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Short title: Dermal γδ T cells enhance CD8+ T cell response
Abstract

A large number of $\gamma\delta$ T cells are located within epithelial tissues including the skin. In mice, epidermal and dermal $\gamma\delta$ T cells consist of distinct subsets and play specific roles in cutaneous immune responses. A recent study demonstrated that $\gamma\delta$ T cells and cutaneous dendritic cells migrate from the skin to the draining lymph nodes (LN). However, it remains unclear whether they regulate the antigen-specific immune response within the LN. Herein, we investigated their properties and role in the LN using the *Mycobacterium bovis* bacille Calmette-Guérin (BCG) infection model. In vivo cell labeling analysis revealed that the most of migratory subset was dermal $V\gamma 4^+$ cells. This population transmigrated from the skin to the LN in a Gi-coupled chemokine receptor-independent manner. By depleting $V\gamma 4^+$ cells, the intranodal expansion of CD8$^+$ T cell against BCG was significantly attenuated. In addition, *in vitro* analysis revealed that $V\gamma 4^+$ cells produced TNF-$\alpha$ and enhanced IL-12 production by dendritic cells. Taken together, these findings suggest that dermal $V\gamma 4^+$ cells are a unique subset that possesses a migratory potency to the skin-draining LN and enhances the dendritic cell function therein.
Introduction

Gamma delta T cells (γδ T cells) are a minor subset of T cells but are the major T cell population in epithelial tissues, including the skin (Hayday, 2000). In contrast to αβ T cells, γδ T cells show less T cell receptor (TCR) diversity and appear to respond to self-molecules that belong to danger signals (Takagaki et al., 1989). Many researchers believe that γδ T cells function in an innate manner. On activation, γδ T cells produce a large amount of inflammatory molecules, such as granulocyte-macrophage colony stimulating factor (GM-CSF), interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, and participate in cutaneous immune surveillance (Macleod and Havran, 2011).

In mice, the skin contains at least three subsets of γδ T cells: epidermal γδ T cells (also known as dendritic epidermal T cells [DETCs]), dermal Vγ4+ cells, and dermal Vγ4− cells (Sumaria et al., 2011). Recent studies emphasize that each subset plays distinct roles in cutaneous immune responses. Strid et al. reported that DETCs secreted interleukin (IL)-13 on activation and are involved in the initiation of cutaneous T helper 2 (Th2)-type responses (Strid et al., 2011). DETCs also play an immune-regulatory role in irritant contact dermatitis and allergic contact dermatitis (Girardi et al., 2002). On the other hand, dermal γδ T cells are known as a main source of IL-17 in mycobacterium infections (Sumaria et al., 2011) and in psoriasiform dermatitis models (Cai et al., 2011; Mabuchi et al., 2011; Yoshiki et al., 2014). These studies suggest that γδ T cells belong “in between” the innate and adaptive immune systems and can modulate acquired immune responses.

Recently, we demonstrated that some αβ T cells in the skin and cutaneous dendritic cells (DCs) migrate to the draining lymph nodes (LNs) and modulate immune events therein (Tomura et al., 2010). This observation suggests that the circulation of immune cells between the skin and the draining LNs is a key mechanism for the modulation of cutaneous immunity. As for γδ T cells, Gray et al. reported that CCR6+ γδ T cells migrated from the skin to the draining LNs in imiquimod-induced skin inflammation (Gray et al., 2013). However, the function of the migratory subset of γδ T cells remains undetermined.

In the present study, we examined the properties of a migratory subset of γδ T cells in the Mycobacterium bovis bacille Calmette-Guérin (BCG) infection model. Dermal γδ T
cells are important for host defense against cutaneous BCG infection (Sumaria et al., 2011). We revealed that dermal Vγ4+ cells are a unique subset that possesses a migratory potency to the draining LNs and modulate immune responses against BCG infection.

Results

Dermal γδ T cells migrate from the skin to the draining LNs

We first analyzed the kinetics of cutaneous γδ T cells that migrated from the skin to the draining LNs. To track cell migration, we used Kaede-transgenic (tg) mice that expressed a photo-convertible Kaede protein throughout the body (Tomura et al., 2010). Before photoconversion, all cutaneous cells in Kaede-tg mice expressed green fluorescence (Kaede-green) (Fig.1a, left panel). Upon violet light exposure to the skin, all cutaneous cells immediately turned their fluorescence to red (Kaede-red) (Fig.1a, right panel). It should be noted that no detectable skin inflammation was induced by the violet light exposure (Tomura et al., 2010).

