment of γδT17 cells from circulation into dermis.

**IRF4 and BATF regulate CCR6 expression in γδT17 cells.** The downregulation of CCR6 upon γδT17 cell activation is surprising, as T cells typically upregulate inflammatory chemokine receptors upon activation. Consequently we investigated the underlying mechanism regulating this process. Ccr6 transcript levels were reduced by approx. fourfold in γδT17 cells within 24 h of stimulation, whereas Ccr2 expression was maintained (Fig. 5a and Supplementary Fig. 6a). This indicated that CCR6 expression is transcriptionally regulated during γδT17 cell activation. We thus examined expression of transcription factors previously implicated directly or indirectly in control of CCR6 expression, including RORγt17, IRF4 (ref. 39), IRF8 (refs 40,41), Blimp1 (refs 39,42), BATF43 and T-bet and Eomes18, Rorc (RORγt) was highly expressed in resting γδT17 cells but was downregulated by 24 h of activation. Batf and Prdm1 (Blimp1) were rapidly upregulated by 24 h, while Irf8 and Irf4 were upregulated by 48 h, although Irf4 was already present in resting γδT17 cells. Expression of Eomes and Tbx21 (T-bet) at rest or following activation was minimal (Fig. 5b and Supplementary Fig. 6b). Therefore, we tested whether RORγt, IRF4, BATF, Blimp1 or IRF8 repressed Ccr6 expression during γδT17 cell activation.

The similar expression kinetics and known Ccr6 regulatory activity of RORγt presented the possibility that its down-regulation may result in loss of CCR6 expression. To test this,
we retrovirally forced Rorc expression in in vitro-expanded γδT17 cells (Fig. 5c). However, this failed to alter CCR6 expression even in the highest GFP-expressing cells, suggesting that RORγt downregulation is not required for repression of CCR6 in activated γδT17 cells (Fig. 5d).

To determine whether IRF4, BATF, IRF8 or Blimp1 actively repress Ccr6 expression, we cultured genetically-marked WT and transcription factor-deficient splenocytes with IL-23 and IL-1β. γδT17 cells were present in all strains although at differing frequencies, and homeostatic CCR6 expression was comparable to WT (Supplementary Fig. 6c). IRF4- and BATF-deficient γδT17 cells were pre-treated with proliferation inhibitor mitomycin C prior to stimulation. Although proliferation was effectively blocked, CCR6 downregulation still occurred upon mitomycin C treatment, suggesting that proliferation and CCR6 downregulation are coincident but independent (Fig. 5f).

Loss of CCR6 promotes γδT17 cell homing to inflamed tissues. Given the constitutive expression of CCL20 in mucocutaneous sites, we hypothesized that repression of CCR6 during activation enables homing of γδT17 cells toward inflammatory lesions by preventing their accumulation in uninflamed skin. To test this, we first compared the trafficking of in vitro-activated WT γδT17 cells with resting WT and Ccr6−/− γδT17 cells upon transfer into uninfomnized hosts. Activated WT γδT17 cells demonstrated the same defect in homing to the dermis as resting Ccr6−/− γδT17 cells, and both pooled in blood compared to resting WT γδT17 cells (Fig. 6a). γδT17 cells lack CD62L and CCR7 expression, and traffic from skin to sLNs in a CCR7-independent manner. Thus γδT17 cell entry to sLNs following adoptive transfer likely occurs via afferent lymph draining from dermis. In keeping with this idea, restoring Ccr6−/− or in vitro-activated WT γδT17 cells, impaired in their ability to home to uninflamed skin, also accumulated less than resting WT γδT17 cells in sLNs (Fig. 6a). These data are consistent with the notion that activation switches off γδT17 cell homeostatic circulation patterns, enabling directed migration toward inflammatory cues.

