Peripheral organs, such as the skin, gastrointestinal tract, and lungs, are barriers against invading microorganisms. The immune system deploys sentinels, including DCs, macrophages, and T lymphocytes, to counteract pathogen invasion upon barrier breach within these organs. Some of these cell lineages, in particular DCs, show strict microcompartmental segregation. Thus, the epidermis harbors Langerhans cells (LCs), whereas the dermis is home to at least three subpopulations of dermal DCs (DDCs). Recently, distinct functions have been ascribed to epidermal and DDC subsets (Heath and Carbone, 2009; Nestle et al., 2009; Ueno et al., 2010), indicating adaptation of these cells to their specific anatomical niches. In mouse epidermis, a prominent population of $\gamma\delta$ TCR-expressing T cells, known as dendritic epidermal T cells (DETCs), can be found. Whether the dermis also contains a discrete population of $\gamma\delta$ T cells and, if so, what role these cells may play in immune defense has been investigated only perfunctorily.

Many studies have documented a remarkable enrichment of $\gamma\delta$ T lymphocytes within the epithelia of peripheral organs; they otherwise represent only a minority of the T cell pool in secondary lymphoid organs. Curiously, these epithelial T cells often display restricted TCR repertoires. Thus, DETCs bear the canonical V$\gamma$5/V$\delta$1 TCR (TCR nomenclature; Heilig and T onegawa, 1986; Asarnow et al., 1988; Havran et al., 1989). Similarly, the lungs, gastrointestinal tract, and genitourinary tract are populated with distinctive subsets of $\gamma\delta$ T cells bearing tissue-specific TCRs with limited or invariant diversity (Carding and Egan, 2002; Xiong and Raulet, 2007; Bonneville et al., 2010). This expression of specific TCRs points toward functional specialization of these cells in distinct microcompartments of the skin.
Based on their localization to the epidermis and their restricted TCR expression, it is debatable whether DETCs actively participate in dermal immunosurveillance. Given the nonredundant role of γδ T lymphocytes in the immune response against pathogens, we wondered whether the dermis also contains resident γδ T cells. In this study, we have identified a robust population of dermal γδ T cells. These cells expressed a mixed panel of TCRs and differed in their survival requirements and functional properties from their epidermal counterparts. Furthermore, dermal γδ T cells regulated antigen-specific CD4+ T cell responses after dermal mycobacterial infection. Together, these results define a population of γδ T cells in the dermis of mice that participates in cutaneous immune responses. Our results further highlight the compartmental specialization of cutaneous T cell populations that exhibit separable functions in the immune surveillance program.

RESULTS
Mouse dermis harbors a prominent population of γδ T cells
To determine the presence of γδ T cells within the individual compartments of the skin, we prepared single-cell suspensions from dermal and epidermal sheets of C57BL/6 mouse ears. Consistent with previous studies (Hayday, 2000; Bonneville et al., 2010), we found that 100% of epidermal CD3+ leukocytes represented γδ T cells, of which ~98% were Vδ5+ (Fig. 1, A and B). Strikingly, we identified an abundant population of γδ T cells in the dermis. γδ T cells constituted 54.1 ± 5.4% of dermal T cells (Fig. 1, A and B), of which 80% were Vδ5– (Fig. 1, A and B) and were therefore distinct from their epidermal counterparts. The Vδ5+ dermal γδ T cell population may represent either epidermal contaminants, e.g., from residual hair follicles in the dermal sheet preparation, or bona fide dermal residents. Further analyses on TCR usage showed that ~30% of dermal γδ T cells expressed Vγ4 TCR (Fig. 1 C), demonstrating that, unlike DETCs, dermal γδ T cells are a heterogeneous population.

Figure 1. The mouse dermis harbors a population of γδ T cells. (A) Flow cytometry profiles of epidermal and dermal γδ T cells from ear skin of WT mice (n ≥ 9/group). Cells were gated on CD45+ (top), CD45+CD3+ (middle), or CD45+CD3+TCRγδ+ (bottom). (B) Percentage of αβ T cells and γδ T cells within dermal and epidermal CD3+ cells of ear skin in WT mice (n = 9). (C) TCRγδ usage of epidermal and dermal γδ T cells from WT mice (n = 9). Results are expressed as a frequency of TCRγδ+ T cells. Data are representative of at least two independent experiments. Data are presented as mean ± SEM.
We next undertook a comprehensive phenotypic characterization of DETCs and dermal γδ T cells in steady-state skin (Fig. 2). Dermal αβ T cells and splenic γδ T cells were included in the analyses for comparison. Dermal αβ and γδ T cells expressed low levels of CD25, whereas DETCs and splenic γδ T cells were CD25−. Although all epidermal and splenic γδ T cells expressed high levels of CD43, the expression of this marker on dermal γδ T cells was bimodal. All cutaneous T cells were CD44+, whereas splenic γδ T cells had bimodal expression of CD44. Remarkably, all cutaneous T cells were CD69+, suggesting a preactivated state. Splenic γδ T cells were CD69−. In addition, cutaneous, but not splenic, T cells expressed CD103 (αE integrin), a ligand for E-cadherin. CD127 (IL-7Rα) was expressed by all skin T cell subsets and, to a lesser extent, by splenic γδ T cells.