Twenty-four hours after the violet light exposure to the footpad, the draining popliteal LNs and non-draining cervical LNs were harvested. In draining LNs, 13.8 ± 2.1% of CD11c+ DCs and 5.3 ± 0.4% of γδ TCR+ cells expressed Kaede-red (Fig. 1b), suggesting that these cells migrated from the skin. Almost no Kaede-red+ cells were found in the non-draining LNs (< 0.1 % of γδ T cells expressed Kaede-red).

We then sought to examine the migratory kinetics of cutaneous γδ T cells in the inflammatory condition. It is well known that γδ T cells play an important role in the surveillance of mycobacterial infection (Belmant et al., 1999). We inoculated Mycobacterium bovis BCG to the footpad and exposed to the violet light. The numbers of Kaede-red+ γδ T cells and Kaede-red+ DCs were significantly increased in the draining LNs after BCG infection (Fig. 1c). These results suggest that cutaneous γδ T cells constantly migrate from the skin to the draining LNs and their migration is enhanced upon BCG infection.

To clarify the γδ T cell migratory ability in other skin inflammation models, we next evaluated the γδ T cell migration in contact hypersensitivity model with
The numbers of Kaede-red$^+$ $\gamma\delta$ T cells were increased after the challenge of DNFB (Supplementary Fig.1). This result suggests that $\gamma\delta$ T cells migration was enhanced not only in the BCG infection but also in other skin inflammation, such as contact hypersensitivity.

**Cutaneous $\gamma\delta$ T cell migration to the draining LNs is independent of Gi-coupled chemokine receptors**

Next, we investigated the mechanism of the cutaneous $\gamma\delta$ T cell migration to the draining LNs. Previous studies have shown that cutaneous DCs and $\alpha\beta$ T cells migrated to the draining LNs in a CCR7-dependent manner (Bromley et al., 2013; Randolph et al., 2008). To determine the CCR7-dependency of cutaneous $\gamma\delta$ T cell migration, we mated Kaede-tg mice with CCR7-deficient mice. As previously reported (Bromley et al., 2013), Kaede-red$^+$ CD11c$^+$ DCs were almost absent in the skin-draining LNs of CCR7-deficient mice (Fig. 2a, b). In contrast, the percentage of Kaede-red$^+$ $\gamma\delta$ T cells in the draining LNs was comparable irrespective of CCR7-deficiency (Fig. 2a, b).

Consistent with this observation, CCR7 expression was absent in cutaneous $\gamma\delta$ T cells and Kaede-red$^+$ skin-derived $\gamma\delta$ T cells (Fig. 2c).

We next examined the involvement of the other Gi-coupled chemokine receptors. With subcutaneous injection of pertussis toxin (PTX), a specific Gi inhibitor, Kaede-red$^+$ DCs in the draining LNs were significantly decreased, whereas the $\gamma\delta$ T cell migration to the draining LNs was not affected (Fig. 2d, e). Taken together, these results suggest that the dermal $\gamma\delta$ T cell migration toward the LNs is independent of Gi-coupled chemokine receptors, including CCR7.

**The migratory subset is exclusively $V\gamma4^+$ dermal $\gamma\delta$ T cells**

To further characterize the migratory property of cutaneous $\gamma\delta$ T cells, we examined which $\gamma\delta$ T cell subset participates in the migratory population. In the skin, all DETCs in the epidermis are $V\gamma5^+$, and dermal $\gamma\delta$ T cells consist of $V\gamma4^+$, $V\gamma5^+$ and $V\gamma4-V\gamma5^-$ subpopulations (Sumaria et al., 2011). Intriguingly, we found that most of Kaede-red$^+$ $\gamma\delta$ T cells in the draining LNs expressed $V\gamma4$, but not $V\gamma5$, in the steady state and after the BCG infection (Fig. 3a). This result suggests that $V\gamma4^+$ dermal $\gamma\delta$ T cells, but not
DETCs possess a capacity to migrate to the draining LNs. Thus, we focused on the $V_\gamma 4^+$ cells and examined what percentage of $V_\gamma 4^+$ cells in the skin-draining LNs were of skin-origin. Twenty-four hours after photoconversion of the footpad, Kaede-red$^+$ cells accounted for 14.5 $\pm$ 5.9% among $V_\gamma 4^+$ cells in the popliteal LNs (Fig. 3b).