To investigate this proposal directly, we studied the migratory patterns of in vitro-activated γδT17 cells retrovirally forced to maintain CCR6 expression. Infection with Ccr6Δ8 virus restored CCR6 expression in activated γδT17 cells, which regained the
ability to migrate toward CCL20 (Fig. 6b,c). Genetically marked control- and Ccr6<sup>−/−</sup>-transduced γδT17 cells were mixed 50:50 and transferred into B16 melanoma-bearing recipients. While the input ratio of transferred GFP<sup>−</sup> cells was maintained in all examined organs as expected, among GFP<sup>+</sup> cells, Ccr6<sup>−/−</sup> γδT17 cells were enriched in the dermis but deficient in tumours (Fig. 6d). Similar results were observed during <i>S. pneumoniae</i> infection: Ccr6<sup>−/−</sup> γδT17 cells were selectively deficient at homing to NP, but accumulated to a greater extent than control-transduced cells in uninflamed dermis (Fig. 6e). Ccr6<sup>−/−</sup> γδT17

Figure 4 | CCR6 regulates homeostatic γδT17 cell recruitment to dermis. (a) Representative flow cytometry and quantitation of CD45<sup>+</sup> CD3<sup>lo</sup>TCR-γδ<sup>+</sup> (γδ<sup>T</sup>) cells from ear skin dermis of naïve wild type (WT) (n = 13), Ccr6<sup>−/−</sup> (n = 11), Ccr2<sup>−/−</sup> (n = 10) and Ccr2<sup>−/−</sup> Ccr6<sup>−/−</sup> mice (n = 5). (b) Representative flow cytometry of Vγ4 expression by dermal γδ<sup>T</sup> cells and quantitation of Vγ4<sup>+</sup> and Vγ4<sup>−</sup> γδ<sup>T</sup> cells in dermis of WT and Ccr6<sup>−/−</sup> mice (n = 7/group). (c) WT or Ccr6<sup>−/−</sup> lymphocytes were transferred i.v. into naïve Ly5.1 mice (n = 4/group). After 36 h, number of CD45.2<sup>+</sup> γδ<sup>T</sup>/γδ<sup>T</sup> cells recovered was expressed as % of number transferred. Representative flow cytometry of dermal CD45.2<sup>+</sup> cells and quantitation of γδT17 cell recovery and Vγ4<sup>+</sup>:Vγ4<sup>−</sup> ratio (normalized to input). (d) Ccl20 mRNA from whole tissues or sorted CD45<sup>+</sup> epidermal keratinocytes (Sca-1<sup>+</sup> Ep-CAM<sup>−</sup> interfollicular epidermis (IE), Sca-1<sup>−/−</sup> Ep-CAM<sup>+</sup> infundibulum and isthmus (IF & IS), Sca-1<sup>−</sup>Ep-CAM<sup>−</sup> double negative (DN)) or CD45<sup>−</sup> dermal populations (CD31<sup>+</sup> CD90<sup>+</sup> CD140a<sup>+</sup> fibroblast, gp38<sup>+</sup> CD31<sup>+</sup> lymphatic endothelial cells (LEC), gp38<sup>+</sup>CD31<sup>+</sup> blood endothelial cells (BEC), CD31<sup>−</sup> CD90<sup>−</sup> CD140a<sup>−</sup> double negative (DN)) from naïve WT mice (pooled from 5 mice/experiment). ND, not detected. See also Supplementary Fig. 5. Mean ± s.e.m. (a,d) Pooled from three experiments, (b,c) representative of two similar experiments. (a) One-way ANOVA with Bonferroni’s multiple comparisons test, (b,c) unpaired two-tailed Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Figure 5 | IRF4 and BATF promote CCR6 downregulation in γδT17 cells. (a) Ccr6 and (b) transcription factor mRNA in sorted γδT17 cells from Il7γCcr6 × Rosa26efffp lymphocytes *ex vivo* or cultured with IL-23/IL-1β for indicated times (pooled from 5 to 7 mice). ND, not detected. (c,d) Expanded γδT17 cells (n = 3) were transduced with empty pMIG or pMIG-Rorc retrovirus. (c) Representative flow cytometry of RORγt expression in GFP*γδT17* cells (gated as in d), relative to isotype (grey) and geometric mean fluorescence intensity (gMFI) relative to GFP fluorescence intensity (FI). (d) Representative flow cytometry of CCR6 expression and quantitation in GFP*γδT17* cells (in γδT17 cells). (e) Splenocytes from Ly5.1 and either wild type (WT), Irf4−/− or Batf−/− mice were 670 dye-labelled, mixed 50:50 and stimulated with IL-23/IL-1β for 72 h. Representative flow cytometry and quantitation of CCR6 expression and proliferation in CD45.1* or CD45.2* γδT17 cells (n = 3/group). (f) Representative flow cytometry and quantitation of CCR6 expression by 670 dye-labelled γδT17 cells from WT splenocytes cultured with IL-23/IL-1β for 72 h with/without mitomycin C pre-treatment (n = 3). See also Supplementary Figs 6 and 7. (a,b) Mean ± s.d., (c-f) Mean ± s.e.m. (a-f) Representative of two similar experiments. (d,e) Paired two-tailed Student’s t-test, (f) one-way paired ANOVA with Bonferroni’s multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001.
cells also homed less efficiently to the CNS during EAE, although subcutaneous complete Freund’s adjuvant immunization precluded analysis of homing to uninflamed skin in this model (Fig. 6f). Together, these experiments demonstrated that activated γδT17 cells with forced CCR6 expression were recruited to uninflamed dermis at the expense of homing to inflamed tissue.