Collectively, these results indicate that the dermis harbors a population of γδ T cells that represents >50% of CD3+ T cells and differs in TCR usage and phenotype from both DETCs and systemic γδ T cells. Dermal γδ T cells are largely radioresistant and proliferate locally in the skin DETCs have previously been shown to be radioresistant (Honjo et al., 1990) and are not replenished from the bone marrow in adult mice. To assess the radiosensitivity of dermal γδ T cells, we generated bone marrow chimeras by transplanting CD45.1+ bone marrow into lethally irradiated congenic CD45.2+ C57BL/6 mice. As expected, 12 wk after transplantation, 100% of Vγ5+ DETCs remained host-derived (CD45.2−; Fig. 3 A). Strikingly, 90.9 ± 2.4% of dermal γδ T cells also remained of host origin, whereas ~10% of dermal Vγ5−γδ T cells were donor-derived (Fig. 3 A). In stark contrast, the vast majority of γδ T cells, including those in the blood, secondary lymphoid organs, and gastrointestinal tract of chimeric mice had been replaced with donor-derived cells (Fig. 3 A). As in other organs, dermal αβ T cells were predominantly of donor origin (Fig. 3 A). These results suggested that, contrary to γδ T cells in other organs and dermal αβ T cells, dermal γδ T cells are largely maintained independent of circulating precursors.

We therefore hypothesized that dermal γδ T cells undergo homeostatic proliferation within the dermis. To test this, we examined BrdU incorporation of skin αβ and γδ T cells. After 6 d of supplementing the drinking water of C57BL/6 mice, we found that 2% of DETCs had incorporated BrdU, whereas 6% of both γδ T cells and αβ T cells were BrdU+. Because BrdU incorporation could have occurred within bone marrow or thymic precursors before their development into γδ T cells, we also analyzed BrdU uptake by dermal γδ T cells in bone marrow chimeras 19 mo after reconstitution. At this point, ~80% of dermal γδ T cells were still host-derived, whereas αβ T cells had been almost completely replaced by donor bone marrow (Fig. 3 C). 6 d after BrdU administration, ~4% of both host- and donor-derived dermal γδ T cells had incorporated BrdU in chimeric mice (Fig. 3 C). These findings indicate that dermal γδ T cells are maintained through their local proliferation in steady-state conditions.

Figure 2. Phenotypic analyses of dermal γδ T cells. Histograms of surface markers expressed by cutaneous and splenic γδ T cells from WT mice (n ≥ 3 per group). Histograms were pregated on CD45+MHC-II−CD3+ cells before gating on γδ+ and γδ− T cell populations. Expression of the indicated markers is also shown for DETCs, dermal αβ T cells, and splenic γδ T cells. Gray lines, isotype control staining; black lines, indicated antibodies. Data are representative of at least two independent experiments.
Dermal γδ T cells display a unique profile of cytokine dependence.

**Figure 3.** Dermal γδ T cells are predominantly radioresistant and proliferate locally in the skin. (A) Degree of chimerism of γδ T cells (left) and αβ T cells (right) in various organs obtained 12 wk after reconstituting lethally irradiated WT mice with B6D2F1 congenic bone marrow. Filled bars, host B6D2F1; open bars, donor B6D2F1. (B, left) Flow cytometry profiles of BrdU incorporation by epidermal and dermal T cells isolated from WT mice (n ≥ 5/group) 6 d after initial BrdU administration (left). (right) Frequency of cutaneous DDU- T cells isolated from WT mice (n = 5). (C, left) Degree of chimerism of cutaneous T cells obtained 19 mo after reconstitution (n = 8). Data are representative of two to four independent experiments.