We then compared the surface markers of $V_\gamma 4^+$ cells in the dermis and in the skin-draining LNs. As previously reported, dermal $V_\gamma 4^+$ cells expressed CCR6 and an E-cadherin ligand CD103 (Gray et al., 2011; Sumaria et al., 2011) (Fig. 3c). In the skin-draining LNs, one third of $V_\gamma 4^+$ cells were CCR6$^+$CD103$^+$ (Fig. 3d) and the majority of Kaede-red$^+$ cells (89.6 $\pm$ 3.1%) belonged to this population (Fig. 3e, f). In turn, Kaede-red$^+$ cells accounted for 33.6 $\pm$ 12.0% of CCR6$^+$CD103$^+$V$\gamma 4^+$ cells in the popliteal LNs 24 h after photoconversion of the footpad (Supplementary Fig. 2a). To evaluate the turnover kinetics of $V_\gamma 4^+$ cells in the LNs, we photoconverted the inguinal LNs. Twenty-four hours later, Kaede-red$^+$ cells accounted for 50.7 $\pm$ 9.1% in CCR6$^+$CD103$^+$ V$\gamma 4^+$ cells (Supplementary Fig. 2b), suggesting that half of this population was retained in the LNs and the other half was replenished in 24 h. Taken together, these results suggest that the majority of CCR6$^+$CD103$^+$ V$\gamma 4^+$ cells in skin-draining LNs were of skin-origin. In line with this observation, CCR6$^+$CD103$^+$ V$\gamma 4^+$ cells were a minor population in the spleen (Fig. 3f).

Administration of anti-$V_\gamma 4$ TCR depleting antibody suppressed CD8$^+$ T cell response against BCG infection

We next sought to examine the function of $V_\gamma 4^+$ cells in the draining LNs. We evaluated IL-17A and TNF-α expression because dermal $\gamma \delta$ T cells produced these inflammatory cytokines upon activation (Supplementary Fig. 3)(Conti et al., 2005; Gray et al., 2013). We found that IL-17A and TNF-α were predominantly expressed by intranodal CCR6$^+$CD103$^+$ V$\gamma 4^+$ cells in the steady state. (Fig. 4a) These results suggest that skin-derived V$\gamma 4^+$ cells are an important source of IL-17A and TNF-α in the draining LNs as well as in the skin.

Next, we examined the role of $V_\gamma 4^+$ cells in the intranodal proliferation of antigen-specific CD8$^+$ T cells, because CD8$^+$ T cells play a pivotal role in the protection
against mycobacterium infection (Winau et al., 2006). Neutralizing anti-Vγ4 antibody- or control antibody-treated mice (Supplementary Fig. 4) (Hahn et al., 2004) were transferred with OT-I tg CD8+ T cells, which specifically recognizes processed peptide of the ovalbumin protein (OVA). These cells were labeled with Cell Trace Violet (CTV) and their proliferation was evaluated via CTV dilution. OVA-expressing BCG (BCG-OVA) was then inoculated. Depletion of Vγ4+ cells significantly reduced the proliferation of OT-I tg CD8+ T cells in the draining LNs (Fig. 4b). These results suggest that Vγ4+ cells are important for the intranodal activation and expansion of antigen-specific CD8+ T cells.

Vγ4+ cells stimulate antigen-specific CD8+ T cell differentiation via modulating DC functions

Immunohistochemical analysis of the draining LNs revealed that γδ T cells, including Kaede-red+ γδ T cells, were distributed in the interfollicular T cell zone (Fig. 5a and Supplementary Fig. 5a), wherein DCs interact with naïve T cells. This observation raised the possibility that skin-derived Vγ4+ cells influence αβ T cells activation via modulating DC functions in the LNs. In fact, such an immunomodulation between γδ T cells and DCs was reported in a human in vitro study (Conti et al., 2005). We therefore compared the properties of intranodal DCs in BCG-infected mice with neutralizing anti-Vγ4 antibody- or control antibody-treatment. No significant difference was observed in the number and co-stimulatory molecule expressions, such as CD80 and CD86, on DCs under a deficiency of Vγ4+ cells (Supplementary Fig. 5b, c), suggesting that Vγ4+ cells contribute little, if any, to the migration and activation of skin-derived DCs.

We next compared the T cell stimulatory properties of intranodal DCs. DCs produce IL-12 that drives CD8+ T cell IFN-γ production and differentiation during pathogen infection (Wilson et al., 2008). DCs were isolated from the skin-draining LNs of anti-Vγ4- or control antibody-treated mice 3 days after BCG-OVA infection. These cells were co-cultured with CTV-labeled OT-I tg cells. We observed significant attenuation of OT-I tg cell proliferation (Fig. 5b) and impaired production of IFN-γ and IL-12p40 (Fig. 5c, d) with depletion of Vγ4+ cells. These results indicate that Vγ4+ γδ T cells play an
essential role during the antigen-specific CD8+ T cell response against BCG infection
possibly via modulating DC functions.

**Vγ4**+ cells stimulate bone marrow-derived DCs to produce IL-12

To further characterize the immune modulation between Vγ4+ cells and DCs, we
isolated CD4+ and Vγ4+ cells from naïve mice and co-cultured them with bone
marrow-derived DCs (BMDCs). In the presence of Vγ4+ T cells, BMDCs produced a
higher amount of IL-12 p40 than with the same number CD4+ cells (Fig. 6a, b and
Supplementary Fig. 6), suggesting that Vγ4+ γδ T cells have the potential to activate
DCs.