**Figure 6 | CCR6 downregulation by γδT17 cells enhances migration to inflamed tissue.** (a) Resting lymphocytes from wild type (WT) (n = 3) or Ccr6−/− (n = 4) mice, or WT lymphocytes stimulated with IL-23/IL-1β for 72 h (n = 4) were transferred i.v. into separate naïve Ly5.1 hosts. After 36 h, number of CD45.2+ γδT0/γδT17 cells recovered was expressed as % of number transferred. sLN, skin-draining lymph node. (b) Representative flow cytometry for CCR6 expression by GFP+ in vitro-expanded γδT17 cells transduced with empty pMIG or pMIG-Ccr6, relative to isotype (grey) (n = 3). (c) Chemotaxis of GFP+ γδT17 cells transduced as in (b) to CCL20 (n = 3). (d) In vitro-expanded γδT17 cells from F1 (CD45.1+CD45.2+) or WT (CD45.2+) mice were transduced with empty pMIG or pMIG-Ccr6, respectively. Equal numbers of mixed GFP+ cells were transferred i.v. into Ly5.1 mice challenged with B16 melanoma 5 days prior and analysed at d7 (n = 5). Representative flow cytometry and ratio of recovered F1 to WT γδT17 cells within transduced (GFP+) and untransduced (GFP−) populations. Recovered values were normalized to input values. TIL, tumour-infiltrating lymphocytes. (e,f) In vitro-expanded γδT17 cells from WT or F1 mice were transduced with empty pMIG or pMIG-Ccr6, respectively. Equal numbers of mixed GFP+ cells were transferred i.v. into Ly5.1 mice either (e) 24 h post-infection with S. pneumoniae (n = 4) or (f) at experimental autoimmune encephalomyelitis (EAE) onset (n = 3) and organs were analysed 48 h later. Ratio of recovered WT to F1 γδT17 cells within transduced (GFP+) and untransduced (GFP−) populations, normalized to input values. CNS, central nervous system; NP, nasal passage. Mean ± s.e.m. (a) Representative of three similar experiments, (b,d) representative of two experiments. (a) One-way ANOVA with Dunnett’s multiple comparisons test relative to resting WT γδT17 cells, (d-f) paired two-tailed Student’s t-test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
Thus, CCR6 downregulation promotes γδT17 cell migration to inflammatory sites.