**Differential cytokine requirements for epidermal and dermal γδ T cells**

Based on the finding of in situ turnover of dermal γδ T cells, we wondered which survival cytokines were controlling their development/maintenance. Epidermal Vγ5+ γδ T cells have been shown to require both IL-7 and IL-15 for development and/or survival in vivo (Moore et al., 1996; Laky et al., 1998; De Creus et al., 2002). To determine whether dermal γδ T cells had similar requirements, we tested IL-7 and IL-15-deficient mice for the presence of these cells. Both DETCs and dermal γδ T cells were dependent on IL-7 because IL-7−/− and IL-15−/− mice for the presence of these cells. Both DETCs and dermal γδ T cells were dependent on IL-7 because IL-7−/− mice were completely devoid of DETCs, and both the frequency and the number of dermal γδ T cells (Vγ5+ TCRγδ+) were significantly reduced (Fig. 4). Thymi of IL-7−/− mice were completely devoid of γδ T cells, whereas the dermis still maintained a minor population of Vγ4− γδ T cells (Fig. S1). This indicates that a subset of dermal γδ T cells is IL-7-independent. The frequency of αβ T cells remained unchanged, but the absolute number of cells was significantly reduced in IL-7−/− mice compared with WT mice (Fig. 4, B and C). In contrast to DETCs, however, the frequency and the number of dermal γδ T cells were unaltered in IL-15−/− mice compared with WT mice (Fig. 4, B and C). Although the frequency of αβ T cells remained unchanged in IL-15−/− mice compared with WT mice, the number of cells was significantly reduced (Fig. 4, B and C). To further test the influence of IL-7 on dermal γδ T cells, we evaluated their frequency in mice with transgenic overexpression of IL-7 (IL-7Tg; Mertsching et al., 1995). As shown in Fig. 4C, this led to a significant increase in the number of dermal γδ and αβ T cells, but not DETCs. Therefore, differential migratory behavior of LCs and DDCs in the ear skin of mice (Ng et al., 2008), i.e., LCs were found to be sessile, whereas DDCs were constitutively migratory. To test whether similar migratory behavior of cutaneous γδ T cells could be observed, we made use of mice with an EGFP knock-in in the Bonzo/CXCR6 locus (CXCR6EGFP mice; Unutmaz et al., 2000). In these mice, CD3+ T cell subsets, including NKT cells, effector and memory CD4+ and CD8+ αβ T cells, and γδ T cells express EGFP (Unutmaz et al., 2000). To evaluate EGFP expression in skin T cells, we analyzed single-cell suspensions prepared from separated epidermis and dermis of heterozygous CXCR6EGFP/+ (CXCR6EGFP) mice by flow cytometry (Fig. 5A). All DETCs, but not LCs, expressed high levels of EGFP (Fig. 5A). Similarly, all dermal γδ T cells were EGFP+ (Fig. 5A). In contrast, only a subset of αβ T cells (10–40%) expressed EGFP, thereby accounting for 5–15% of EGFP+ dermal cells. γδ T cells can express several chemokine receptors (Glatzel et al., 2002). However, unlike a role for CCR9 in tissue homing of γδ T cells, studies in CXCR6-deficient mice do not demonstrate an obvious phenotype (Unutmaz et al., 2000). Consistently, we did not find any difference in number or phenotype of γδ T cells in homozygous CXCR6EGFP/EGFP mice, indicating that this chemokine receptor is not required for the trafficking of these cells to the skin (Fig. S2).

Next, we investigated the distribution of EGFP+ T cells in the skin by performing multiphoton imaging of the ear skin of CXCR6EGFP mice (Fig. 5B). Using second harmonic generation signals, we were able to distinguish between the extracellular matrix–free epidermis and the collagen-rich dermis (blue
crossed the CXCR6EGFP mice onto a TCRβ knockout background lacking all αβ T cells (Fig. 5, C and D). Phenotype, density, and morphology of skin γδ T cells in these mice were identical to CXCR6EGFP mice (Fig. 5, C and D, and not depicted). These mice allowed us to further interrogate the migratory behavior of cutaneous γδ T cells using time-lapse in vivo multiphoton microscopy in ear skin. Similarly to DCs, DETCs were sessile (mean migratory velocity <1 µm/min; Fig. 5 E; Ng et al., 2008). Dermal EGFP+ γδ T cells exhibited variable migratory behavior, with some cells migrating and others remaining sessile (mean velocity of 2.0 ± 0.3 µm/min; Fig. 5 B). Vertical scans of the ear skin revealed a network of EGFP+ cells localized to the epidermis ~20 µm below the stratum corneum. These cells exhibited long dendrites, characteristic of DETCs (Bergstresser et al., 1983; Tschachler et al., 1983). In the superficial dermis, we detected numerous EGFP+ cells that were morphologically different to the DETCs (Fig. 5 B). These cells were mostly round or amoeboid in shape with no visible dendrites (Fig. 5 B). The overall density of DETCs was greater than that of dermal EGFP+ T cells (Fig. 5 B).

Because in these studies we were unable to distinguish between dermal EGFP-expressing γδ and αβ T cells, we

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**Figure 4.** Epidermal and dermal γδ T cells exhibit differential cytokine requirements for development/maintenance. (A) Flow cytometry profiles of cutaneous T cells isolated from WT, IL-15−/−, IL-7−/−, and IL-7Tg mice (n ≥ 3/group). Cells were gated on CD45+ CD3+. (B) Percentage of DETCs, dermal γδ T cells, and αβ T cells in the CD45+ leukocyte population in the ear skin of the various groups of mice. (C) Absolute numbers of DETCs, dermal γδ T cells, and αβ T cells in the ear skin of the various groups of mice. Data are representative of two to three independent experiments with at least three mice per group.
the case for γδ T cells, we analyzed their numbers in CCR7-deficient mice. Consistently, a significant increase in αβ T cell numbers was observed in the dermis of CCR7−/− mice compared with WT mice (Fig. 6). In contrast, the number of both DETCs and dermal γδ T cells remained unchanged (Fig. 6). In skin-draining LN, however, both αβ and γδ T cells were reduced in numbers, consistent with a requirement of circulating T cells for CCR7 to traffic to LNs (Fig. 6). These data suggest that, unlike memory αβ T cells, dermal γδ T cells do not exit the skin under homeostatic conditions.