As mentioned in Fig. 4a, skin derived CCR6+CD103+Vγ4+ cells produced a
substantial amount of IL-17A and TNF-α. IL-17A and TNF-α are important for DC
activation (Papadakis and Targan, 2000; Sutton et al., 2009). Thus, we examined
whether IL-17A and/or TNF-α from Vγ4+ cells activated DCs. We co-cultured BMDCs
with Vγ4+ cells in the presence or absence of neutralizing antibody against IL-17A or
TNF-α. We found that IL-12 p40 production by BMDCs was not attenuated by the
blockade of IL-17A, but was significantly inhibited by the neutralization of TNF-α (Fig.
6c). Next, we checked the *in vivo* TNF-α production in the skin draining LNs after BCG
infection. We also found that the number of Vγ4+ cells producing TNF-α was much
higher than that of DCs producing TNF-α in the draining LNs upon BCG infection (Fig.
6d). These results suggest that Vγ4+ cells stimulate intranodal DCs to produce IL-12p40
via producing TNF-α.

**Discussion**

In this study, we identify a novel function of dermal γδ T cells that migrate to the
draining LNs. Vγ4+ dermal γδ T cells egressed from the skin to the draining LNs in a
Gi-coupled receptor independent manner, and produced IL-17A and TNF-α therein.
Following BCG infection, Vγ4+ γδ T cells enhanced CD8+ T cell activation in the
draining LNs. In addition, Vγ4+ cells led to a significant up-regulation of IL-12
production by DCs through a TNF-α-dependent mechanism.
Our previous study showed that cutaneous αβ T cell migration to the LNs was enhanced upon contact hypersensitivity (Tomura et al., 2010). Herein we have demonstrated that Vγ4+ dermal γδ T cell migration to the LNs is enhanced upon BCG infection. Therefore, cutaneous T cells, as well as DCs, have the potential to accumulate in the LNs, particularly after cutaneous inflammations. The skin-derived regulatory T cells represent a stronger immune regulatory potential than LN-resident populations and play an important role in the resolution of cutaneous inflammation (Tomura et al., 2010). Taken together, our findings suggest that skin-derived T cell is highly-activated in nature and the skin might be an important organ as a site for T cells activation.

The distribution of immune cells is directly affected by the signaling from Gi-coupled chemokine receptors. In cutaneous DCs and αβ T cells, their migration toward the draining LNs is largely dependent on CCR7 (Bromley et al., 2013). Analysis of skin-draining lymph has demonstrated that bovine γδ T cells migrated from the skin to the draining lymph nodes in a CCR7 independent manner (Vrieling et al., 2012). Consistently, in this report, we demonstrated that murine Vγ4+ dermal γδ T cell migration to the LNs is independent of Gi-coupled receptors. Chemokine-independent migration was previously reported in plasma cells in lymphoid follicles (Fooksman et al., 2010). Plasma cells undergo a persistent random walk until they find the medullary cords, where plasma cells are retained by local chemokines for their differentiation. Further studies are required to reveal how dermal γδ T cells egress from the skin.

Another novel finding in this study is that skin-derived Vγ4+ γδ T cells participate in intranodal TNF-α production and DC activation. Leslie et al. demonstrated that human peripheral blood γδ T cells interact with human monocyte-derived DCs and induce DC maturation in vitro (Leslie et al., 2002). In addition, Conti et al. showed that peripheral blood γδ T cells secrete TNF-α and IFN-γ and activate DC functions (Conti et al., 2005). We and others have shown that Vγ4+ γδ T cells produce TNF-α, but not IFN-γ (Narayan et al., 2012), which suggests that both blood γδ T cells and skin-derived γδ T cells activate DCs in a TNF-α-dependent manner. TNF-α is a potent proinflammatory and immunomodulatory cytokine implicated in inflammatory conditions. Treatment with neutralizing anti-TNF-α antibody is effective for several
diseases, including psoriasis, Crohn’s disease, and rheumatoid arthritis. However, anti-TNF-α therapy, but not anti-IL-17A therapy, has been linked to an increased risk of granulomatous infections such as tuberculosis (Hueber et al., 2010; Keane et al., 2001). Our study, in line with these clinical observations, suggests that TNF-α is essential for the host response against mycobacterium.

In our examination, the anti-Vγ4 mAb treatment suppressed OT-I proliferation modestly, but significantly reduced the production of IL-12 by DCs, which promotes IFN-γ production by T cells (Okamura et al., 1998). These results suggest that Vγ4+ cells modulate the cytokine expression by DCs rather than directly regulate CD8+ T cell proliferation.

