Development of a new monoclonal antibody specific to mouse Vy6 chain

Shinya Hatano1, Xiun Tun1, Naoto Noguchi1, Dan Yue1,2, Hisakata Yamada1, Xun Sun2, Mitsuru Matsumoto3, Yasunobu Yoshikai1

There are seven Vy gene segments in the TCR γ chain loci of mice. We developed monoclonal antibodies (mAbs) specific to the Vy6 chain (Heilig & Tonegawa nomenclature). By immunizing Vy4/6 KO mice with complementarity-determining region peptides in Vy6 chains, we generated three hybridomas. These hybridomas produced mAbs capable of cell surface staining of Vy6/V61 gene–transfected T-cell line lacking TCR as well as of Vy1~Vy4~ Vy5~Vy7 γδ T cells and the CD3high TCRIγδT cells in various organs. The location of Vy6 γδ T cells, which peaked in the newborn thymus, was associated with mTEC. In vivo administration of clone 1C10-1F7 mAb impaired protection against Klebsiella pneumoniae infection but ameliorated psoriasis-like dermatitis induced by imiquimod treatment. These new mAbs are useful to elucidate the development, location, and functions of Vy6 γδ T cells in mice.

DOI 10.26508/lsa.201900363 | Received 1 March 2019 | Revised 28 April 2019 | Accepted 29 April 2019 | Published online 7 May 2019

Introduction

TCR γ chain loci have three functional Cγ genes (Cγ1, Cγ2, and Cγ4) and one nonfunctional pseudo Cγ gene (pCγ3), four joining segments, including one pseudogene (Jγ1, Jγ2, pJγ3, and Jγ4), and seven variable (Vγ) gene segments (Saito et al., 1984). The Vy genes are Vy1, Vy2, Vy3, Vy4, Vy5, Vy6, and Vy7, using the Heilig & Tonegawa nomenclature (Heilig & Tonegawa, 1986), which we used here, or Vy1.1, Vy1.2, Vy1.3, Vy2, Vy3, Vy4, and Vy5, using the Garman nomenclature (Garman et al., 1986). Gene rearrangement of γδ TCR loci occurs at an early stage in the fetal thymus before αβ TCR genes rearrange in the thymus. Mouse fetal development is characterized by producing waves of γδ T-cell populations that use different Vy chains (Chien et al., 1987; Ito et al., 1989). During embryonic development, the first T cells to appear from approximately embryonic day 12 (E12) to E16 carry γδ TCR composed of Vy5 and V61 chains (Vy5y1 and V61D52I62), which populate the epidermis, and these T cells, which become wedged among keratinocytes and adopt a dendritic-like form, are termed dendritic epidermal T cells (dETCs) (Asarnow et al., 1988; Havran et al., 1989, 1990). The second T cells appearing from E14 to birth carry Vy6 paired with Vδ1 of γδ TCR (Vy6y1 and Vδ1D62I62), which home to the epithelia of the reproductive tract, tongue, lungs, peritoneal cavity (PEC), skin dermis, colon-lamina propria lymphocytes (c-LPLs) and adipose tissue as tissue-associated cells (Itohara et al., 1990; Mokuno et al., 2000; Roark et al., 2004; Cai et al., 2011; Sun et al., 2013; Kohlgruber et al., 2018). These two subsets bear truly invariant TCRs without junctional diversity, even no nucleotides in the TCR gene junction, and are essentially an oligoclonal population of cells. The following waves are Vy4+ T cells from E16 onward and Vy1+ T cells from E18 onward, all of which show junctional diversity in complementarity-determining region (CDR) 3. At the periphery, most of the spleen and LN γδ T cells express Vy1 and Vy4, whereas Vy7-expressing γδ T cells are more prevalent in intestinal intraepithelial cells (i-IELs) (Goodman & Lefrancois, 1989). This bias in Vy usage has led to the suggestion that Vy-encoded residues enable these T cells to respond to Ag unique to their resident tissues. Recently, Vy7+ i-IELs are reported to respond to epithelial butyrophilin-like (Btrl) protein of the B7 superfamily using germ line–encoded motifs distinct from CDRs within the Vy7 chain (Di Marco Barros et al., 2016; Melandri et al., 2018). Thus, the bias of Vy usage in various mucosal tissues has led to the suggestion that Vy-encoded residues enable these T cells to respond to agonists unique to their resident tissues.

All monoclonal antibodies (mAbs) specific to Vy chains, except for Vy3 and Vy6, are currently available for cell surface staining (Goodman & Lefrancois, 1989; Havran et al., 1989, 1990; Dent et al., 1990; Goodman et al., 1992; Pereira et al., 1995; Mallick-Wood et al., 1998; Grigoriadou et al., 2002). We have detected Vy6 γδ T cells indirectly by expressing Vy6-encoding mRNA (Mokuno et al., 2000; Murakami et al., 2016). Roark et al reported that 17D1 mAb, which was first thought to detect dETCs bearing Vy5/V61 (Mallick-Wood et al., 1998), could also bind Vy6/V61 γδ T cells if their TCR was first complexed to an anti-Cδ mAb (GL3) (Roark et al., 2004). Furthermore, Paget et al identified IL-17A–producing Vy6/V61 γδ T cells as CD3high γδ T cells by anti-CD3ε mAb. However, detailed characteristics of Vy6 γδ T cells remain obscure because of the lack

1Division of Host Defense, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan 2Department of Immunology, China Medical University, Shenyang, China 3Division of Molecular Immunology, Institute for Enzyme Research, Tokushima University, Tokushima, Japan

Correspondence: hatano@bioreg.kyushu-u.ac.jp

© 2019 Hatano et al. https://doi.org/10.26508/lsa.201900363 vol 2 no 3 e201900363 1 of 12
of Vy6-specific mAb. In this study, we developed new mAbs specific to the murine Vy6 chain and report the successful production, characterization, and in vitro effects of a novel anti-Vy6 mAb with potential applications in elucidating roles of Vy6 γδ T cells in infection and inflammation in mice.