**Discussion**

In the present study we define the molecular regulation of γδT17 cell trafficking between resting and activated states. Our data are consistent with a model in which γδT17 cells are imprinted with expression of both CCR6 and CCR2 during thymic development. CCR6 coordinates steady-state recruitment of circulating γδT17 cells into the dermis, where CCL20 is constitutively expressed, thus orchestrating their homeostatic recirculation. Upon activation, γδT17 cells rapidly downregulate CCR6 in an IRF4- and BATF-dependent manner. CCR2 expression is maintained and drives homing of activated γδT17 cells to inflamed tissue during autoimmunity, cancer and infection. CCR6 downregulation is required to promote optimal recruitment of activated γδT17 cells to inflamed tissue during ongoing inflammation, rendering these cells unable to home to inflammatory sites.

These data identify a novel mode of lymphocyte trafficking that facilitates both γδT17 cell innate-like surveillance of host tissues and their rapid recruitment to distal sites of ongoing inflammation.

Our data demonstrate that CCR6 coordinates homeostatic recirculation of Vγ4+ and Vγ6+ γδT17 cells through blood, skin and sLNs. Given that γδT17 cells likely populate sLNs via afferent lymph12,14, our data suggest that entry is contingent on first trafficking through the dermis, as Ccr6-deficient γδT17 cells fail to localize to both organs. Thus, in addition to driving infiltration of skin, CCR6 ultimately regulates γδT17 cell homing to sLNs. These findings complement a recent report that CCR6 positions sLN Vγ4+ γδT17 cells in the subcapsular sinus13. Our findings are also in agreement with previous reports of steady-state γδT17 cell circulation, although we show that in addition to Vγ4+, Vγ6+ γδT17 cells also undergo constitutive trafficking10,13,14. While most Vγ4+ γδT17 cells in sLNs do not recirculate within a 2-week period, approximately 25% undergo extensive circulation11. The non-recirculating population of sLN γδT17 cells is likely those positioned in interfollicular zones and subcapsular sinus, which were proposed to scan for sLN-invading microbes11,16. However, the function of the recirculating population of γδT17 cells is unclear. As γδT17 cells do not need to scan sLNs for antigen like conventional T cells, they why they adopt a constitutive CCR6-dependent circulation loop between tissues and sLNs remains to be resolved.

We found that programmed expression of CCR2 equips γδT17 cells with the ability to rapidly home from circulation into diverse inflammatory environments. Our description of CCR2-driven γδT17 cell infiltration of the autoimmune CNS reflects other reports during psoriasis and arthritis15,20. Additionally, CCR2-mediated infiltration of both Vγ4+ and Vγ6+ γδT17 cells into B16 melanomas suggests that this axis is a universal inflammatory homing signal for γδT17 cells. Our data reveal a redundant function for CCR6 in these processes, due to its immediate downregulation upon γδT17 cell activation. However, these data are inconsistent with reports of CCR6-driven migration of γδT17 cells during skin and liver inflammation19,47. Tissue-specific signals directing maintained CCR6 expression in activated γδT17 cells in these particular scenarios could possibly explain discrepancies between these reports and our data, although this requires further investigation. The function of tissue-infiltrating γδT17 cells during inflammation in barrier tissues, where a resident γδT17 cell population already exists, is unclear. This phenomenon was previously reported during psoriasis, although did not appear to affect disease14,48. Here, we show that γδT17 cells expand in draining LNs and infiltrate nasal mucosa via CCR2 during bacterial infection. In absence of endogenous γδ T cells, transferred circulating γδT17 cells are able to control infection in a CCR2-dependent manner. Thus, we propose that LN-expanded γδT17 cells home to lesions via CCR2 to supplement the local γδT17 cell pool during ongoing tissue inflammation.

