CD200R1 promotes the development of murine γδ17 T cells

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Research Article

CD200R1 promotes the development of murine γδ17 T cells

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Abstract

γδ T cells are enriched at barrier sites such as skin, gut, and lung, where they protect against cancer and infections, and promote healing. They detect diverse ligands in T-cell receptor–dependent or independent manners, producing large quantities of pro-inflammatory cytokines. γδ T cells develop in fetal thymi in temporally controlled waves where, unlike αβ T cells, many γδ T cells adopt their effector fate, becoming either IFN-γ or IL-17A producers (γδ17 T cells).

CD200R1 suppresses myeloid cell activity but has also been shown to promote innate lymphoid cell IL-17A production, enhancing psoriasis-like skin inflammation. γδ17 T cells are potent IL-17A producers in skin. Therefore, the effect of CD200R1 on IL-17A production by γδ17 T cells was investigated using CD200R1KO mice. CD200R1 was revealed to promote IL-17A production by γδ T cells in skin and lymphoid organs. Although CD200R1 is not expressed by adult γδ T cells, it is expressed by immature developing γδ T cells in fetal thymus, where it supports the development of γδ17 T cells, enhancing γδ17 T-cell and RORγt$^+$ γδ T-cell numbers in fetal thymic organ cultures. This identifies CD200R1 as an important novel regulator of γδ17 T-cell development in early life, a key process for ensuring immunity, particularly at barrier sites.

Keywords: γδ T cells, CD200R1, development, IL-17A, skin

1. Introduction

γδ T cells are rare in many tissues but are enriched at barrier sites such as the skin, lung, and gut, where they are crucial for wound healing and protecting against infections and cancer.[1–5] However, they also can drive inflammatory disease.[6] γδ T cells are potent and rapid producers of inflammatory cytokines, being activated in ways either dependent or independent of T-cell receptors (TCRs). Like conventional αβ T cells, γδ T cells rearrange their *Tcr* genes in the thymus; however, γδ TCRs are less variable than αβ TCRs[5] and lack major histocompatibility complex (MHC) restriction. Instead, γδ TCRs recognize a range of ligands, including stress-induced MHC-like molecules, phosphorylated metabolites, and lipid antigens presented by CD1 molecules.[7] The ability of γδ T cells to be activated via innate-like receptors allows the rapid induction of immune defense against pathogens, even to those not previously encountered. γδ T cells also maintain tissue homeostasis, are important in tissue surveillance at barrier sites, and play crucial antitumor roles.[4,8] These functions of γδ T cells are principally fulfilled via cytokine secretion and cytotoxic activity, but these cells can also act as antigen-presenting cells, driving adaptive immune responses.[9]

γδ T cells can be classified based on their effector capabilities, being either IFN-γ or IL-17A producers, with effector phenotypes conferred by the lineage-defining transcription factors T-bet or RORγt, respectively. In mice, the markers CD27 and CD44 correlate relatively well with IFN-γ– and IL-17A–producing capability, respectively. Concomitantly with expressing rearranged γδ TCR chains, γδ T cells also acquire their effector fate, with murine Vγ1 and Vγ5 cells (Tonegawa nomenclature[10]) mainly gaining IFN-γ–producing potential, and Vγ4 and Vγ6 T cells largely gaining IL-17A–producing potential.[11] Although the Vγ TCR chain expressed is not the determining factor in effector fate acquisition,[12,13] *Vγ* gene order on the *Tcrγ* locus is important.[14]

In mice, the development of γδ T cells occurs in ordered waves comprising different subsets, beginning at embryonic day 15 of gestation with the appearance of Vγ5 (dendritic epidermal T cells [DETCs]), which home to the epidermis.[15] This is followed by the development of Vγ6 cells, which home to dermis, uterus, and the peritoneal cavity. Vγ4 cells develop shortly after and home to lung, dermis, and lymph nodes (LNs).[16] Then, perinatally, Vγ7 (intraepithelial lymphocytes [IEL]) develop and home to the intestine. Perinatal progenitors are required for generating IL-17–producing γδ (γδ17) T cells in the absence of inflammation[13]; however, γδ17 T cells can be generated de novo from adult bone marrow–derived precursors in response to inflammation,[17] and Vγ4 and Vγ1 T cells continue to develop later in life but are "adaptive γδ T cells," retaining the ability to adopt multiple effector fates in the periphery.[18] Factors governing the sequential development of γδ T-cell subsets are not completely understood, but both TCR and environmental signals are important.