In conclusion, our study has shown that dermal Vγ4+ γδ T cells play an important role in the draining LNs. Vγ4+ γδ T cells distribute in other epithelial tissues, such as the lung and vagina, and produce IL-17 and TNF-α against infections (Okamoto Yoshida et al., 2010; Rakasz et al., 1998). Thus Vγ4+ γδ T cells might have the capacity to migrate to the draining LNs and augment adaptive immunity via enhancing DC functions in the several epithelial tissues. Clarification of these issues might enable the modulation of systemic immune responses through regulating local immunity.
Materials and Methods

Mice

Seven to ten-week-old C57BL/6N and OT-I tg mice were purchased from SLC (Shizuoka, Japan) and Jackson Laboratory (Bar Harbor, ME), respectively. Kaede-tg, CCR7-deficient, and TCRδ-H2B-eGFP mice were described previously (Forster et al., 1999; Prinz et al., 2006; Tomura et al., 2008). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

Antibodies and flow cytometry

Antibodies used in this study were described in Supplementary Table 1. For intracellular staining, cells were stimulated for 3 h with 50 ng/ml PMA (phorbol myristate acetate; Sigma-Aldrich, St Louis, MO) and 1 μg/ml ionomycin (Wako, Osaka, Japan) in GolgiStop (BD Biosciences, San Diego, CA), then fixed and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences). Flow cytometry was performed using LSRFortessa (BD Biosciences) and analyzed with FlowJo (TreeStar, San Carlos, CA).

Single cell preparation from ear skin

The ear splits were incubated with 0.25% trypsin/EDTA (Life Technologies, Gaithersburg, MD) for 30 min at 37°C. Then, the epidermis and dermis were separated. Dermis was minced and digested with 1000 U/ml collagenase type II (Worthington Biochemical, Lakewood, NJ) containing 0.1% DNase I (Sigma-Aldrich) for 60 min at 37°C. The cell suspensions were filtered with a 40 μm cell strainer.

In vivo Vγ4+ T cell depletion

Hamster anti-Vγ4 antibody UC3 hybridoma was obtained from American Type Culture Collection (Rockville, MD). Vγ4+ cell depletion was achieved by intraperitoneal injection of 200 μg of anti-Vγ4 antibody 3 days before the BCG infection. Depletion was monitored as previously described (Sumaria et al., 2011).

OT-I tg CD8+ T cell isolation, labeling, and adoptive transfer
CD8+ T cells were isolated from OT-I tg mice by positive selection using auto MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). CD8+ T cell purity was routinely >95% as assessed by flow cytometry. For proliferation assays, purified T cells were labeled with CTV (Life Technologies) according to the manufacturer’s protocol. Control or anti-Vγ4 antibody-treated mice received 2 × 10^6 CTV-labeled OT-I tg CD8+ T cells intravenously via the tail vein.

**BCG generation and infection**

BCG-OVA was generated as described previously (Saito et al., 2006). Mice were anesthetized by isoflurane and 10^6 CFU of BCG suspended in 60 μl phosphate buffered saline was injected into the footpad. Mice that received CTV-labeled OT-I tg CD8+ T cells were infected 24 h after the adoptive transfer of cells. Six days after infection, popliteal LNs were harvested and analyzed by flow cytometry.

**Photoconversion and PTX treatment**

Photoconversion of the skin was performed (Tomura et al., 2008). Briefly, mice were anesthetized and exposed to violet light at 95 mW/cm² with a 436-nm bandpass filter using Spot UV curing equipment (SP500; USHIO, Tokyo, Japan). For photoconversion of inguinal LNs, Kaede tg mice were anesthetized and the abdominal skin was cut at the midline to visualize the inguinal LNs. The surrounding tissue was covered with aluminum foil, and then the LNs was exposed to violet light through a hole in the foil with continuous instillation of warmed phosphate buffered saline at 37°C. Pertussis toxin (PTX) (1 μg/mouse; Kaketsuken, Kumamoto, Japan), or phosphate buffered saline was subcutaneously injected into the abdominal skin.

**Cell proliferation, beads array and ELISA**

For antigen specific CD8 T cell proliferation, OT-I tg CD8+ T cells were sorted from the spleen and LNs using auto MACS (Miltenyi Biotec) (purity >95% respectively), and labeled with the CTV. CD11c+ DCs were sorted from popliteal LNs 3 days after infection with BCG-OVA using auto MACS (purity >95%) and co-cultured with OT-I tg CD8+ T cells. A total of 2 × 10^5 DCs and 2 × 10^5 T cells per well were incubated in a 96
well plate for 4 days, and the supernatants were collected for ELISA and beads array assays. The amounts of IFN-γ in the culture medium were measured by enzyme-linked immunosorbent assay (ELISA) (BD Biosciences). The amounts of IL-12 p40 were measured using a cytometric beads array system (BD Biosciences). T cell proliferation was measured by flow cytometric analysis of CTV-labeled cells.