Along with our previous work, this study identifies a shared chemokine receptor program used by IL-17-producing cells. We show that like Th17 cells18, γδT17 cells express CCR6 early during their effector program, but lose CCR6 expression via IL-23 signalling during ongoing inflammation. Instead, CCR2 is the defining IL-17-program inflammatory homing signal. Whether CCR6 repression in Th17 cells occurs to disrupt barrier tissue-homing and promote recruitment to inflamed tissue, as we show in γδT17 cells, is unclear. Despite γδT17 cell development occurring independently of IRF4 and BATF49,50, we report that these factors suppress Ccr6 expression in γδT17 cells upon IL-23- and IL-1β-driven activation. This is likely to be cooperative, as has been shown during Th17 cell development45. Moreover, we identified a common binding site for IRF4 and BATF in the Ccr6 promoter, suggesting that repression of Ccr6 expression is directly mediated by these factors. It remains to be seen if a similar mechanism operates in Th17 cells. Therefore, while γδT17 and Th17 cells both lose CCR6 expression during inflammation, the mechanism and function of this process may differ between these populations.

Investigating the trafficking of human γδT17 cells is of clinical relevance as they are increasingly implicated in autoimmunity and cancer, and it will be important to determine whether the model we have established here for murine γδT17 cells applies to humans. Of interest, CCR6 is known to be expressed by both Vδ1+ and Vδ2+ γδT17 cells in humans, as well as by circulating Vδ2+ cells with a skin-homing CLA+ phenotype51-53. CCR2 expression has been identified in human γδ T cells, but to our knowledge this has not been examined specifically in IL-17+ cells54,55. Whether human γδT17 cells also undergo activation-induced CCR6 downregulation to enhance inflammatory homing has yet to be determined. While the human dermal γδT17 cell compartment is relatively small compared to the murine system, the relative abundance of circulating skin-homing Vδ2+ cells suggests that similar recirculation mechanisms may also operate in humans. These issues await further experimental resolution.

Our description of γδT17 cell trafficking between homeostasis and inflammation presents a novel mode of lymphocyte migration. Conventional T cells downregulate the homeostatic recirculation signal CCR7 and induce expression of inflammatory chemokine receptors upon differentiation into effector subsets, a slow process. In contrast, we show that γδT17 cells constitutively co-express homeostatic and inflammatory homing receptors. The switch in γδT17 cell trafficking from homeostatic to inflammatory programs is solely driven by downregulation of the homeostatic receptor CCR6, rather than induction of additional inflammatory homing receptors. This model likely facilitates immediate homing to inflammatory sites in addition to homeostatic scanning behaviour, consistent with the ‘activated-but-resting’ phenotype of γδT17 cells. Use of CCR6 expression to distinguish resting and activated states may facilitate future investigation of γδT17 cell biology. We conclude that γδT17 cells exhibit a unique bi-phasic trafficking program driven by programmed changes in homing receptor expression to facilitate tissue sentinel responses and rapid homing to distal inflammatory sites.

**Methods**

Mice. C57Bl/6 (WT) and Ly5.1 mice were purchased from Animal Resource Centre (WA, Australia) or bred at the University of Adelaide animal facility. NOD.Cg-IL7rα<sup>−/−</sup> <sup>+</sup> × Rosa26<sup>YFP</sup>, Ccr6<sup>−/−</sup>, Ccr2<sup>−/−</sup>, Ccr2<sup>−/−</sup>/Ccr6<sup>−/−</sup>, Ter1<sup>−/−</sup> and...
C57Bl/6 × Lyt-1.2 (F1) mice were bred at the University of Adelaide animal facility. Ifny−/−, Ispf−/−, Batf−/− and Lck−/−/Pdym−/− mice were bred at the WEHI animal facility. Male mice of age and gender similar to those used at 6–14 month of age. Experiments were conducted with approval of the University of Adelaide Animal Ethics Committee.