### Dermal γδ T cell numbers are normal in CCR7-deficient animals

CCR7 is a crucial regulator of αβ T cell exit from peripheral tissues via migration into lymphatic vessels (Bromley et al., 2005; Debes et al., 2005). To determine whether this is also

### Antigen-specific CD4+ T cell priming is reduced in γδ T cell–deficient mice

Having identified and characterized a population of resident γδ T cells in the mouse dermis, we next sought to demonstrate
a role for these cells in immune responses. To do this, we made use of a *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) infection model because γδ T cells have been previously reported to play a role in immunity to mycobacteria (Hayday, 2009; Cua and Tato, 2010). As a readout, we measured the expansion of antigen-specific T cells in the retroauricular LNs after intradermal (i.d.) BCG infection of ear skin. WT and TCRΔδ/− mice were adoptively transferred with CFSE-labeled P25-TCR transgenic CD4+ T cells, which recognize an immunodominant epitope (peptide 25) from *Mycobacterium tuberculosis* antigen 85B (Tamura et al., 2004). 24 h later, mice were infected intradermally (i.d.) BCG infection of ear skin. WT and TCRΔδ/− mice were infected with a low dose of BCG (10^5 CFU). At days 5 and 6 after infection, BCG in TCRΔδ/− mice induced significantly lower expansion of CD44+CFSElo/+ P25 T cells as compared with WT mice (Fig. 7 A). Because TCRΔδ/− mice not only lack γδ T cells in the skin but in all organs, it remained possible that the observed difference in CD4+ T cell expansion was caused by the absence of γδ T cells in draining LN rather than in the skin. To address this question, we investigated the P25 T cell response to intraperitoneal BCG infection, in which dermal γδ T cells would not be involved. At day 6 after infection, no difference in the expansion of P25 CD4+ T cells was observed between WT and TCRΔδ/− mice (Fig. 7 B). These data suggested that cutaneous γδ T cells rather than circulating γδ T cells regulated the downstream CD4+ T cell response to skin challenge. Because in this model the pathogen was directly introduced into the dermis, and thus physically separated from DETCs, our data further suggest that dermal γδ T cells play the major, if not exclusive, role in this effect. In addition, we found a subtle but significant up-regulation of the early activation marker CD69 on dermal γδ T cells, but not DETCs, 24 h after BCG infection (Fig. S3).

**Early recruitment of neutrophils to sites of BCG infection is compromised in γδ T cell–deficient mice**

Several mechanisms may underlie the observed deficiency in T cell expansion in TCRΔδ/− mice. For example, γδ T cells may influence DC development and/or maturation. We therefore examined the frequencies and absolute numbers of conventional and migratory DCs in TCRΔδ/− mice, but observed no differences to WT controls (unpublished data). To test whether skin DCs could be adequately activated in the absence of γδ T cells, we analyzed the expression of co-stimulatory molecules by DCs after subcutaneous immunization with CFA. Expression of CD80 and CD86 by DC from both WT and TCRΔδ/− mice was comparable at day 3 after CFA administration (Fig. S4), indicating normal activation of these cells in the absence of γδ T cells.

Some subsets of γδ T cells are potent producers of the proinflammatory cytokine IL-17 and, based on this function, have been implicated in the recruitment of neutrophils in response to bacterial pathogens including *E. coli* and *Staphylococcus aureus* (Shibata et al., 2007; Cho et al., 2010; Cua and Tato, 2010). Given that cutaneous γδ T cells are well positioned to respond rapidly to skin invading pathogens, we examined whether these cells were capable of producing IL-17 after i.d. infection with BCG. As shown in Fig. 8 (A and B), ~6% of dermal γδ T cells secreted IL-17 rapidly after i.d. BCG inoculation, whereas both DETCs and αβ T cells exhibited very low IL-17 production.

Next, we determined neutrophil infiltration in the skin after i.d. infection with BCG in WT and TCRΔδ/− mice. Consistent with previously reported findings (Abadie et al., 2005), we observed a rapid infiltration of Gr1+ neutrophils in the skin of WT mice after infection with BCG, which reached a peak at 24 h after infection (Fig. 9, A and B). Confocal imaging of whole-mount dermal sheets revealed that Gr1+ neutrophils clustered around the i.d. BCG deposit, and were frequently found to contain intracellular mCherry-tagged bacteria (Fig. 9 A). Importantly, BCG-containing neutrophils were detected in dermal lymphatic vessels, indicating their role in bacteria transport to draining LN (Fig. 9 A; Abadie et al., 2005). Using flow cytometry, we determined that neutrophils were the predominant leukocyte population (83 ± 1.8%) to have internalized BCG at this early time point (Fig. 9 A), whereas the rest of BCG-containing cells were MHC IIlo cells, which are most likely macrophages. DDCs represented only a minor population of cells to pick up BCG (~1%).