Results and Discussion

Production of mAbs against Vy6 TCR available for cell surface staining

The V part of an Ab, including the unique Ag-binding site, is known as the idiotype. When one Ab binds to an idiotype of another Ab, it is referred to as an anti-idiotypic Ab (Jerne, 1974). An anti-idiotypic network exists in autoimmune diseases, regulating the production of autoantibodies, or the idiopathic response (Menshikov et al, 2015). However, in a healthy state, because of tolerance to self Ag (Kappler network exists in autoimmune diseases, regulating the production of these autoantibodies. The most likely immunogenic epitopes for staining the V part of the TCR by mAbs should include the CDR3 of the Vγ6 chain, which was transfected with Vγ6/Vδ1 mouse T-cell line (Sussman et al, 1988; Ohno et al, 1991), which was developed to produce anti-idiotype Abs against the Vδ6 chain. However, the CDR3 of the Vγ6 chain of the invariants Vγδ6 chain of the invariants Vγδ6 chain. Therefore, we used Vγ4/δ6 KO mice. To further select mAbs available for cell surface staining, we screened hybridomas that secreted mAbs specific for Vy6 γδ T cells. After a consecutive booster, iliac LN were collected from immunized C57BL/6 mice; clones: 1C10-1F7, 2G2-2A3, and 5E10-C12 producing mAbs, were selected. The Vγ6/Vδ1 mAbs also stained Vy6 γδ T cells in the PEC and c-LPL from C57BL/6 mice (Fig 2D). We selected 1C10-1F7 and 5E10-C12 mAbs showing different CDR3 sequences. The isotypes of 1C10-1F7 and 5E10-C12 mAbs were absent in the PEC from Vy6 KO mice (Fig 2C). The Vy6 γδ T cells stained with 1C10-1F7, 2G2-2A3, or 5E10-C12 mAbs were absent in the PEC from Vy4/δ6 KO mice. The Vy6 γδ T cells stained with 1C10-1F7 mAb (Fig 2A) and 5E10-C12 mAb (Fig 2B) were stained with 1C10-1F7 mAb (Fig 2A). The Vy6 γδ T cells stained with 1C10-1F7, 2G2-2A3, or 5E10-C12 mAbs were absent in the PEC from Vy4/δ6 KO mice (Fig 2D). We selected 1C10-1F7 mAb (IgG1, κ) with the highest affinity for further experiments. The Vy6 γδ T cells in the PEC were not stained with 1C10-1F7 mAb (Fig 2D). DECTs, which express Vy5 exclusively (Asarnow et al, 1988; Havran & Allison, 1990; Havran et al, 1989) and i-EL, which contains abundant levels of Vy7 γδ T cells (Goodman et al, 1992; Pereira et al, 1995), were not stained with 1C10-1F7 mAb (Fig 2C and D). To further confirm that 1C10-1F7 mAb recognizes the Vy6 γδ T cells, we stained Vy6 γδ T cells in the PEC and examined the expression of TCR Vy6 gene by RT-PCR and nucleotide sequences. The Vy6 γδ T cells sorted from the PEC expressed Vy6-specific transcripts, and all Vy6 γδ T cells showed no junctional diversity (Fig S2). This resulted in in-frame invariant canonical sequences, which are preferentially expressed in Vy6 γδ T cells in the fetal thymus (Laflaile et al, 1989), reproductive organs (Itohara et al, 1990), and the PEC (Mokuno et al, 2000).

We next examined the location of Vy6 γδ T cells by immuno-histochemical analysis with 1C10-1F7 mAb in the uterine cervix, in which most of Vy6 γδ T cells were positively stained with 1C10-1F7 mAb. Consistent with a previous report (Itohara et al, 1990), the Vy6 γδ T cells are relatively abundant in the epithelia of the PEC, reproductive organs (vagina/uterine cervix), lungs, and c-LPL as tissue-associated cells (Itohara et al, 1990; Mokuno et al, 2000; Murakami et al, 2016; Sun et al, 2013). We stained for Vy6 γδ T cells in the nonlymphoid tissues from C57BL/6, BALB/c, and Vy4/δ6 KO mice with 1C10-1F7, 2G2-2A3, or 5E10-C12 mAbs. Most of the Vy7 γδ T cells in the reproductive organs and large proportions of the Vy4 γδ T cells in the PEC and c-LPL from C57BL/6 were positively stained with 1C10-1F7 mAb (Figs 2A and 2B). The Vy6 γδ T cells stained with 1C10-1F7, 2G2-2A3, or 5E10-C12 mAbs were absent in the PEC from Vy4/δ6 KO mice (Fig 2D). We selected 1C10-1F7 mAb (IgG1, κ) with the highest affinity for further experiments. The Vy7 γδ T cells in the PEC were not stained with 1C10-1F7 mAb (Fig 2D). DECTs, which express Vy5 exclusively (Asarnow et al, 1988; Havran & Allison, 1990; Havran et al, 1989) and i-EL, which contains abundant levels of Vy7 γδ T cells (Goodman et al, 1992; Pereira et al, 1995), were not stained with 1C10-1F7 mAb (Fig 2C and D). To further confirm that 1C10-1F7 mAb recognizes the Vy6 γδ T cells, we stained Vy6 γδ T cells in the PEC and examined the expression of TCR Vy6 gene by RT-PCR and nucleotide sequences. The Vy6 γδ T cells sorted from the PEC expressed Vy6-specific transcripts, and all Vy6 γδ T cells showed no junctional diversity (Fig S2). This resulted in in-frame invariant canonical sequences, which are preferentially expressed in Vy6 γδ T cells in the fetal thymus (Laflaile et al, 1989), reproductive organs (Itohara et al, 1990), and the PEC (Mokuno et al, 2000).