In-vitro culture of BMDCs with Vγ4+ cells

Mouse BMDCs were generated as previously described (Otsuka et al., 2011). CD4+ and Vγ4+ T cells were sorted from naïve murine LNs and the spleen using auto MACS (Miltenyi Biotec). BMDCs (2 × 10⁵) were cultured for 24 h with CD4+ or Vγ4+ T cells (5 × 10⁴ each) in 96-well round-bottom plates in IL-17RFc (2 μg/ml; R&D Systems), anti-mouse TNF-α (MP6-XT22) (10 μg/ml; eBioscience), or control Rat IgG (eBRG1) (10 μg/ml; eBioscience) antibodies. Golgistop was added for the last 4 h of culture (BD Biosciences).

Statistic analysis

All data were statistically analyzed using Student’s t-test. P value of less than 0.05 was considered to be significant. Bar graphs are presented as mean ± standard deviation (SD).

Immunohistochemical staining, Quantitative polymerase chain reaction analysis and Contact hypersensitivity protocol

These methods were described in the Supplementary Material and Method.
Acknowledgments

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Figure legends

**Figure 1. Migration of γδ T cells from the skin to the draining LN.**

(a) Flow cytometry of the skin of Kaede-tg mice before (left) and immediately after (right) the violet light exposure. (b) Flow cytometry of Kaede-red+ cells in the draining popliteal LNs 24 h after the photoconversion of the footpad. Cells were gated on CD11c+ (left) or γδ TCR+ (right) cells. (c) The number of Kaede-red+ CD11c+ (left) or γδ TCR+ cells (right) in the draining LNs 3 days after the intra-dermal injection of BCG. The cells in the footpad were photoconverted 24 h before the analysis. Data are representative of three experiments (n=3) and are presented as means ± SD. *P < 0.05.

**Figure 2. γδ T cells migrate from the skin to the draining LNs in a Gi-independent manner.**

(a, b) Flow cytometry of CD11c+ (left) and γδ TCR+ (right) cells in the skin-draining LNs of WT (upper panel) and CCR7-deficient (lower panel) Kaede-tg mice 24 h after the photoconversion of the skin. The % frequencies of Kaede-red+ cells are shown (b). (c) Flow cytometry of CCR7 expression on Vγ4+ T cells in the skin (left panel) and in the skin-draining LN 24 hours after the photoconversion (right panel). (d, e) Flow cytometry of CD11c+ (left) and γδ TCR+ (right) cells in the draining LNs of Kaede-tg mice 24 hours after the photoconversion of the skin with PTX- or phosphate buffered saline-treatment. The % frequencies of Kaede-red+ cells were shown (e). Data are representative of three experiments (n=3) and are presented as means ± SD. *P < 0.05.

**Figure 3. Skin derived γδ T cells are Vγ4+ dermal γδ T cells.**

(a) Flow cytometric analysis of Kaede-red+ γδ T cells in the draining LNs 24 h after the photoconversion of the skin. The % frequencies of Vγ4+ cells are shown in the right panel. (b) Flow cytometric analysis of Vγ4+ cells in the skin-draining LNs of Kaede-tg mice 24 h after photoconversion of the skin cells. (c) Flow cytometric analysis of Kaede-red+ Vγ4+ cells in the dermis. (d) Flow cytometric analysis of Vγ4+ cells in the skin-draining LNs. (e) Flow cytometric analysis of Kaede-red+ Vγ4+ cells in the skin-draining LNs 24 hours after the photoconversion of the skin. (f) The % frequency of CCR6+CD103+ cells among Vγ4+ cells in the dermis, LN, and spleen. Data are
Figure 4. \(\gamma^4\) cells enhance the intranodal expansion of CD8\(^+\) T cells against BCG  
(a) Relative amount of Il17a and Tnfa mRNA expression in each subset of intranodal  
\(\gamma^4\) cells. For each subset of \(\gamma^4\) cells, equal amounts of total RNA were pooled from  
five mice. ND, not detected. (b) Flow cytometric analysis of CTV-labeled OT-I tg T  
cells from control (Ctrl)- or neutralizing anti-\(\gamma^4\) antibody- treated mice 6 days after  
injection of BCG-OVA. Number of CTV\(^{low}\) cells is shown in lower panel. Data are  
representative of three experiments (n=4) and are presented as means ± SD. *P < 0.05.  