TCR signal strength plays a crucial role in murine γδ T-cell development. CD4$^-$ CD8$^-$ double-negative cells receiving strong TCR

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signals commit to the γδ lineage, and strong TCR signaling is key for IFN-γ–producing γδ T-cell development.[19,20] However, weaker TCR signals are required for γδ17 T-cell development,[16,19–22] where distinct TCR-signaling pathways are engaged, specifying γδ17 T-cell fate.[23] Negative regulators of TCR signaling, such as the SRC family kinase BLK, aid the development of γδ17 T cells,[24] as does the transcription factor cMAF, which drives expression of the lineage-defining transcription factor RORγt. TCR signal strength regulates cMAF levels, with strong TCR signals reducing, and weaker TCR signals increasing, cMAF levels and thus driving RORγt and reinforcing γδ17 T-cell effector fate.[25]

Cytokines also regulate γδ T-cell development, with IL-7Rα required for V-J *γδ Tcr* recombination.[26–30] IL-7 signaling particularly affects γδ17 T cells, because high levels of IL-7/IL-7Rα signaling favor IL-17 production,[31,32] and IL-7Rα is required for the homeostasis of γδ17 T cells.[33] Lymphotoxin signaling is key for the development of both IFN-γ–producing and γδ17 T cells, where lymphotoxin βR drives the NF-κB family member RELB, which, in turn, maintains RORγt expression.[34] TGF-β also drives γδ T-cell IL-17 production in early life,[35] although the mechanism is not yet understood.

Medullary thymic epithelial cells (mTECs) and cortical TECs (cTECs) are crucial for conventional αβ T-cell development, supporting positive and negative selection and providing NOTCH signals and IL-7.[36] However, less is known about their role in supporting γδ T-cell development. In mice, cTECs promote Vγ4 T-cell activity but are not required for Vγ6 T cells.[37] Conversely, mTECs are important for Vγ5 DETC development because they provide signals via the butyrophilin-related protein SKINT-1.[38] Thymic dendritic cells (DCs) also contribute to conventional αβ T-cell development, but a role in γδ T-cell development has not been defined. Despite advances in our understanding of how γδT-cell development is regulated, factors involved in this process remain incompletely understood.

CD200R1 is a cell surface receptor expressed on many immune cell types, including T cells (both αβ and γδ), natural killer cells, antigen-presenting cells, subsets of granulocytes, and innate lymphoid cells (ILCs) in human skin[39]; and on mast cells, ILCs macrophages, monocytes, DCs, Langerhans cells, and a subset of both αβ and γδ T cells in murine skin; as well as being expressed on neutrophils in murine bone marrow.[39,40] CD200R1 expression is particularly high on type 2 immune cells, including basophils, and ILC2 and Th2 T cells.[41] Interestingly, it was recently shown that *Cd200r1* is downregulated in Th17 cells by *Staphylococcus aureus* α hemolysin,[42] suggesting that *Cd200r1* expression levels are regulated by microbial factors. CD200R1 inhibits pro-inflammatory cytokine production and mast cell degranulation in myeloid cells.[43,44] However, conversely, CD200R1 is required for efficient IL-17 production by ILCs, and CD200R1 promotes inflammation in a psoriasis model and controls cutaneous fungal infections,[39,40] demonstrating that CD200R1 has diverse functions in different cell types, including promoting IL-17 production.

Given that γδ T cells are crucial for producing IL-17 in skin and drive psoriasis-like and antifungal immune responses,[45–47] the role of CD200R1 in regulating murine γδ T-cell IL-17 production was investigated. We show that CD200R1-deficient (KO) mice have impaired IL-17 production by γδ T cells, due to reduced γδ17 T cells, with IFN-γ–producing cells not being affected. Although CD200R1 is not expressed by γδ T cells in adults, it is expressed on subsets of developing γδ T cells in neonates during the developmental window for γδ17 T cells. The reduction in γδ17 T cells is also observed during this period, demonstrating a role for CD200R1 in supporting the development of γδ17 T cells. Therefore, here we identify, for the first time to our knowledge, CD200R1 as a key factor required for the development of murine γδ17 T cells, shaping immunity at barrier sites.

2. Materials and methods

2.1 Mice

All animal experiments were locally ethically approved and performed in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986. C57BL/6 (WT) mice were obtained from Charles River Laboratories. CD200R1KO mice[48] on a C57BL/6 background, were bred and maintained in specific pathogen-free conditions in-house. Mice were male and 7 to 12 wk old at the start of procedures, unless otherwise stated. Where neonatal tissues and fetal thymi were used, sex was not determined.