We next examined the location of Vy6 γδ T cells by immuno-histochemical analysis with 1C10-1F7 mAb in the uterine cervix, in which most of Vy6 γδ T cells were positively stained with 1C10-1F7 mAb. Consistent with a previous report (Itohara et al, 1990), the Vy6 γδ T cells are relatively abundant in the epithelia of the PEC, reproductive organs (vagina/uterine cervix), lungs, and c-LPL as tissue-associated cells (Itohara et al, 1990; Mokuno et al, 2000; Murakami et al, 2016; Sun et al, 2013). We stained for Vy6 γδ T cells in the nonlymphoid tissues from C57BL/6, BALB/c, and Vy4/δ6 KO mice with 1C10-1F7, 2G2-2A3, or 5E10-C12 mAbs. Most of the Vy7 γδ T cells in the reproductive organs and large proportions of the Vy4 γδ T cells in the PEC and c-LPL from C57BL/6 were positively stained with 1C10-1F7 mAb (Figs 2A and 2B). The Vy6 γδ T cells stained with 1C10-1F7, 2G2-2A3, or 5E10-C12 mAbs were absent in the PEC from Vy4/δ6 KO mice (Fig 2D). We selected 1C10-1F7 mAb (IgG1, κ) with the highest affinity for further experiments. The Vy7 γδ T cells in the PEC were not stained with 1C10-1F7 mAb (Fig 2D). DECTs, which express Vy5 exclusively (Asarnow et al, 1988; Havran & Allison, 1990; Havran et al, 1989) and i-EL, which contains abundant levels of Vy7 γδ T cells (Goodman et al, 1992; Pereira et al, 1995), were not stained with 1C10-1F7 mAb (Fig 2C and D). To further confirm that 1C10-1F7 mAb recognizes the Vy6 γδ T cells, we stained Vy6 γδ T cells in the PEC and examined the expression of TCR Vy6 gene by RT-PCR and nucleotide sequences. The Vy6 γδ T cells sorted from the PEC expressed Vy6-specific transcripts, and all Vy6 γδ T cells showed no junctional diversity (Fig S2). This resulted in in-frame invariant canonical sequences, which are preferentially expressed in Vy6 γδ T cells in the fetal thymus (Laflaile et al, 1989), reproductive organs (Itohara et al, 1990), and the PEC (Mokuno et al, 2000).
cells were abundantly present sub-epithelially, just under the cervical epithelium (Fig 2E).

Ontogenic wave of Vγ6 γδ T cells

γδ T cells expressing Vγ5, Vγ6, Vγ4, Vγ1, and Vγ7 TCR develop sequentially in this order in the fetal thymus around E12 and E16 (Chien et al, 1987; Ito et al, 1989). We consistently found that Vγ5+ γδ T cells were abundant in the fetal thymus at earlier stages of development, and the percentage decreased from E16 onward during embryonic development, reaching a peak at neonatal stage from birth to day 3 (Fig 3A–C). These results are consistent with previous data showing a peak of Vγ6 γδ T cells at birth (Ito et al, 1989).

Reconstitution of lethally irradiated adult mice with BM or fetal liver (FL) resulted in failure to generate Vγ5+ γδ T cells, implying that the development of fetal type γδ T cells requires an embryonic thymus per se (Vantourout & Hayday, 2013; Cai et al, 2014). We also examined whether Vγ6+ γδ T cells in lethally irradiated mice reconstituted with BM or FL cells and Vγ6+ γδ T cells in the periphery. Vγ4+ γδ T cells were detected in the PEC of either of these reconstituted mice, but Vγ6+ γδ T cells were not (Fig S3A–C). These

Figure 1. New mAbs are available for cell surface staining for Vγ6 TCR.

(A) TG60 introduced with the Vγ6/Vδ1 gene (Vγ6Vδ1-rCD2) or the Vγ5/Vδ1 gene (Vγ5Vδ1-rCD2) were stained with mAbs from 1C10-1F7, 2G2-2A3, and 5E10-C12 or anti-Vγ5 mAbs. Histograms show expression of Vγ5 and Vγ6 on Vγ6Vδ1-rCD2 or Vγ5Vδ1-rCD2 after gating on TCRδ’ CD3ε’. (B) Vγ6Vδ1-rCD2 and Vγ5Vδ1-rCD2 were stained with 1C10-1F7 or 17D1 with or without prestaining with GL3. The zebra plot shows 1C10-1F7 staining and 17D1 staining of Vγ6Vδ1-rCD2 and Vγ5Vδ1-rCD2, respectively. (C) V-D-J genes of the H chain, V-J genes of the L chain, and aa sequences of each CDR3 of 1C10-1F7, 2G2-2A3, and 5E10-C12 mAbs.
results suggest that Vγ6γδT cells are of the fetal type, and the development of γδT cells may require an embryonic thymus per se. We recently reported that IL-17–producing γδT cells developed at the CD4−CD8−double-negative (DN)2b stage, which is located in the medulla (Shibata et al., 2014). Consistent with this report, immunohistochemical staining with 1C10-1F7 mAb revealed that Vγ6+γδT cells were located at the medulla of the neonatal thymus (Fig 3D). Double staining with 1C10-1F7 mAb and medullary thymic epithelial cell (mTEC)–specific mAb ER-TR5 (Van Vliet et al., 1984) suggested cross talk between Vγ6 and mTEC for selection (Fig 3E). It has been reported that IL-17+ Vγ6+Vδ1+T cells are enriched in several organs of mice deficient in autoimmune regulator (Aire) gene, which is expressed by the mTEC (Fujikado et al., 2016). Nitta et al recently reported that IL-17+ Vγ6+T cells were substantially enhanced in TN mice, which have no mature cortical TECs (cTECs) and substantially reduced number of mTECs in thymus (Nitta et al., 2015). Taken together, it is suggested that mTECs negatively regulate the development of IL-17+ Vγ6+γδT cells in the thymus. However, this is only speculation and further experiments need to clarify the significance of interaction of Vγ6+γδT cells and mTECs.