When we then compared the recruitment of neutrophils in BCG-infected TCRΔδ/− mice, we observed a significant decrease in the number of neutrophils accumulating in the skin (Fig. 6).
skin of TCRδ−/− mice at 24 h after infection compared with WT controls (Fig. 9 C). This decrease was not caused by differences in the number of neutrophils in circulation in WT and TCRδ−/− mice in the steady-state (unpublished data). Importantly, using quantitative RT-PCR, we found a significantly lower amount of BCG in the draining LN of TCRδ−/− mice at day 3 after infection compared with WT controls (Fig. 9 D), indicating impaired antigen transport via lymphatic vessels. Together, these data show that dermal γδ T cells are the predominant source of IL-17 in the skin after BCG infection and that these cells regulate the composition of the cutaneous inflammatory infiltrate under these conditions.

DISCUSSION

The skin represents the outermost defense against microbial and environmental insults. The importance of cutaneous integrity is emphasized by the multitude of strategies that counteract pathogen invasion, including the physical barrier provided by keratinocytes, the production of antimicrobial...
mediators by skin-resident cells, and the localization of immune cells in the different layers of the skin. It has become increasingly clear that epidermis and dermis do not merely represent anatomically distinct compartments, but rather directly and specifically shape the immune milieu to support the activities of resident leukocytes. In this study, we have identified an abundant population of resident dermal γδ T cells that displays a unique phenotypic profile, survival requirements, and migratory behavior as compared with its epidermal and systemic counterparts. Our results thus expand on the concept of microcompartamental specialization of distinct immune cell subsets within the skin. They further emphasize the need to define the function of dermal γδ T cells in skin immunity and for the study of the environmental and molecular cues that regulate these cells during homeostatic and inflammatory conditions.

It is quite surprising that dermal γδ T cells in the mouse have received little or no attention in the past, given that DETCs have served as a paradigm for studying γδ T cell biology. Nakagawa et al. (1993) reported on the migration of Vγ5− γδ T cells from whole-skin explants of mice, but did not further investigate their original localization. Tamaki et al. (1996) identified Thy-1+ dermal cells of dendritic shape that expressed Vγ5; however, these cells also contained melanosomes and therefore may have represented autofluorescent dermal macrophages. In mice with transgenic IL-7 overexpression in basal keratinocytes, increased numbers of cutaneous γδ and αβ T cells were observed (Williams et al., 1997). The authors speculated that this increase may have been caused by an expansion of precursor cells present within the dermis, but did not characterize these cells any further. More recently, Kisielow et al. (2008) reported on the presence of

![Figure 9](https://example.com/figure9)

**Figure 9.** Neutrophil recruitment to the site of BCG infection is compromised in the absence of IL-17-producing γδ T cells. (A, top left) Single-plane image from a dermal whole-mount stain depicting Gr1+ neutrophils at the site of BCG infection in ear skin of WT mice 24 h after infection. Bar, 500 µm. (top right) Higher magnification of the boxed area in the top left panel depicting Gr1+ neutrophils around a deposit of BCG. Arrows point to BCG-containing Gr1+ neutrophils. Boxed inset shows a single Gr1+ neutrophil containing intracellular BCG bacilli. Bar, 50 µm. (bottom left) Extended focus image from a dermal whole-mount stain 36 h after BCG infection depicting BCG-incorporated Gr1+ neutrophils (arrows) in the lumen of a lymphatic vessel stained with anti-LYVE-1. Bars, 25 µm. (bottom right) Flow cytometry profiles of CD45+ leukocytes isolated from WT ear skin, 24 h after i.d. infection with BCG-mCherry. CD45+ BCG-mCherry+ cells were further evaluated for Ly6G and MHC II expression. Data are representative of two to four independent experiments. (B) Flow cytometry profiles of Gr1+ cells infiltrating the skin of WT mice (n = 4), 24 h after i.d. injection with BCG or PBS. Cells were gated on CD45+ CD3−. Data are representative of 3 independent experiments. (C) Absolute cell number of Gr1+ neutrophils isolated from ear skin of WT and TCRδ−/− mice (n = 14–17 per group), 24 h after i.d. infection with BCG. Data are representative of three independent experiments. (D) Copy number of 16S rRNA from BCG isolated from auricular draining LN of WT and TCRδ−/− mice (n = 5 per group), 3 d after i.d. infection. Data are representative of two independent experiments.
T cells and MHC II+ cells have previously been found in the SCART2+ Vγ5− γδ T cells in the dermis. However, no further phenotypic or functional characterization of the dermal population was performed. Human dermis has been reported to contain an oligoclonal population of γδ T cells that differs in their TCR repertoire from circulating γδ T cells (Bos et al., 1990; Foster et al., 1990; Holtmeier et al., 2001). Our data support the concept of oligoclonality demonstrated by the broader repertoire of TCRs expressed by murine dermal γδ T cells as compared with DETCs, suggesting that they are capable of responding to a more diverse spectrum of antigens. Therefore, the predominant function of DETCs may be their role as “lymphoid stress-sensors” (Hayday, 2009), that is the recognition of self-encoded stress molecules on keratinocytes upon epithelial disturbance such as trauma or epidermal infection. Dermal γδ T cells on the other hand, may act as direct pathogen sensors that rapidly respond to foreign antigens upon their introduction into the dermis. The immediate production of proinflammatory cytokines, most prominently IL-17, then triggers an inflammatory cascade to facilitate clearance of the pathogen. Clearly, future studies will need to further dissect the role individual γδ T cell subpopulations play after the introduction of pathogens, including bacteria, fungi or viruses, into the epithelium or dermis.