2.2 Skin cell isolation

Ears were split in half and floated on 0.8% weight per volume trypsin (Sigma-Aldrich) at 37 °C for 30 min, then were chopped and digested in 0.1 mg/mL (0.5 Wunch units/mL) Liberase (Roche) at 37 °C for 1 h. The fat was removed from dorsal skin before floating on trypsin, as was done with ears. Tissue was chopped and digested with 1 mg/mL Dispase II (Roche) at 37 °C for 1 h. Digested skin tissue suspensions were passed through 70-μm cell strainers, washed, and counted.

2.3 LN, thymus, and spleen cell isolation

Inguinal, axillary, and brachial LNs, thymi, or spleens were passed through 70-μm cell strainers and washed. Red blood cells present in the splenocyte samples were lysed on ice for 3 min with ACK lysing buffer (Lonza) before cells were washed and counted.

2.4 Flow cytometric analysis of cells

Cells were incubated with 0.5 μg/mL anti-CD16/32 (2.4G2, BD Bioscience) and either Blue or Near-IR Dead Cell Stain (Invitrogen) (except when annexin V/7AAD staining was performed) prior to staining with fluorescently labeled antibodies. Cells were fixed with the FOXP3/Transcription Factor Buffer Staining Set (eBioscience) for between 30 min and 16 h at 4 °C.

For cytokine analysis, 10 μM Brefeldin A was added to cell cultures for 4 h prior to staining for cell surface markers, as described above. After overnight fixation, cells were permeabilized with the FOXP3/Transcription Factor Buffer Staining Set (eBioscience) and were stained with antibodies against intracellular markers or cytokines. Cells were analyzed on a Fortessa, LSRII (both BD Biosciences), or Cytoflex (Beckman Coulter) flow cytometer. Data were analyzed using FlowJo (TreeStar). Antibodies are detailed in Table S1.

For annexin V/7AAD staining, cells were stained on ice for cell surface markers, then were stained with annexin V (BioLegend) following the manufacturer's instructions. Then 7AAD (BioLegend) was added and cells were analyzed within 2 h.

For pSTAT3 staining, inguinal, axillary, and brachial LN cells were stained for surface markers, then stimulated with 100 ng/mL IL-23 (BioLegend) for 15 min. Cells were fixed with Phosflow Fix Buffer I (BD Biosciences) at 37 °C for 10 min.

For pERK staining, skin cells were isolated and cultured for 30 min to acclimatize. Cells were then stimulated for 10 min with either 50 ng/mL phorbol myristate acetate (PMA) (Sigma-Aldrich) or 1 μg/mL eF450-conjugated anti-TCR γδ, then were stained with extracellular antibodies on ice for 15 min.

Cells were washed and fixed with Phosflow Lyse/Fix Buffer (BD Biosciences).

After fixation, cells were permeabilized at 4 °C in Phosflow Perm Buffer III (BD Biosciences) for 30 min before staining with either phycoerythrin-conjugated pSTAT3 (pY705) (BD Bioscience, clone 4/P-STAT3) or pERK1/2 (pT202/pY204) (eBiosciences, clone MILAN8R) at room temperature for 30 min, before washing and analyzing by flow cytometry, as detailed above.

2.5 In vitro γδ T-cell activation

Mouse dorsal skin cells were cultured in complete Roswell Park Memorial Institute (RPMI) medium (RPMI 1640 supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin solution, 2 mM L-glutamine, 1 mM sodium pyruvate, 20 mM HEPES, 1× nonessential amino acid solution, and 25 nM 2-mercaptoethanol [Sigma-Aldrich]) and stimulated with 40 ng/mL IL-23 (BioLegend) for 16 h. Cells were cultured with 10 μM Brefeldin A (Sigma-Aldrich) for 4 h before staining for flow cytometric analysis. Alternatively, dorsal skin cells, thymocytes, splenocytes, or LN cells were stimulated with 50 ng/mL PMA and 500 ng/mL ionomycin (Sigma-Aldrich) and 10 μM Brefeldin A for 4 h before staining for flow cytometric analysis.

For co-culture of wild-type (WT) and CD200R1KO cells, the cells were isolated from dorsal skin, then either the WT or CD200R1KO cells were labeled with 10 μM eF450-conjugated cell proliferation dye (eBioscience) for 10 min in the dark at 37 °C before washing and co-culturing with unlabeled cells.