Figure 5. \(\gamma^4\) \(\gamma\delta\) T cells stimulate antigen-specific CD8\(^+\) T cell differentiation by  
enhancement of DC functions.  
(a) Immunohistochemical staining of the skin-draining LNs 24 h after the  
photoconversion of the skin. B220\(^+\) (white) and \(\gamma\delta\) TCR\(^+\) (green) cells are shown. Right  
panel shows the higher magnification view of the boxed area in the left panel. Red  
signals represent Kaede-red. T, T cell zone; B, B cell zone. Arrowheads in the right  
panel indicate Kaede-red\(^+\) \(\gamma\delta\) T cells (right). Scale bars = 100 \(\mu\)m (left) and 50 \(\mu\)m  
(right). (b-c) The number of CTV\(^{low}\) (as an indication of cell proliferation) OT-I tg T  
cells (b) and IFN-\(\gamma\) producing cells (c). CTV-labeled OT-I tg T cells were cocultured  
with CD11c\(^+\) DCs from BCG-OVA-sensitized mice treated with control (Ctrl) or  
neutralizing anti-\(\gamma^4\) antibody. (d) The protein levels of IFN-\(\gamma\) and IL-12p40 in the  
coculture supernatant. Data are representative of three experiments (n=3–4) and are  
presented as means ± SD. *P < 0.05.  

Figure 6. \(\gamma^4\) \(\gamma\delta\) T cells stimulate BMDCs to produce IL-12p40.  
(a) The % frequency of IL-12p40\(^+\) BMDCs cultured with or without CD4\(^+\) T cells or  
\(\gamma^4\) cells. (b) The mean fluorescence intensity (MFI) of IL-12p40 expression in  
BMDCs cultured with or without \(\gamma^4\) cells. (c) The % frequency of IL-12p40\(^+\) BMDCs  
cultured with or without \(\gamma^4\) \(\gamma\delta\) T cells in the presence of isotype control (Ctrl),  
IL-17RFc and anti-TNF-\(\alpha\) antibodies. (d) The number of TNF-\(\alpha\)\(^+\) CD11c\(^+\) DCs (open  
column) and TNF-\(\alpha\)\(^+\) \(\gamma^4\) cells (filled column) in the skin-draining LNs 3 days after
treatment without (day0) or with BCG (day3). Data are representative of three experiments (n = 3) and are presented as means ± SD. *P < 0.05.

Supplementary figure legends

Supplementary Figure 1. Skin-derived γδ T cells into the draining LNs were increased in contact hypersensitivity response.

The number (left) and subset (right) of Kaede-red+ γδ TCR+ cells in the draining LNs 3 days after the elicitation. The cells in the footpad were photoconverted 24 h before the analysis. Data are representative of two experiments (n=3) and are presented as means ± SD.

Supplementary Figure 2. The majority of CCR6+ CD103+ Vγ4+ cells in the LNs are replaced from the skin.

(a) Flow cytometric analysis of CCR6+CD103+Vγ4+ cells in the skin-draining LNs 24 h after the photoconversion of the skin. (b) The % frequency of Kaede-red+ Vγ4+ cells in the skin-draining LNs of Kaede-tg mice. Data are representative of three experiments (n=3) and are presented as means ± SD. *P < 0.05.

Supplementary Figure 3. Vγ4+ γδ T cells produce IL-17 and TNF-α in the skin

The number of IL-17 (left) and TNF-α (right) producing cells in the skin. Data are representative of three experiments (n=4) and are presented as means ± SD.

Supplementary Figure 4. Depletion of Vγ4+ cells in vivo.

FACS plots of skin and LN cells 9 days after anti-Vγ4 or control antibody treatment. The percentages in γδ TCR+ cells were indicated. Data are representative of three experiments (n=4) and are presented as means ± SD.

Supplementary Figure 5. Vγ4+ cells do not affect migration and activation of DCs.

(a) Immunohistochemical staining of the LNs of TCRδ-H2B-eGFP mice 24 h after the photoconversion of the skin. B220+ cells (white) and TCR-β+ cells (red) are shown.
Green represents $\gamma\delta$ TCR$^+$ cells. T, T cell zone; B, B cell zone. (b, c) The number (b) and MFI of CD80 and CD86 expression (c) of migratory (MHC II$^{hi}$ CD11c$^{int}$) and resident (MHC II$^{int}$ CD11c$^{hi}$) DCs isolated from the draining LNs of control- or anti-V$\gamma$4 antibody-treated mice 3 days after the inoculation with BCG. Data are representative of three experiments (n=3~4) and are presented as means ± SD. *$P$ <0.05.