Figure 2. New mAbs are useful for analyzing Vγ6γδT cells in various tissues. (A) 1C10-1F7 mAb staining in C57BL/6 mice. Representative upper dot plots are shown after gating γδT cells and lower dot plots are shown after gating Vγ1−Vγ4+γδT cells in indicated organs. (B) 2G2-2A3 or 5E10-C12 mAbs staining in C57BL/6 mice. Representative dot plots are shown after gating Vγ1−Vγ4+γδT cells in indicated organs. (C) 1C10-1F7 mAb staining in BALB/c mice. Representative dot plots are shown after gating Vγ1−Vγ4+γδT cells in indicated organs. (D) Dot plots are shown after gating γδT cells in PEC from WT (C57BL/6) or Vγ4/6 KO mice. Histograms show the expression of 1C10-1F7, 2G2-2A3, or 5E10-C12 on Vγ1−Vγ4+γδT cells of PEC from WT or Vγ4/6 KO mice. (E) Paraformaldehyde-fixed paraffin section of uterine cervix from WT mice was stained with Alexa Fluor 647–conjugated 1C10-1F7 mAb (green) and DAPI (blue). Right panel shows higher magnification image in the red square region of left panel. All scale bars represent 100 μm.
Evaluation of in vivo effect of 1C10-1F7 mAb on Vγ6γδ T cells

Paget et al (2015) reported that CD3^high TCRδ^int γδ T cells were IL-17A–producing Vγ6/Vδ1 γδ T cells and that CD3^int TCRδ^high γδ T cells were IL-17A–producing Vγ4 γδ T cells. We analyzed γδ T cells in the lungs from mice after they were inoculated intratracheally with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) (Umemura et al, 2007) and stained with anti-CD3ε, TCRδ (clone: GL3), and 1C10-1F7 mAbs. Consistent with the previous report, we confirmed that IL-17A–producing CD3^high TCRδ^int γδ T cells were Vγ6^+ γδ T cells and that IL-17A–producing CD3^int TCRδ^high γδ T cells were Vγ4^+ γδ T cells (Fig 4A).
We next examined the effect of in vivo administration of 1C10-1F7 mAb on Vy6 γδ T cells. Koenecke et al (2009) reported an in vivo application of mAb directed against γδ T cells (clone GL3, Armenian hamster IgG), leading to prolonged TCR internalization lasting at least 14 d, without clearance of the actual γδ T cells. As shown in Fig 4B–D, we found that Vy6 TCR γδ T cells became invisible in the PEC, reproductive organs, and lungs on day 3 after in vivo administration of 1C10-1F7 mAb (mouse IgG1, κ), whereas γδ T cells with a low intensity of Vy6 TCR recovered in these organs by day 6 after administration (Fig 4B–D). To ensure that the 1C10-1F7 mAb is not a depleting mAb but is internalized by target cells, we used Alexa Fluor 647–conjugated 1C10-1F7 mAb for in vivo administration and found CD3ε−Alexa Fluor 647+ cells, which internalized Vy6 TCR, on day 3 after administration of Alexa Fluor 647–conjugated 1C10-1F7 mAb.
Further deleterious contributions of IL-17A–producing γδ T cells were observed in models of psoriasis (Chien et al., 2014), ischemic brain injury (Shichita et al., 2009), experimental autoimmune encephalomyelitis (Sutton et al., 2009), and collagen-induced arthritis (Roark et al., 2007). In all these cases, IL-17A+ γδ T cells are known to be major contributors of inflammation and associated disease pathology. Previous studies have demonstrated that IL-17A+ γδ T cells play a crucial role in psoriasis-like dermatitis induced by imiquimod (IMQ) (Cai et al., 2011, 2014; Gray et al., 2013). Upon IMQ treatment, IL-17A+ γδ T cells specifically expand in the draining LN and recirculate to inflamed skin (Gray et al., 2013). However, we observed that IMQ-induced skin inflammation was significantly attenuated in mice that received 1C10-1F7 mAb (Fig 5C–G). These results indicate that at least γδ T cells contributed to pathogenesis of psoriasis such as dermatitis induced by IMQ. Constitutive TCRδ KO mice were reported to show similar IMQ pathology, whereas conditional TCRδ KO mice showed an attenuated pathology as compared with WT mice, suggesting that the pathological role of IL-17A+ γδ T cells may be compensated by other IL-17A+ cells in constitutive TCRδ KO mice (Sandrock et al., 2018). Block of γδ TCR by in vivo administration of 1C10-1F7 mAb may be useful for investigation of the role of γδ T cells in various inflammatory diseases, similar to conditional TCRδ KO mice (Sandrock et al., 2018). However, the γδ T cells are still present after administration of...
1C10-1F7 mAb and could potentially still react, for example, in a TCR-independent manner with cytokines via cytokine receptors and Toll-like receptors (Nakamura et al, 2008; Dejima et al, 2011).

In conclusion, we have successfully developed new mAbs specific to Vy6 γδ T cells. These mAbs are available for flow cytometry, immunohistochemistry, and in vivo function analysis. Vy6 γδ T cells play important roles in protection against microbial infection and in pathogenesis of inflammatory diseases such as colitis and autoimmune diseases by producing IL-17A (Chien et al, 2014). Our mAbs may be useful for elucidating the roles of Vy6 γδ T cells in these inflammatory diseases.

Materials and Methods

Mice

C57BL/6 or BALB/c female mice were purchased from Japan KBT. Vy4/6 KO mice were generated as previously described (Sunaga et al, 1997). All mice were maintained under specific pathogen-free conditions and provided food and water ad libitum. Age- and gender-matched mice were used for all experiments. This study was approved by the Committee of Ethics on Animal Experiments of the Faculty of Medicine, Kyushu University. Experiments were carried out according to local guidelines for animal experimentation.

Immunization and fusion protocols

For immunization, two types of peptides were synthesized from truncated regions of CDR1 (Vy631–35) and CDR2 (Vy610–24) in Vy6 chains. Vy4/6 KO mice were immunized with the KLH-conjugated peptides emulsified with CFA. These mice were reimmunized with the KLH-conjugated peptides without CFA 17 d after the first immunization. Iliac LNs were collected 21 d after the last immunization and were fused with SP2/0-Ag14 using polyethylene glycol.