2.6 Quantitative PCR to detect *Cd200r1* in neonatal and adult γδ T cells

Two-day-old neonatal body skin and adult dorsal skin was removed from euthanized C57BL/6 mice and cells were isolated as detailed in section 2.3. Cells were stained with a dead-cell dye and antibodies, as described in section 2.4, before isolation of the dermal γδ T cells (live $CD45^+$ $CD3^{mid}$ $TCR\gamma\delta^{mid}$) by flow cytometric sorting on a MA900 cell sorter (Sony). RNA (from 1,000 to 6,000 cells per sample) was isolated using the RNeasy plus micro kit (QIAGEN) before cDNA was synthesized using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems), following the manufacturer's instructions. Quantitative PCR (qPCR) was carried out in duplicate using the cDNA equivalent of 3.6 to 14 ng RNA per reaction and PowerUp SYBR Green Master Mix (Applied Biosystems) with 100 nM primers and cycles of 50 °C for 2 min, 95 °C for 3 min, then 40 cycles of 95 °C for 30 s, 55 °C for 45 s, and 60 °C for 90 s on a CFX Opus machine (Bio-Rad). Primers used for *Cd200r1* were 5′-TGTGAGACAGTAACA CCTGAAGG-3′ and 5′-TGCCATTGCCTCACACACTGCA-3′ and for *Hprt* were 5′-GCTGACCTGCTGGATTACATTAA-3′ and 5′-TGATC ATTACAGTAGTCTTTCAGTCRGA-3′. Melt curves were checked for single peaks at the correct melting temperature, and relative gene expression was calculated using the comparative cycle threshold method.

2.7 Fetal thymic organ cultures

Fetal thymic organ cultures (FTOCs) were set up and analyzed as described previously.[16] Briefly, E15-15.5 embryos were obtained from WT or CD200R1KO timed matings. Fetal thymic lobes were dissected and cultured on nucleopore membrane filters (Whatman) (4 to 5 per filter) in complete RPMI (as described in section 2.5) for 8 d. Where necessary, cells were stimulated with 50 ng/mL PMA (Sigma-Aldrich) and 500 ng/mL ionomycin (Sigma-Aldrich) with 10 μM Brefeldin A (Sigma-Aldrich) and 2 μM monensin (eBioscience) for 4 h. Thymic lobes were homogenized using a syringe plunger and were stained and analyzed by flow cytometry, as described above. To count cells, Precision count beads (BioLegend) were added to each FTOC sample prior to analysis.

2.8 Statistical analysis

Data were analyzed for normal distribution by Shapiro-Wilks test and were then analyzed by appropriate statistical tests. Statistically significant differences were determined using Student's *t* tests, or Mann-Whitney *U* tests for nonparametric data. Where groups of data had unequal variance, a Welch's *t* test was used. All statistical tests were performed using Prism software (GraphPad Software Inc.). Values of $P < 0.05$ were considered significant. All experiments were performed at least twice, with at least 3 independent samples per group.

3. Results

3.1 CD200R1 is required for efficient IL-17A production by γδ T cells

CD200R1-deficient mice have a reduced ability to produce IL-17A in both psoriasis and cutaneous fungal infection models.[39] Given the importance of γδ T cells for IL-17A production in these models, we measured IL-17A production by KO cutaneous γδ T cells (Fig. 1A), specifically examining the TCR $\gamma\delta^{int}$ dermal population containing IL-17–producing cells, in contrast to TCR $\gamma\delta^{hi}$ DETCs, which are less capable of IL-17 production.[49] This analysis demonstrated that CD200R1 is required for efficient IL-17A production by murine dermal γδ T cells in response to either IL-23 or PMA and ionomycin stimulation (Fig. 1B).

CD200R1 suppresses cytokine production in myeloid cells; therefore, we hypothesized that KO cultures may contain an overactive cell type that inhibits γδ T-cell IL-17 production. To test this, KO murine dorsal skin cells were fluorescently labeled and co-cultured with unlabeled WT cells before stimulation. KO γδ T cells retained an impairment in IL-17A production when co-cultured (Fig. 1C), demonstrating that KO cultures do not contain factors that inhibit IL-17 production by γδ T cells. Therefore, γδ T cells are impaired in IL-17A production when isolated from KO skin.

To determine if CD200R1 affects γδ T-cell cytokine production in a tissue-specific or γδ T-cell subset–specific manner, cells from spleen, LN. and thymus were stimulated with PMA and ionomycin, and IL-17A and IFN-γ production by γδ T cells was measured by flow cytometry. WT and KO γδ T cells had a similar ability to produce IFN-γ, but KO γδ T cells were impaired in IL-17A production in each tissue examined (Fig. 1D), showing that CD200R1 promotes IL-17A production by γδ T cells but does not affect IFN-γ production.