**Disease models.** Mice were immunized for chronic EAE by subcutaneous injection of 100 μg of MOG35-55 (GL Biochem) in phosphate-buffered saline (PBS) emulsified 1:1 in complete Freund’s adjuvant, coupled with i.p. injection of 300 ng Pertussis toxin (Sapphire Bioscience) on days 0 and 2. Mice were analysed at clinical scores of 0.5 (asymptomatic) and 2–3 (peak) in wild type (WT) mice, where scoring criteria were: 0.5, tremor; 1, partially limp tail; 2 fully limp tail, 2.25 unable to right, 2.5 sprawled hindlimbs, 2.75 one hindlimb paralysed, 3 both hindlimbs paralysed, 3.5 one forelimb paralysed. B16.F10 melanoma cells (provided by Prof. Mark Smyth, QIMR Berghofer, mycoplasma free and verified by short tandem repeat) were cultured in RPMI 1640 containing 10% fetal calf serum (FCS) and 5 mM L-glutamine, pen/strep, L-glutamine, 1 ml PBS washes. Tumour and NP supernatants from digested samples and supernatants of WT or Ccr2−/− T cells were collected by 3 ml PBS washes. T cells were cultured in complete RPMI for 20–24 h, re-seeded on fresh plastic at 100,000 cells per well using antibodies and staining, cells were incubated in Foxp3 kit perm buffer (eBioscience) for 30 min to stain Foxp3. After fixation with 20% paraformaldehyde, cells were washed in PBS then incubated in 5 mM EDTA for 40 min at 37 °C, then washed and re-seeded in 20 ng ml−1 -IFN-γ (BioXcell) in 96-well round-bottom plates coated with 1 μg ml−1 –α-TCR-γ (clone GL3, Biologend) for 3 days. Cells were washed and re-seeded on fresh plastic at 1 × 10^6 cells per well for a further 3 days as above without TCR-γ stimulation. Cells were then washed and re-seeded in 20 ng ml−1 -IFN-γ (BioXcell) for a further 3 days. Pmig, Pmig-Rorc and Pmig-Ly5.1 (clone from mouse C56/CDNA) were transfected into EcoPack 2 293 cells (Clontech; mycoplasma free) with Lipofectamine 2000 (ThermoFisher), and supernatant collected after 48 h. γT17 cells at days 4 and 5 of culture were centrifuged at 2,500 r.p.m. (30 °C for 1.5 h) in supernatant with 8 μg ml−1 polybrene (Sigma) in flat-bottom 96 well trays before being returned to culture.

**Adaptive transfers.** For Ccr2−/− and Ccr6−/−γT17 cell transfers, 1–2 × 10^7 of each of in vitro-expanded F1 (CD45.1+CD45.2−) and Ccr2−/− (CD45.2+), or transduced control and Ccr6−/−γT17 cells (F1, or WT), were mixed and transferred i.v. into Ly5.1 (CD45.1+) recipient mice d5 post-challenge with B16 melanoma, d8–10 post EAE induction or 24 h post-S. pneumoniae infection. γT17 cell infiltration of target organs was analysed 24–48 h post-transfer, and CD45 congenic ratios were normalized to input sample. For S. pneumoniae Tcd−/− reconstitution, in vitro-expanded WT and Ccr2−/−γT17 cells were further purified by MACs (Miltenyi Biotec) before 3 × 10^6 cells were transferred into separate Tcd−/− hosts 24 h prior to infection. For naive dermis trafficking experiments, 5–10 × 10^6 fresh WT or Ccr6−/−/lymphocytes or 3 × 10^3 72 h IL-23/IL-1β-stimulated WT lymphocytes were transferred into separate unimmunized Ly5.1 mice and analysed 36 h later. Number of recovered cells was normalized to γT17 cells transferred.

**ELISA.** Tumour and NP supernatants from digested samples and supernatants from filtered CNS were supplemented with protease inhibitors (Sigma) and stored at −80 °C. Mouse CCL2 Duoset ELISA (R&D) was conducted according to the manufacturer’s instructions.