Cell lines

TG40 is a variant T-cell hybridoma cell line lacking the expression of TCR-α and TCR-β chains, which has been used as recipient cells for TCR transfection (Sussman et al, 1988; Ohno et al, 1991). TG40 cell lines were introduced with the Vy5V61 or Vy6V61 genes using a retroviral bicistronic vector containing an internal ribosomal entry site (IRES) and rat CD2 (rCD2) (pMX-IRES-rCD2).

Purification of new anti-Vy6 mAbs

Hybridomas were cultured in Hybridoma-SFM (Thermo Fisher Scientific) including 1 ng/ml recombinant human IL-6 (R&D systems). After 7–10 d of culture, culture supernatants were collected. For flow cytometry and immunohistochemical analysis, new anti-Vy6 mAbs were purified from the hybridoma supernatant using the mouse TCS purification system (Abcam) and conjugated with Alexa Fluor 647 using a labeling kit (Invitrogen). For the 1C10-1F7 administration experiment, 1C10-1F7 mAb was purified from the hybridoma supernatant using the Protein G Spin kit (Thermo Fisher Scientific).

Sequence analysis of the heavy and light chain variable regions of Vy6-specific mAbs

Total RNA was isolated and purified from hybridomas with RNeasy Plus Universal Mini Kit (QIAGEN). Total RNA was converted to complementary DNA (cDNA) with Superscript III reverse transcriptase (Invitrogen). Next, BCR genes were amplified using adaptor ligation-mediated PCR (Kitaura et al, 2017). High-throughput sequencing was performed using the Illumina Miseq paired-end platform (2 × 300 bp) (Illumina). V-D-J genes and CDR3 sequences were identified using IgBlast (NCBI: National Center for Biotechnology Information) (Ye et al, 2013).

Cell preparations from various tissues

Single-cell suspensions were isolated from the thymus, PEC, reproductive organs (vagina/uterine cervix), i-IEL, c-LPL, and lungs as previously described (Shibata et al, 2008). Epidermal sheets were isolated from ears (Haas et al, 2012) and dETCs were isolated from the epidermal sheets by centrifugation at 600 g for 20 min in a 40% and 70% Percoll (GE Healthcare Bio-Sciences AB) gradient.

Flow cytometry analysis

Cells were stained for 20 min at 4°C with mAbs. We added 1 μg/ml propidium iodide (Sigma-Aldrich) to the cell suspension just before flow cytometry to detect and exclude dead cells from the surface staining analysis. To measure cytokine production, the cells were stimulated with 25 ng/ml PMA (Sigma-Aldrich) and 1 μg/ml ionomycin ( Sigma-Aldrich) for 5 h at 37°C; 10 μg/ml Brefeldin A (Sigma-Aldrich) was added for the last 4 h of incubation. After the cells were stained with various mAbs, intracellular staining was performed according to the manufacturer’s instructions (BD Biosciences): 100 μl BD Cytofix/Cytoperm solution (BD Biosciences) was added to the cell suspension with gentle mixing and incubated for 20 min at 4°C. Fixed cells were washed twice with 250 μl 10% BD Perm/Wash solution (BD Biosciences) and then stained intracellularly for 30 min at 4°C. Stained cells were analyzed on a FACSVerse flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (Tree Star). Abs for flow cytometric analysis used in this study: PerCP-Cy5.5–conjugated anti-MHC class II (M5/114,15.2), APC-Cy7–conjugated anti-CD3ε (145-2C11), PE-Cy7–conjugated anti-CD45.2 (104), anti-Vy4 (UC3-10A6) V500-conjugated anti-MHC class II (M5/114,15.2) mAbs, and streptavidin were purchased from BD Biosciences. PE-conjugated Hamster IgG isotype control (HTK888), PerCP-Cy5.5–conjugated anti-IL-17A (ebio17B7), biotin-conjugated anti-CD45.1 (A20), Purified Mouse IgG1 κ isotype control (P3), and Purified Mouse IgG2b κ isotype control (eBMG2b) mAbs were all purchased from eBioscience. FITC-conjugated, anti-MHC class II (M5/114,15.2), anti-Vy1 (2.11), anti-Vy4 (UC3-10A6), PE-conjugated anti-Vy1 (2.11), anti-Vy4 (UC3-10A6), anti-mouse IgG (Poly4053), APC-conjugated, anti-TCR6 (GL3), anti-Vy4 (UC3-10A6), Alexa Fluor 647–conjugated anti-CD3ε (17A2), Mouse IgG1 κ isotype control.
(MOPC-21), anti-mouse IgG (Poly4053), PE-Cy7–conjugated anti-TCRδ (GL3), V421–conjugated anti-TCRδ (GL3), and biotin–conjugated anti-rat IgM (MRM-47) mAbs were purchased from BioLegend. PE-conjugated anti-Vγ5 (536) mAb was purchased from Santa Cruz Biotechnology. Anti-Vγ7 (F2.67) and 17D1 mAbs were collected from F2.67 and 17D1 hybridoma culture supernatant.

γδ T-cell sorting, RNA purification, RT-PCR, and sequencing of Vγ6

Single-cell suspensions were isolated from PEC and stained with mAbs. 1C10–1F7 γδ T cells were sorted using FACS Aria (BD Bioscience). Total RNA was purified from sorted 1C10–1F7 γδ T cells using an RNeasy Mini kit (Qiagen), and cDNA was synthesized using Superscript II (Invitrogen) according to the manufacturer’s instructions. PCR was performed on a PCR thermal cycler (Takara Corp.). RT-PCR products were analyzed by blotting in 1.8% agarose gels. For RT-PCR analysis of Vγ6 TCR gene, combinations of following primers were used. Forward primers: Vy6, 5’-GGAACTCAAAGAAMCATGTTCT-3’. Reverse primers: Cγδ, 5’-TCCATAGGAGTTGATCGC-3’. Forward primers: β-actin, 5’-TGAATCTCGTGGACATGAAAC-3’. Reverse primers: β-actin, 5’-TAAACCGAGCTCAGTAAAATCCG-3’. Purified Vy6 PCR products of 1C10–1F7 γδ T cells from PEC were sequenced using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and 3500xL Genetic Analyzers (Applied Biosystems).