3.2 CD200R1 deficiency reduces γδT17 cell populations and impairs their ability to produce IL-17

To determine if γδ T-cell population numbers are affected by CD200R1, γδ T-cell subsets were examined in thymus, spleen, LN, and dermis, based on $CD44^{hi}$ and $CD27^+$ expression, corresponding largely to IL-17A and IFN-γ producers, respectively.[21] In all these tissues, the proportion of $CD44^{hi}$ γδ T cells was reduced in KO mice (Fig. 2A), demonstrating that CD200R1 is required to maintain the balance between γδ17 T cells and IFN-γ–producing γδ T-cell populations. Examining γδ T-cell numbers showed reduced γδ T cells in LNs and dermis in CD200R1KO mice, but similar overall numbers in thymus and spleen (Fig. 2B). γδ T cells take on

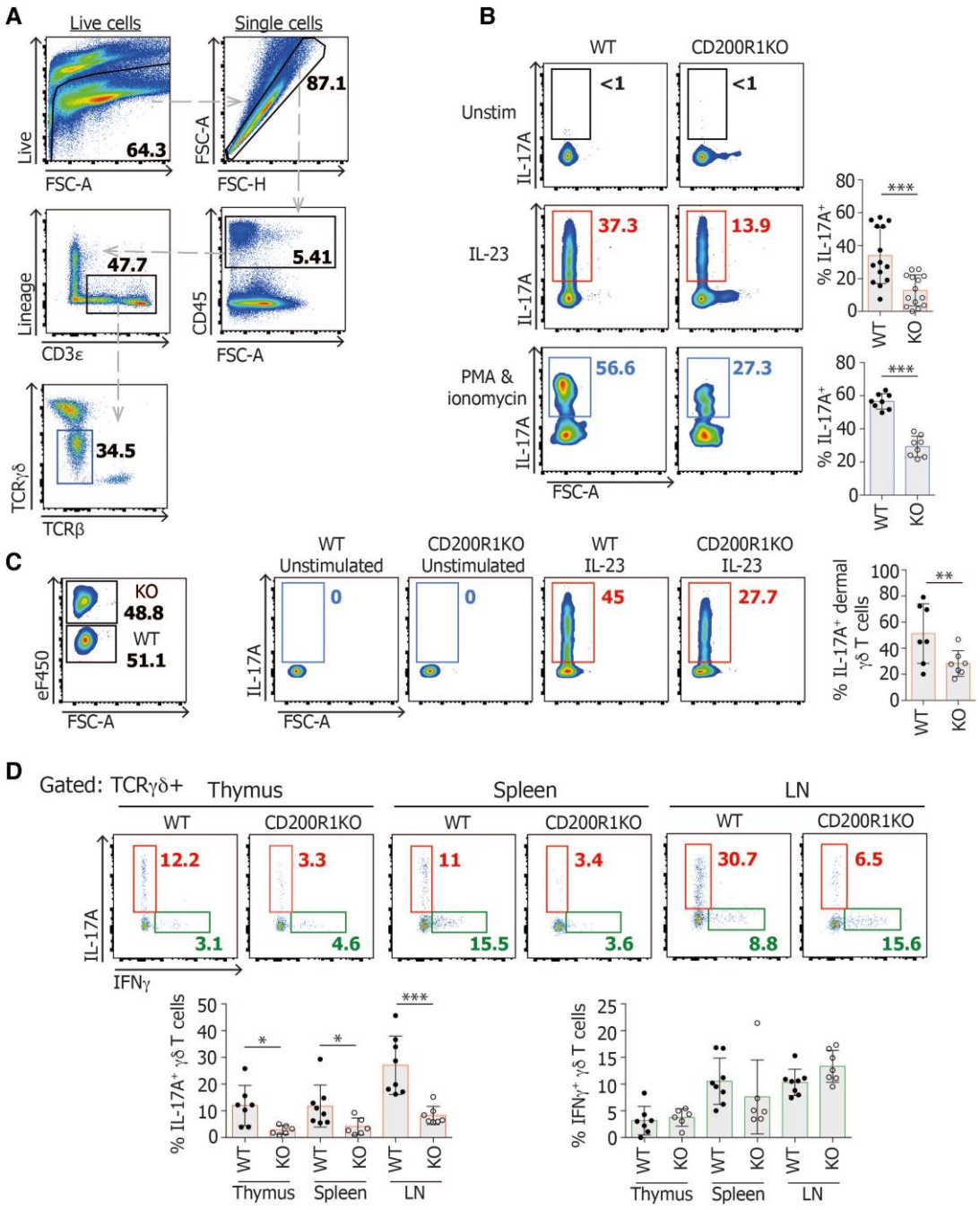

Fig. 1. γδ T cells from KO mice have an impaired ability to produce IL-17A. Cells were isolated from WT or KO mouse dorsal skin, then were placed in culture and stimulated with 40 ng/mL IL-23 overnight, or 50 ng/mL PMA and 500 ng/mL ionomycin for 4 h. IL-17A production within the TCRγδ^{low} cells was analyzed by flow cytometry. A) Gating strategy for TCRγδ^{low} cells. B) IL-17A production by TCRγδ^{low} cells. IL-23 stimulation, $n = 14$ per group; PMA/ionomycin stimulation, $n = 8$ per group. C) Prior to culture and stimulation, KO cells were labeled with a fluorescent dye and then mixed with unlabeled WT cells. $n = 7$ per group. D) Cells were isolated from thymus, spleen, and LNs and were stimulated with 50 ng/mL PMA and 500 ng/mL ionomycin for 4 h, before flow cytometric analysis for IL-17A and IFN-γ production by γδ T cells. For thymus WT, $n = 6$; KO, $n = 7$; and $n = 8$ per group for spleen and LN. Data points indicate individual mouse data. Numbers on flow plots show percentages of cells in each gate. Data are pooled from at least 2 independent experiments. Student's t test was used to determine statistically significant differences except where data are not normally distributed, in which case Mann-Whitney U tests were used [in (D), for IL-17 and IFN-γ production in spleen and IL-17 production in LN]. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Unstim, unstimulated.

their effector fate during development in the thymus, when they rearrange their *Tcr* loci and express specific Vγ and δ TCR chains, with Vγ4$^+$ cells being the major γδ17 T-cell subset in skin. To determine if CD200R1 affects specific populations of γδ T cells, inguinal LN cells were gated into Vγ4$^+$, Vγ1$^+$, and non-Vγ4– or Vγ1-expressing populations, demonstrating that the Vγ4$^+$ subset specifically was reduced in the absence of CD200R1 (Fig. 2C). Similarly, the number of Vγ4$^+$ cells was reduced in KO skin (Fig. 2D). Therefore, CD200R1 promotes either the generation or maintenance of Vγ4$^+$ cells.

No increase in the proportion of apoptotic Vγ4$^+$ T cells or the proliferation of these cells was observed in the absence of