**qPCR.** γT cells from Il17a+ × Rose2eTPP mice were enprinted by MACs using mouse TCRδ−/− isolation kit (Miltenyi Biotec, # 130-092-125), and then sorted using a BD FACSAria. Naïve CD4+ T cells were sorted from WT splenocytes, and skin stromal populations were sorted from digested epidermal and dermal suspensions from WT mice. Sorting strategies are detailed in Supplementary Figure 9. RNA was extracted from sorted cells using Qiagen RNeasy Micro kit (# 74104). For epimeris and dermis, tissues were snap frozen in liquid nitrogen, crushed with mortar and pestle and RNA purified using Qiagen RNeasy Minit kit (# 74104) according to the manufacturer’s instructions. cDNA was generated with the Roche Transcriptor First Strand cDNA synthesis kit (# 0489866001). qPCR was performed with Roche LightCycler 480 SYBR Green 1 master mix (# 0488835201) using primer sequences in Supplementary Table 2 on 10 ng ml−1 mRNA. Relative gene expression was calculated as 2−ctargetCT control reference where reference was Rpp60.

**Chemotaxis.** Splenocytes were rest in complete RPMI for 3–4 h at 37 °C, washed and suspended in chemotaxis buffer (RPMI 0.5% bovine serum albumin 20 mM HEPES). γT17 cells from culture were washed and suspended in chemotaxis buffer. CCL2 or CCL20 (from the late Prof. Ian Clark-Lewis) were diluted in chemotaxis buffer and loaded into the lower chambers of 96-well 5 μm pore transwell plates (Corning). 2 × 10^5 splenocytes or 2 × 10^5 γT17 cells were suspended in 200 μl of chemotaxis buffer and loaded into the upper chambers of transwell plates. 24 h later, wells were harvested and stained for flow cytometry. CountBright beads (Invitrogen) were added to samples prior to acquisition to normalize event counts. Chemotaxis index was calculated as number of gated events divided by number in 0 chemokine control.

**In vitro stimulation.** Splenocytes were cultured in complete IMDM (10% FCS, pen/strep, L-glutamine, β-mercaptoethanol) at 2.5 × 10^6 cells per ml with 10 ng ml−1 –α-TCR-γ (BioXcell) for up to 72 h at 37 °C. For myeloid cells, pre-treatment, cells were first incubated with 10 μg ml−1 polybrene (Sigma) in complete IMDM at 2 × 10^6 cells per ml for 2 h at 37 °C before extensive washing.

**Chip-seq analysis.** Chip-seq data for IRF4 in CD8+ T cells45, BATF in CD8+ T cells44 and IRF4/BATF in Th17 cells46,47 were previously published and were acquired on a BD LSR II or FACSAria and analysed with Flowjo (Treestar). Gating strategies are detailed in Supplementary Fig. 8.
obtained from NCBI database using accession codes GSE49930, GSE49149 and GSE69198, respectively. BAM files were loaded and displayed using the IGB genome browser.

**Statistics.** Data were analysed with GraphPad Prism 6. Appropriate statistical tests were two-sided and used as indicated in figure legends. *P* < 0.05, **P** < 0.01, ***P** < 0.001, ****P** < 0.0001. All replicates are biological except in qPCR experiments, where technical replicates are denoted. Sample sizes were determined empirically to ensure adequate power. **/** * host mice were randomly assigned to groups before receiving adoptive transfers in Fig. 3E. No blinding was utilized. Minimal variance was generally observed between groups; Welch’s correction was used in t-tests where standard deviations were significantly different. Most data sets were normally distributed; Mann–Whitney and Kruskal–Wallis tests were utilized where data were not normally distributed.

**Data availability.** Relevant data are available from authors upon reasonable request.

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Author contributions

D.R.M., E.E.K., I.C. and S.R.M. designed experiments, analysed data and wrote the manuscript; D.R.M. performed most experiments; E.E.K., C.R.B., T.S.T., K.A.F., C.E.G. and J.J.W. performed experiments; R.B. and J.C.P. contributed to *S. pneumoniae* experiments; A.K. and S.L.N. contributed to transcription factor experiments; A.B. contributed to retrovirus experiments; M.M. provided key reagents; J.C.P., A.K., S.L.N., A.B. and M.M. edited the manuscript; I.C. and S.R.M. supervised the study and attained funding.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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