Immunohistochemistry

Uterine cervixes from 8-wk-old WT mice and thymus from 0-d-old WT mice were fixed with phosphate-buffered 4% paraformaldehyde (Nacalai Tesque) overnight and embedded in paraffin. Paraffin sections were stained with Alexa Flour 647–conjugated 1C10–1F7 mAb, DAPI (BioLegend), and H&E. Frozen thymus from 0-d-old WT mice was embedded in OCT compound (Sakura Finetek) and frozen sections were fixed with acetone. Frozen sections were stained with Alexa Flour 647–conjugated 1C10–1F7 mAb and ER-TR5 mAb followed by Alexa Flour 488–conjugated anti-rat IgG (Thermo Fisher Scientific). For multicolor confocal analysis, slides were mounted in ProLong Gold AntiFade reagent (Invitrogen) and analyzed with a Zeiss LSM700 confocal microscope (Carl Zeiss). H&E–stained slide was analyzed with All-in-One Fluorescence Microscope BZ-9000 (Keyence).

Generation of BM and FL chimera

BM cells were extracted from 8-wk-old WT mice (Ly5.2/5.2) by flushing femurs and tibias and were then depleted of T cells using anti-CD3 mAb (17A2; BioLegend) and anti-rat IgG Dynabeads (Invitrogen). FL cells were extracted from the liver of embryonic day (ED) 14 WT mice (Ly5.2/5.2). 2 × 10⁶ BM cells or 5 × 10⁶ FL cells were intravenously injected into lethally irradiated (10 Gy) recipient 8-wk-old WT mice (Ly5.1/5.1). After 8 wk, reconstitution was confirmed.

Microorganisms and bacterial infection experiment

Lyophilized M. bovis BCG (Tokyo strain) was purchased from Kyowa Pharmaceuticals and dissolved in 7H9 broth (Difco) supplemented with albumin–dextrose–catalase enrichment (BD Biosciences). Single colonies were grown with vigorous shaking at 37°C in Middlebrook 7H9 broth supplemented with 10% albumin–dextrose–catalase, 1% glyceral (Sigma-Aldrich), and 0.5% Tween 80 (Wako) until the optical density at 600 nm (OD₆₀₀) reached 1. Bacteria were stored at −80°C in 50% glyceral as single-use aliquots. Mice were intratracheally injected with 1 × 10⁶ CFUs of M. bovis BCG (Tokyo strain) intranasally inoculated with K. pneumoniae at 1 × 10⁴ CFUs on day 0.

IMQ experiments

8-wk-old WT mice were intraperitoneally injected with 200 μg of 1C10–1F7 or mouse IgG1 isotype control mAb on days −3, 0, and 3. These mice were applied daily a topical dose of 62.5 mg of commercially available IMQ cream (5%; Aldara; 3M Pharmaceuticals) on the shaved back for five consecutive days, translating to a daily dose of 3.125 mg of the active compound. Paraffin–fixed back skin of IMQ treatment on day 5 was fixed with phosphate-buffered 4% paraformaldehyde, embedded with paraffin, and stained with H&E. The back skin thickness was measured on days 0–5. The erythema score or scaling score of the back skin was scored on days 0–5 on a scale from 0 to 4.

Statistical analysis

Statistical significance was evaluated using Prism software (GraphPad). The t test was used when only two groups were compared, and the survival curve was assessed by the log-rank test. P values <0.05 were considered to represent significant differences.

Supplementary Information

Supplementary information is available at https://doi.org/10.26508/lsa.201900363.

Acknowledgements

The authors are grateful to Dr. Y Matsumura and Dr. K Shibata for providing reagents and protocols and Y Kitada and A Yano for helping to prepare the manuscript. This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas (JSPS KAKENHI no. JP 16H06496 and a Grant-in-Aid for JSPS Research Fellow no. JP 17J03389.

Author Contributions

S Hatano: conceptualization, resources, data curation, software, formal analysis, supervision, funding acquisition, validation,
investigation, visualization, methodology, project administration, and writing—original draft, review, and editing.
X Tun: validation and investigation.
N Noguchi: validation and investigation.
D Yue: validation and investigation.
H Yamada: methodology.
X Sun: methodology.
M Matsumoto: resources and methodology.
Y Yoshikai: conceptualization, supervision, funding acquisition, methodology, project administration, and writing—original draft, review, and editing.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