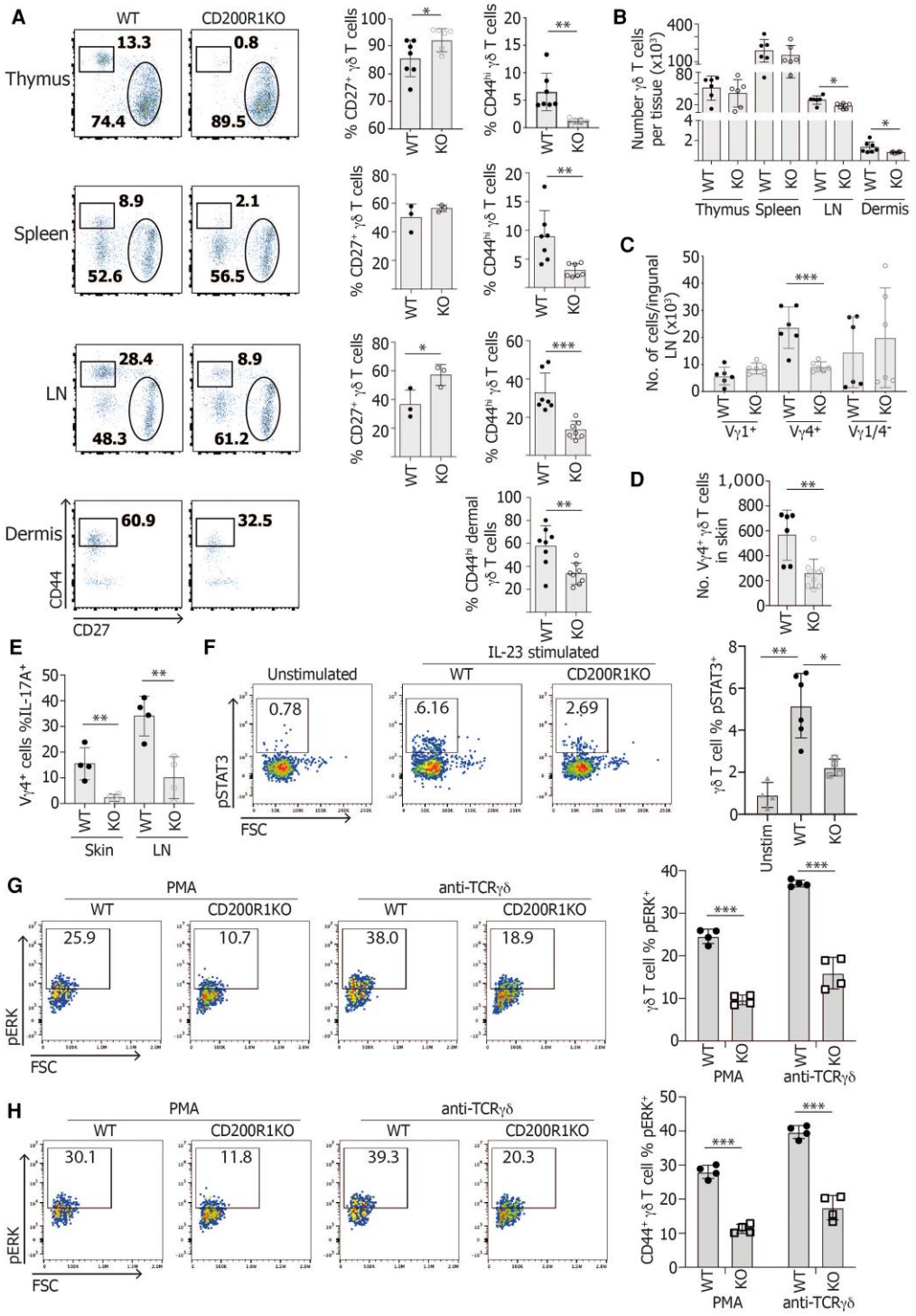

Fig. 2. CD200R1-deficient mice have a reduced population of γδ17 T cells. Cells were isolated from thymus, spleen, LN (inguinal axillary and brachial), and skin of WT and KO mice and were analyzed by flow cytometry. A) Proportion of γδ T cells expressing high levels of CD44 (γδ17 T cells) or expressing CD27 (cells with potential for IFN-γ production). For the $CD27^+$ population, thymus, $n = 7$, and spleen and LN, $n = 3$; for $CD44^{hi}$, $n = 7$, and $n = 8$ for dermis. B) Numbers of γδ T cells in thymus, spleen, and LN, or $TCR\gamma\delta^{low}$ T cells in skin. $n = 6$ per group. C) Numbers of γδ T cells within each Vγ subset in 1 inguinal LN. $n = 6$ per group. D) The numbers of $V\gamma4^+$ T cells in skin: WT, $n = 6$; KO, $n = 10$. E) Proportion of $V\gamma4^+$ cells producing IL-17A after PMA and ionomycin stimulation. $n = 4$ per group. F) LN cells were stimulated with IL-23 for 10 min and analyzed by flow cytometry for pSTAT3 within the γδ T-cell gate. $n = 4$ to 6 per group, as indicated by data points. G and H) Skin cells were stimulated with either PMA or anti-TCR γδ antibodies for 10 min and analyzed by flow cytometry for pERK1/2. G) Gated on γδ T cells. H) Gated on $CD44^{hi}$ γδ T cells. $n = 4$ per group. Data points indicate individual mouse data. At least 2 independent experiments were performed, except in (G and H), which report data from 1 experiment. Numbers on flow plots show percentages of cells in each gate. Student's *t* test was used to determine statistically significant differences except where data are not normally distributed, in which case Mann-Whitney *U* tests were used [namely, $CD44^{hi}$ data in spleen