**Supplementary Figure 6. $V\gamma4^+\gamma\delta$ T cells stimulate BMDCs to produce IL-12p40.**

FACS plots of IL-12p40$^+$ BMDCs cultured with or without CD4$^+$ T cells or V$\gamma$4$^+$ cells. Cells were gated on CD11c$^+$ cells. Data are representative of three experiments (n=3).

**Supplementary Table 1. List of antibodies used in flow cytometry**
Figure 1

(a) Flow cytometry scatter plots showing the percentage of photoconverted cells.

(b) Quantification of Kaede-red+ CD11c+ and Kaede-red+ γδ TCR+ cells.

(c) Bar graphs showing the number of Kaede-red+ CD11c+ and Kaede-red+ γδ TCR+ cells with and without BCG treatment.
Figure 2
Figure 3
Figure 4

(a) CCR6: + – –
CD103: + + –

(b) Ctrl

Anti-Vγ4 antibody

CTVlow OT-I (x10^2)

Antibody Ctrl Vγ4
Red: Kaede-red, Green: γδTCR, White: B220

**Figure 5**

**Figure 5a:** Microscope images showing the distribution of B cells (B), T cells (T), and other immune cell populations.

**Figure 5b:** Bar graph showing the number of OT-I 

**Figure 5c:** Bar graph showing the number of IFN-γ producing cells.

**Figure 5d:** Bar graph showing the concentration of IFN-γ and IL-12p40.
**Figure 6**

(a) Graph showing the percentage of IL-12p40+ DCs (dendritic cells) under different conditions: CD4, Vγ4, and Vγ4 (control). The error bars indicate the standard error of the mean (SEM).

(b) Bar graph illustrating the mean fluorescence intensity (MFI) of IL-12p40 in Vγ4 cells under control (−) and stimulated (+) conditions.

(c) Graph depicting the percentage of IL-12p40+ DCs with antibody treatment: Vγ4, control (Ctrl), IL-17, and TNF-α.

(d) Bar graph showing the number of TNFα+ cells on Day 0 and Day 3, with data for DCs and Vγ4 cells indicated by different colors and error bars for SEM.

* denotes statistical significance.
Materials and Methods

Immunohistochemical staining

Immunohistochemical staining of LNs was carried out as described previously (Kabashima et al., 2003). Briefly, LN samples were immersed in 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan) for 3 h, embedded in OCT compound (Sakura, Torrance, CA), frozen, and then sectioned. After treatment with Image-iT FX Signal Enhancer (Life Technologies), the sections were incubated with biotin-conjugated anti-mouse γδTCR (eBioGL3) (eBioscience), eFluor 450-conjugated anti-mouse B220 (RA3-6B2) (eBioscience), PE-conjugated anti-mouse TCR-β (H57-597) (eBioscience) and APC-conjugated anti-mouse B220 (RA3-6B2) (eBioscience) antibody for 1 h and then with goat anti-rat IgG-Alexa350 and streptavidin-Alexa647 (Life Technologies) for 30 min. The slides were mounted using ProLong Antifade (Life Technologies) and observed under a fluorescent microscope (BZ-900, Keyence, Osaka, Japan).

Quantitative polymerase chain reaction analysis

Cells were sorted with a FACS Aria II cell sorter (BD Biosciences) and total RNA was extracted using a CellAmp Whole Transcriptome Amplification Kit (Takara Bio, Shiga, Japan). Quantitative reverse transcription polymerase chain reaction analysis was performed with SYBR Green I (Roche, Basel, Switzerland) using a Light Cycler 480 (Roche) according to the manufacturer’s instructions. The primer sequences used in this study were as follows: Gapdh, 5’-GGCCTCACCCCATTTGATGT-3’ (forward) and 5’-CATGTTCAGTGACTCCACTC-3’ (reverse); IL-17A, 5’-CTCCAGAAGGCCTCAGACTAC-3’ (forward), 5’-GGGTCTTCATTTGCGGTTG
-3’ (reverse); and TNF-α, 5’- CAGGCGGTGCCTATGTCTC -3’ (forward), 5’- CGATCACCCCGAAGTTAGTAG -3’ (reverse). Fold expression was calculated by the ΔΔCT method and Gapdh was used as a reference gene.

**Contact hypersensitivity protocol**

The ear of Kaede mice was sensitized with 20 μl 0.5% (w/v) dinitrofluorobenzene (DNFB; Nacalai Tesque) in acetone/olive oil (4:1) (Nacalai Tesque). Five days after the sensitization, the footpad was challenged with an application of 20 μl 0.3% DNFB. The number of Kaede-red⁺ γδ TCR⁺ cells in the draining LNs was measured 3 days after the challenge. The cells in the footpad were photoconverted 24 hours before the analysis.
Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 3
Supplementary Figure 4
Supplementary Figure 5
Supplementary Figure 6
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Supplementary Table 1