References

Asarnow DM, Kuziel WA, Bonyhadi M, Tigelaaer RE, Tucker PW, Allison JP (1988) Limited diversity of gamma delta antigen receptor genes of Thy-1+ dendritic epidermal cells. Cell 55: 837–847. doi:10.1016/0092-8674(88)90139-0
Cai Y, Shen X, Ding C, Qi C, Li K, Li X, Jala VR, Zhang HG, Wang T, Zheng J, et al (2011) Pivotal role of dermal IL-17-producing γδ T cells in skin inflammation. Immunity 35: 596–610. doi:10.1016/j.immuni.2011.08.001
Cai Y, Xue F, Fleming C, Yang J, Ding C, Ma Y, Liu M, Zhang HG, Zheng J, Xiong N, et al (2014) Differential developmental requirement and peripheral regulation for dermal Vγ4 and Vγ6T17 cells in health and inflammation. Nat Commun 5: 3986. doi:10.1038/ncomms4986
Chien YH, Iwashima M, Wettstein DA, Kaplan KB, Elliott JF, Born W, Davis MM (1987) T-cell receptor delta gene rearrangements in early thymocytes. Nature 330: 722–727. doi:10.1038/330722a0
Chien YH, Konigshofer Y (2007) Antigen recognition by gammadelta T cells. Immune Rev 215: 46–58. doi:10.1111/j.1600-065x.2006.00470.x
Chien YH, Meyer C, Bonnivelle M (2014) γδ T cells: First line of defense and beyond. Annu Rev Immunol 32: 121–155. doi:10.1146/annurev-immunol-032013-110216
Dent AL, Matis LA, Hooshmand F, Widdack SM, Bluestone JA, Hedrick SM (1990) Self-reactive gamma delta T cells are eliminated in the thymus. Nature 343: 714–719. doi:10.1038/343714a0
Dejima T, Shibata K, Yamada H, Hara H, Iwakura Y, Naito S, Yoshikai Y (2011) A protective role of naturally occurring IL-17A-producing γδ T cells in the lung at the early stage of systemic candidiasis in mice. Infect Immun 79: 4503–4510. doi:10.1128/aii.05799-11
Di Marco Barros R, Roberts NA, Dart RJ, vantourout P, Jamdke A, Nussbaum O, Deban L, Cipolat S, Hart R, Lannitto ML, et al (2016) Epithelia use butyrophilin-like molecules to shape organ-specific γδ T cell compartments. Cell 167: 203–218. doi:10.1016/j.cell.2016.08.030
Fujikado N, Mann AO, Bansal K, Romito KR, Ferre EMN, Rosenzweig SD, Lionakis MS, Benoist C, Mathis D (2016) Aire inhibits the generation of a perinatal population of interleukin-17A-producing γδ T cells to promote immunologic tolerance. Immunity 45: 999–1012. doi:10.1016/j.immuni.2016.10.023
Garman RD, Doherty PJ, Raulet DH (1986) Diversity, rearrangement, and expression of murine T cell gamma genes. Cell 45: 733–742. doi:10.1016/0092-8674(86)90787-7
Goodman T, LeCorre R, Lefrancois L (1992) A γT-cell receptor gamma delta-specific monoclonal antibody detects a V gamma 5 region polymorphism. Immunogenetics 35: 65–68. doi:10.1007/BF00216631
Goodman T, Lefrancois L (1989) Intraepithelial lymphocytes. Anatomical site, not T cell receptor form, dictates phenotype and function. J Exp Med 170: 1569–1581. doi:10.1084/jem.170.5.1569
Gray EE, Ramirez-Valle F, Xu Y, Wu S, Wu ZH, Kajalainen KE, Cyster JG (2013) Deficiency in IL-17-committed Vγ(γδ)4− γδ T cells in a spontaneous Sox11-mutant CD45.1+ congenic mouse strain provides protection from dermatitis. Nat Immunol 14: 584–592. doi:10.1038/ni.2585
Grigoriadou K, Boucontet L, Pereira P (2002) γT cell receptor-gamma allele-specific selection of V gamma 1/7 delta 4 cells in the intestinal epithelium. J Immunol 169: 3736–3743. doi:10.4049/jimmunol.169.7.3736
Guo Y, Sun X, Shibata K, Yamada H, Muta H, Podack ER, Yoshikai Y (2013) CD30 is required for activation of a unique subset of interleukin-17A-producing γδ T cells in innate immunity against Mycobacterium bovis Bacillus Calmette-Guerin infection. Infect Immun 81: 3923–3934. doi:10.1128/aii.00887-13
Haas JD, Ravens S, Düber S, Sandrock I, Oberdörfer L, Kashani E, Chennupati V, Föhse L, Naumann R, Weiss S, et al (2012) Development of interleukin-17-producing γδ T cells is restricted to a functional embryonic wave. Immunity 37: 48–59. doi:10.1016/j.immuni.2012.06.003
Hamada S, Umemura M, Shiono T, Tanaka K, Yahagi A, Begum MD, Oshiro K, Okamoto Y, Watanabe H, Kawakami K, et al (2008) IL-17A produced by gammadelta T cells plays a critical role in innate immunity against listeria monocytogenes infection in the liver. J Immunol 181: 3456–3463. doi:10.4049/jimmunol.181.5.3456
Havran WL, Allison JP (1990) Origin of Thy-1+ dendritic epidermal cells of adult mice from fetal thymic precursors. Nature 344: 68–70. doi:10.1038/344068a0
Havran WL, Grell S, Duwe G, Kimura J, Wilson A, Krüsebk AM, O’Brien RL, Born W, Tigelaaer RE, Allison JP (1989) Limited diversity of T-cell receptor gamma-chain expression of murine Thy-1+ dendritic epidermal cells revealed by V gamma 3-specific monoclonal antibody. Proc Natl Acad Sci U S A 86: 4185–4189. doi:10.1073/pnas.86.11.4185
Heilig JS, Tonegawa S (1986) Diversity of murine gamma genes and expression in fetal and adult T lymphocytes. Nature 322: 836–840. doi:10.1038/322836a0
Ito K, Bonnivelle M, Takagaki Y, Nakashima N, Kanagawa O, Kreccko EG, Tonegawa S (1989) Different gamma delta T-cell receptors are expressed on thymocytes at different stages of development. Proc Natl Acad Sci U S A 86: 631–635. doi:10.1073/pnas.86.1.631
Itohara S, Farr AG, Lafaille JJ, Bonnivelle M, Takagaki Y, Haas W, Tonegawa S (1990) Homing of a gamma delta thymocyte subset with homogeneous T-cell receptors to mucosal epithelia. Nature 343: 754–757. doi:10.1038/343754a0
Itohara S, Nakashima N, Kanagawa O, Kubo R, Tonegawa S (1989) Monoclonal antibodies specific to native murine T-cell receptor gamma delta: Analysis of gamma delta T cells during thymic ontogeny and in peripheral lymphoid organs. Proc Natl Acad Sci U S A 86: 5094–5098. doi:10.1073/pnas.86.13.5094
Jerne NK (1974) Towards a network theory of the immune system. Ann Immunol (Paris) 125C: 373–389.
Kappler JW, Starecz U, White J, Marrack PC (1988) Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. Nature 332: 35–40. doi:10.1038/332035a0
Kitaura K, Yamashita H, Ayabe H, Shini T, Matsutani T, Suzuki R (2017) Different somatic hypermutation levels among antibody subclasses disclosed by a new next-generation sequencing-based antibody repertoire analysis. Front Immunol 8: 389. doi:10.3389/fimmu.2017.00389
Koennecke C, Chennupati V, Schmitz S, Malissen B, Förster R, Prinz I (2009) In vivo application of mAb directed against the gammadelta TCR does not deplete but generates “invisible” gammadelta T cells. *Europ J Immunol* 39: 372–379. doi:10.1002/eji.200838741