and LN in (A), and the $V\gamma1/V\gamma4^-$ population in (C)]. Where variances were significantly different [ie thymus $CD44^{hi}$ in (A) and skin data in (E)], Welch correction was used. Where there are 3 data groups (F), a Browne and Forsythe and Welch ANOVA was used. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. FSC, forward scatter; Unstim, unstimulated.

CD200R1 (Fig. S1), suggesting that CD200R1 affects the generation of Vγ4$^+$ cells rather than their maintenance.

To determine if CD200R1 promotes γδ T-cell IL-17 production independent of its effects on the numbers of these cells present, skin and LN Vγ4 T cells specifically were examined for IL-17A production capability. A profound reduction in the proportion of Vγ4 T cells producing IL-17 was observed in the absence of CD200R1 (Fig. 2E), revealing that CD200R1 promotes IL-17 production by these cells not merely via increasing the numbers of these cells.

To gain mechanistic insight into how CD200R1 promotes IL-17 production by γδ T cells, signaling downstream of activation was examined. LN cells were stimulated with IL-23 and the resulting phosphorylation of STAT3 within γδ T cells was examined by Phosflow. We found that in the absence of CD200R1, γδ T cells were less able to signal downstream of IL-23 stimulation (Fig. 2F). Similarly, in the absence of CD200R1, stimulation of skin cells with either PMA or anti-TCR γδ antibody led to a blunted pERK response in either the total γδ T-cell population (Fig. 2G) or, indeed, in the CD44hi γδ T-cell subset (Fig. 2H). Together, these findings demonstrate that CD200R1 promotes signaling in response to IL-23 or TCR activation in IL-17–producing subsets of γδ T cells.