Another novel finding in this study is the capacity of dermal γδ T cells to proliferate in situ. Strikingly, in chimeric mice, 80% of these cells were still host derived even 19 mo after bone marrow reconstitution, indicating that the dermal γδ T cell population is largely self-renewing under homeostatic conditions. These data contrast with the paradigm of cutaneous αβ T cell immunosurveillance, which has traditionally been believed to occur by means of continuous trafficking of circulating cells through the skin. Thus, antigen-experienced αβ T cells expressing skin homing receptors, such as E-selectin ligands, enter the skin and, in the absence of antigen, exit in a CCR7-dependent manner via lymphatics (Yoshida et al, 1998; Bromley et al., 2005; Debès et al., 2005). Consistently, we found that cutaneous αβ T cells are replaced in bone marrow chimeras, indicating different survival signals for dermal αβ and γδ T cells. The difference in screening behavior of the two T cell subsets is further corroborated by the fact that dermal γδ T cells exhibited very little interstitial migration. Although we did not visualize dermal αβ T cells in our system, previous studies have shown that naive αβ T cells in the LN and effector/memory T cells in peripheral tissues, including the skin, migrate at much higher velocities (~10 μm/min) in the absence of cognate signals than dermal γδ T cells (Miller et al., 2002; Mrass et al., 2006; Matheu et al., 2008). This difference may be at least partly accounted for by the observation that one third of dermal γδ T cells were in close contact with MHC II+ cells. Interactions between γδ T cells and MHC II+ cells have previously been found in the lungs (Wands et al., 2005). Although we do not know the molecular basis of these interactions, it is conceivable that dermal γδ T cells recognize self-antigens on APCs. In this context, it is interesting to note that TCRδ−/− mice develop spontaneous dermatitis, at least on certain genetic backgrounds (Girardi et al., 2002). Thus, the APC–γδ T cell crosstalk in the dermis under noninflammatory conditions may serve an immunoregulatory function.

At the functional level, we have found that cutaneous γδ T cells regulate the downstream CD4+ T cell response to i.d. BCG infection. These results are in line with previous studies demonstrating that γδ T cells are involved in protective immunity against mycobacterial infections (Hayday, 2009; Cua and Tato, 2010). Importantly, our experiments suggest that dermal γδ T cells are the predominant source of IL-17 in the skin, whereas DETCs displayed negligible production. Our findings are somewhat in contrast with a recent study that demonstrated IL-17 production by DETCs in the skin after S. aureus infection (Cho et al., 2010). Despite the fact that S. aureus was applied i.d., dermal cells produced considerably less IL-17 than epidermal cells. However, this study did not use single-cell labeling, but rather determined IL-17 levels in the entire CD3+ dermal and epidermal populations after in vitro restimulation for 24 h. In our experience, dermal γδ T cells die within a few hours after their isolation, which may be a confounding factor for the analysis of culture supernatants. Finally, it is possible that Gram-positive cocci and mycobacteria induce different response programs in dermal γδ T cells. In sum, we show that dermal γδ T cells resemble the previously described subset of “innate” γδ T cells defined by their production of IL-17 (Jensen et al., 2008; Shibata et al., 2008; Ribot et al., 2009). Future studies will focus on determining the full profile of cytokines, chemokines, and other mediators produced by these cells in response to pathogens.

IL-17 has been implicated in the recruitment of neutrophils to sites of infection and inflammation (Kolls and Lindén, 2004; Lindén et al., 2005; Cua and Tato, 2010), and our data showed that neutrophil recruitment was decreased in TCRδ−/− mice in response to i.d. BCG infection. Neutrophils have previously been shown to be the first blood-borne cells to arrive at the site of dermal BCG infection in mice, where they subsequently “pick up” and shuttle live bacilli to the skin-draining LN (Abadie et al., 2005). Because DC numbers and phenotype were identical in WT and TCRδ−/− mice, a likely mechanism for the reduced priming of mycobacteria-specific CD4+ T cells in TCRδ−/− mice is the decreased availability of antigen in LNs caused by decreased neutrophil trafficking. In addition, because IL-17 also activates neutrophils, the reduced availability of this cytokine may also influence the phagocytic capacity of these cells. Indeed, BCG was much less abundant in the draining LN of TCRδ−/− mice, most likely relating to decreased antigen transport by neutrophils via dermal lymphatic vessels. Future studies will evaluate the distribution of fluorescently tagged BCG in draining LNs, as well as the capacity of DCs to activate antigen-specific T cells.

Collectively, dermal γδ T cells emerge as new players in the regulation of skin homeostasis and immunity. Although we have identified one of their functions, namely the response to mycobacterial infection, it remains to be determined whether they fulfill roles similar to those ascribed to DETCs.
during wound repair and surveillance for stressed and tumor cells. Nevertheless, it is conceivable that some of the activities previously attributed to DETCs may in fact be performed by their dermal cousins.