Kohlgruber AC, Gai-Oz ST, LaMarche NM, Shimazaki M, Duquette D, Koay HF, Nguyen HN, Mina AI, Paras T, Takavoli A, et al. (2018) γδ T cells producing interleukin-17A regulate adipose regulatory T cell homeostasis and thermogenesis. *Nat Immunol* 19: 464–474. doi:10.1038/s41590-018-0094-2

Lafaille JJ, DeCloux A, Bonneville M, Takagaki Y, Tonegawa S (1989) Junctional sequences of T cell receptor gamma delta genes: Implications for gamma delta T cell lineages and for a novel intermediate of V-(D)-J joining. Cell 59: 859–870. doi:10.1016/0092-8674(89)90609-0

Mallick-Wood CA, Lewis JR, Richie LJ, Owen MJ, Tigelhaar RE, Hayday AC (1998) Conservation of T cell receptor conformation in epidermal gammadelta cells with disrupted primary Vγγ gene usage. *Science* 279: 1729–1733. doi:10.1126/science.279.5377.1729

Melandri D, Zlatareva I, Chaleil RAG, Dart RJ, Chancellor A, Nussbaumer O, Menshikov I, Beduleva L, Frolov M, Abisheva N, Khramova T, Stolyarova E, Ohno H, Ushiyama C, Taniguchi M, Germain RN, Saito T (1991) CD2 can mediate lymphocytes in normal mice. *Nature* 349: 1729–1732. doi:10.1038/3491729a0

Murakami T, Hatano S, Ikawa Y, Yoshikai Y (2016) Two types of interleukin-17-producing γδ T cells in protection against pulmonary infection with *Klebsiella pneumoniae*. *J Infect Dis* 214: 1752–1761. doi:10.1093/infdis/jiw443

Murphy AO, O’Keeffe KM, Lalor SJ, Maher BM, Mills KH, McLoughlin RM (2014) Staphylococcus aureus infection of mice expands a population of memory γδ T cells that are protective against subsequent infection. *J Immunol* 192: 3697–3708. doi:10.4049/jimmunol.1303420

Nakamura R, Shibata K, Yamada H, Hara H, Kishihara K, Yoshikai Y (2007) Resident Vδ1+ γδ T cells control early inﬁltration of neutrophils after *Escherichia coli* infection via IL-17 production. *J Immunol* 178: 5940–5947. doi:10.4049/jimmunol.181.05940

Shibata K, Yamada H, Nakamura M, Katsuragyi Y, Kominami R, Yoshikai Y (2014) Identification of CD25+ gamma delta T cells as fetal thymus-derived naturally occurring IL-17 producers. *J Immunol* 192: 2210–2218. doi:10.4049/jimmunol.1302145

Shibata K, Yamada H, Nakamura M, Hatano S, Katsuragyi Y, Kominami R, Yoshikai Y (2014) IFN-γ-producing and IL-17-producing γδ T cells differentiate at distinct developmental stages in murine fetal thymus. *J Immunol* 192: 2210–2218. doi:10.4049/jimmunol.1302145

Sonnag S, Maki K, Komagata Y, Miyazaki J, Ikuta K (1997) Developmentally ordered Vδ1− Vδ1+ recombination in mouse T cell receptor gamma locus is not perturbed by targeted deletion of the Vγm6 gene. *J Immunol* 158: 4223–4228.

Sun X, Shibata K, Yamada H, Guo Y, Muta H, Podack ER, Yoshikai Y (2013) CD30L/CD28 is critical for maintenance of IL-17A-producing gamma delta T cells bearing Vγ6 in mucosa-associated tissues in mice. *Mucosal Immunol* 6: 1191–1201. doi:10.1038/mi.2013.18

Sussman BI, Saito T, Shevach EM, Germain RN, Ashwell JD (1988) Thy-1- and Ly-6-mediated lymphokine production and growth inhibition of a T cell hybridoma require co-expression of the T cell antigen receptor complex. *J Immunol* 140: 2520–2526.

Sutton CE, Lalor SJ, Sweeney CM, Breerton CF, Lavelle EC, Mills KH (2009) Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* 31: 331–341. doi:10.1016/j.immuni.2009.08.001

Umemura M, Yahagi A, Hamada S, Begum MD, Watanabe K, Kawakami K, Suda T, Sudo K, Nakae S, Ikawa Y, et al. (2007) IL-17-mediated regulation of innate and acquired immune response against pulmonary *Mycobacterium* bovis bacille Calmette-Guerin infection. *J Immunol* 178: 3786–3796. doi:10.4049/jimmunol.178.6.3786

van Vliet E, Melsis M, Van Ewijk W (1984) Monoclonal antibodies to stromal cell types of the mouse thymus. *Eur J Immunol* 14: 524–529. doi:10.1002/eji.1803140608

Vautour G, Hayday A (2013) Six-of-the-best: Unique contributions of γδ T cells to immunology. *Nat Rev Immunol* 13: 88–100. doi:10.1038/nri3384

World Health Organization-International Union of Immunological Societies (WHO-IUIS) (1995) Nomenclature for T-cell receptor (TCR) gene
segments of the immune system. WHO-IUIS Nomenclature Sub-Committee on TCR Designation. *Immunogenetics* 42: 451–453.

Ye J, Ma N, Madden TL, Ostell JM (2013) IgBLAST: An immunoglobulin variable domain sequence analysis tool. *Nucleic Acids Res* 41: W34–W40. doi:10.1093/nar/gkt382

Yokoyama C, Katoh-Fukui Y, Morohashi K, Konno D, Azuma M, Tachibana T (2010) Production and characterization of monoclonal antibodies to mouse germ cells. *Hybridoma (Larchmt)* 29: 53–57. doi:10.1089/hyb.2009.0101

License: This article is available under a Creative Commons License (Attribution 4.0 International, as described at https://creativecommons.org/licenses/by/4.0/).