MATERIALS AND METHODS

Mice. C57BL/6 (WT) and B6.SJL/Ptpcr (CD45.1) mice were purchased from the Animal Research Centre (Perth, Australia). Peptide-25 TCR transgenic (P25 TCR-Tg) mice have been described previously (Tamura et al., 2004). IL-7Tg mice, were bred and maintained in specific pathogen-free conditions at the Centenary Institute animal facility. All animal experiments were performed in accordance with the Animal Ethics Committee at the University of Sydney.

Generation of bone marrow chimeras. To create bone marrow chimeras, C57BL/6 (CD45.2) hosts were lethally irradiated with 1,200 Gy split-dose irradiation (2 doses of 600 Gy each, 4 h apart). 24 h later, the irradiated hosts received 8–10 × 10⁶ bone marrow cells from B6.SJL/Ptpcr (CD45.1) donor mice intravenously via the tail vein. Chimeric mice were given a course of antibiotics in sterile drinking water for 3 wk after irradiation to prevent infections. All chimeric mice were allowed to reconstitute for at least 3 mo before use in experiments.

Tissue processing and flow cytometry. Isolation of skin cells involved the following process. Ears were split into dorsal and ventral halves using forceps and subjected to enzymatic digestion as follows: first step, 5 U/ml dispase I (BD) in PBS for 90 min at 37°C to allow for epidermal and dermal separation; second step, 2 mg/ml collagenase type IV (Sigma-Aldrich) in PBS for 45 min at 37°C to release cells. In certain experiments where cell surface markers of interest were dispase sensitive and would potentially be cleaved, ears were only digested with 2 mg/ml collagenase type IV in PBS for 60 min at 37°C. To obtain single-cell suspensions, the tissues were filtered through an 80-µm stainless steel mesh. Isolation of leukocytes from the spleen and LN involved teasing the organs apart, followed by filtering through an 80-µm stainless steel mesh to obtain single-cell suspensions.

Cell suspensions were incubated with anti-CD16/32 (2.4G2; BD) to block Fc receptors and stained with fluorochrome-conjugated antibodies (purchased from BD, eBioscience, or BioLegend) against the following cell surface molecules were used: CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD25 (PC61), CD43 (S7), CD44 (IM7), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CD69 (H1.2F3), CD80 (16-10A1), CD86 (GL1), CD103 (2E7), CD127 (A7R34), B220 (RA3-6B2), Gr1 (RB6-8C5), Ly6G (1A8), MHC II (M5/114), TCRβ (H57-597), TCRγδ (GL3), Vγ5 (S36), and Vγ4 (UC3-10A6). After staining, cell suspensions were resuspended in FACS buffer containing 0.5 µg/ml DAPI (Invitrogen) for dead cell exclusion. For intracellular anti-BrdU staining, samples were first stained for cell surface molecules and aquafluorescent reactive dye (Invitrogen) for dead cell exclusion after fixation. Samples were acquired using an LSR-II flow cytometer (BD) and data were analyzed using FlowJo software (Tree Star, Inc.).

BrdU labeling in vivo. Mice were injected i.p. with 1 mg BrdU (BD) to block Fc receptors and stained with fluorochrome-conjugated antibodies diluted in “FACS buffer” (PBS containing 5% FCS, 2 mM EDTA, and 0.02% sodium azide). Fluorochrome-conjugated antibodies (purchased from BD, eBioscience, or BioLegend) against the following cell surface molecules were used: CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD25 (PC61), CD43 (S7), CD44 (IM7), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CD69 (H1.2F3), CD80 (16-10A1), CD86 (GL1), CD103 (2E7), CD127 (A7R34), B220 (RA3-6B2), Gr1 (RB6-8C5), Ly6G (1A8), MHC II (M5/114), TCRβ (H57-597), TCRγδ (GL3), Vγ5 (S36), and Vγ4 (UC3-10A6). After staining, cell suspensions were resuspended in FACS buffer containing 0.5 µg/ml DAPI (Invitrogen) for dead cell exclusion. For intracellular anti-BrdU staining, samples were first stained for cell surface molecules and aquafluorescent reactive dye (Invitrogen) for dead cell exclusion after fixation. Samples were acquired using an LSR-II flow cytometer (BD) and data were analyzed using FlowJo software (Tree Star, Inc.).

BrdU labeling in vivo. Mice were injected i.p. with 1 mg BrdU (BD) to block its immediate availability, and then given 0.8 mg/ml BrdU in sterile drinking water that was changed daily. Epidermal and dermal cell suspensions were prepared at day 6 after initial BrdU administration. Cell suspensions were stained for surface markers as described in the previous section, and BrdU+ cells were identified using the BrdU Flow kit (BD) according to the manufacturer’s protocol.

P25-TCR transgenic CD4+ T cell isolation, labeling, and adoptive transfer. Spleens and LN were harvested from P25-TCR transgenic mice and single-cell suspensions were obtained. Erythrocytes were osmotically lysed in NH4Cl, and the cells were washed twice in RPMI 1640 (Invitrogen) supplemented with 10% FCS. CD4+ T cells were magnetically purified by negative selection using PE-conjugated anti-B220 (RA3-6B2), CD11b (M1/70), CD11c (HL3), NK1.1 (PK136), and TCRβγ (GL3) antibodies (BD or eBioscience) and anti-PE immunomagnetic beads (Miltenyi Biotec). CD4+ T cell purity was routinely >80% as assessed by flow cytometry. For proliferation assays, purified T cells were labeled with CFSE (Invitrogen) by resuspending the P25-TCR transgenic T cells in serum-free RPMI 1640 containing 5 µM CFSE. Cells were incubated with CFSE for 10 min at 37°C with intermittent mixing every 3–4 min. Labeling was stopped by adding ice-cold FCS and the cells were washed twice in RPMI 1640 with 10% FCS. WT and TCRβγ mice host received 5 × 10⁶ CFSE-labeled P25-TCR transgenic CD4+ T cells intravenously via the tail vein.

BCG generation and infection. Fluorescently tagged BCG was generated as described previously (Tricas et al., 1999). Mice were anesthetized by i.p. injection of Ketamine/Xylazine (80/10 mg/kg), and 10⁵ CFU of BCG (in 2 µl saline) was injected i.d. into the pinnae of ears using a 35-gauge Hamilton syringe as described previously (Ng et al., 2008). For systemic infection, 10⁵ CFU of BCG (in 100 µl saline) was injected i.p. Ears were harvested at various time points after infection and analyzed by flow cytometry or confocal microscopy. Mice that received CFSE-labeled P25-TCR transgenic CD4+ T cells were infected 24 h after adoptive transfer of cells. On day 5 or after infection, organs were harvested and analyzed by flow cytometry.

Cytokine secretion assay. 24 h after i.d. BCG (10⁵ CFU) infection, epidermal and dermal cells isolated from ear skin of mice (as described in Tissue processing and flow cytometry) were further stimulated ex vivo by culturing in RPMI 1640 containing 10% FCS, PMA (100 ng/ml), and Ionomycin (500 ng/ml) for 2 h at 37°C. IL-17 secretion by epidermal and dermal cells was analyzed using the Cytokine Secretion Assay Detection kit (Milenyi Biotec) according to the manufacturer’s protocol. Cell suspensions were then stained for surface markers as described above and analyzed by flow cytometry.

Quantitation of bacterial gene expression. WT and TCRβγ mice were infected i.d. with 10⁵ CFU BCG, and 3 d after infection auricular draining LN was isolated and resuspended in TRIzol (Invitrogen). Cells were then disrupted by a Mini-Beadbeater-16 with 0.1 mm zirconia/silica beads (BioSpec Products). Homogenates were extracted with chloroform/isooamyl alcohol, and the pellet was resuspended in 100 µl of DEPC-treated water (Invitrogen). The solution was treated with TURBO DNase according to the manufacturer’s instructions (Applied Biosystems), and RNA was additionally purified by treatment with phenol/chloroform/isooamyl alcohol. Total RNA was dissolved in 20 µl of DEPC-treated water and stored at −80°C until needed.

For quantitation of M. tuberculosis gene expression, cDNA was prepared by reverse transcribing 1 µg of total RNA using a gene-specific primer (5’-GCCCGACGCTTACAGGTAAG-3’) for the mycobacterial housekeeping gene 16S rRNA encoded by M. tuberculosis rrs (5’-GCCCGACGCTTACAGGTAAG-3’) and Superscript III reverse transcription (Invitrogen) according to the manufacturer’s recommendations. The number of amplicons was measured by real-time PCR using the following primer pair: rrs forward 5’-AGGCCAGCAGTTGGGAATA-3’; rrs reverse 5’-CTACCGTCAATCCCAGAGAAG-3’. 2 µl of cDNA, 62.5 µl SYBR green 1 PCR Master Mix (QIAGEN), and 5 µl of the rrs primer pair was added to each reaction to a total volume of 12.5 µl with DEPC-treated water. The reaction profile consisted of 30°C for 2 min and 95°C for 2 min, followed by 80 cycles at 95°C for 15 s, 55°C for 15 s, and 72°C for 15 s. PCR reactions were run on a Rotor-Gene 6000-series sequence detector (Rotor-gene; Corbett Life Science) and were performed in duplicate for each sample. 10-fold dilutions of known amounts of plasmid DNA encoding M. tuberculosis rrs (7.2 × 10³ to 7.2 × 10⁸ copies) were used to generate a standard curve. The copy number in each sample was calculated according to the formula N = (Ct − b)/m,

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where \( N \) is the copy number, \( b \) is the intercept, and \( m \) is the slope of the standard curve line.

Confocal microscopy of mouse ear dermis. Epidermis and dermis were separated as described in Tissue processing and flow cytometry, and the dermal half was fixed in 4% paraformaldehyde/10% sucrose for 30 min at room temperature. Fixed tissue was washed in PBS and blocked in 5% FCS in PBS for 2 h at room temperature. In some experiments, dermal tissue was treated with EDTA and Triton X as described previously and analyzed by flow cytometry. Agitation. Single cell suspensions were obtained and stained for surface markers.

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