3.3 CD200R1 is not expressed by adult γδ T cells but is expressed by these cells during their development

Previously, only low levels of CD200R1 expression were observed in murine dermal γδ T cells,[39,40] but specific subsets were not examined. To determine if CD200R1 expression is specific to Vγ4$^+$ cells, flow cytometric staining for CD200R1 was carried out, revealing that both cutaneous Vγ4$^+$ and Vγ1$^+$ T cells express exceptionally low levels of CD200R1 (Fig. 3A). This suggests that CD200R1 does not directly affect mature Vγ4$^+$ T cells but may instead have either indirect effects via another cell type or may have effects during the development of these cells if CD200R1 is expressed earlier in development. In support of the effects of CD200R1 being via development, IL-17 production by γδ T cells was affected in thymus (Fig. 1D), and the maintenance of Vγ4$^+$ T cells, measured by apoptosis and proliferation frequency, was not affected by CD200R1 (Fig. S1). Therefore, the thymus, as the site of γδ T-cell development, was examined to determine CD200R1 expression. CD200R1 was observed on a proportion of both CD11b$^+$ and CD11c$^+$ thymic DCs (Fig. 3B) but was absent from both cortical and medullary thymic epithelial cells (Fig. 3C), suggesting that effects of CD200R1 on adult thymus may be via thymic DCs.

Although γδ T cells can develop in adult mice, the most significant generation of these cells, certainly in the absence of inflammation, occurs early in life. Vγ4$^+$ and Vγ6$^+$ T cells, subsets capable of IL-17 production, develop in specific waves during fetal development and seed the skin to become resident populations. Indeed, CD200R1 expression on γδ T cells is inversely correlated with age, with high CD200R1 levels on γδ T cells from neonatal mice (Fig. 3D), suggesting that CD200R1 may have important effects in early life. Interestingly, this difference in CD200R1 expression at the protein level is not mirrored by differences in *Cd200r1* mRNA levels between neonatal and adult skin γδ T cells (Fig. 3E), suggesting that regulation of CD200R1 levels may be post-transcriptional.

To determine which cells types express CD200R1 during γδ17 T-cell development, FTOCs were examined. Similar to adult thymus, CD200R1 was only expressed at low levels by thymic epithelial cells (Fig. 3F) but was expressed more highly by populations of thymic DCs, particularly those expressing high levels of MHC class II (Fig. 3G). In contrast to the negligible expression of CD200R1 on adult γδ T cells, CD200R1 was expressed by developing fetal γδ T cells, particularly the CD44^{hi} subset of less mature (CD24$^+$) γδ T cells (Fig. 3H). In addition to these very early γδ T cells, CD200R1 was expressed by more mature developing γδ T cells (CD24^{neg}), including IFN-γ–producing subsets as indicated in Fig. 3H (population B, CD44^{neg} CD45RB$^+$; and population C, CD44$^+$ CD45RB$^+$) and IL-17–producing subsets (population D, CD44$^+$ CD45RB^{neg}) after stimulation. Together, these findings revealed that CD200R1 is expressed in fetal thymus by DCs and developing γδ T cells, and expression levels are influenced by T-cell activation, suggesting a role for CD200R1 during this developmental window.

3.4 CD200R1 promotes fetal γδT17 cell development

The impact of CD200R1 on fetal γδ T-cell development was examined using FTOCs. Total numbers of γδ T cells were not affected by CD200R1 deficiency (Fig. S2A), but the overall number of γδ17 T cells and both proportions and numbers of RORγt-expressing γδ T cells were reduced in KO FTOCs (Fig. 4A), showing that CD200R1 supports developing γδT17 cell expression of RORγt and IL-17.

To determine the stage of γδT17 development affected by CD200R1, cells were gated based on CD24 expression. Examination of the less mature CD24$^+$ populations showed distinct populations based on CD44 expression (Fig. 4 and Fig. S2). In KO FTOCs, the CD24$^+$ CD44^{hi} population was reduced in both proportion and number, and KO FTOCs also had reduced proportions and numbers of RORγt-expressing cells and reduced numbers of IL-17–producing CD24$^+$ γδ T cells (Fig. 4B), demonstrating that CD200R1 supports the early development of γδ17 T cells.

The CD24$^-$ γδ T-cell population contains more mature developing cells and, again, although the absence of CD200R1 largely did not affect the numbers of developing CD24^{neg} γδ T cells in each subpopulation (Fig. S2C), CD200R1 promoted IL-17 production by these cells (Fig. 4C). Overall, these data demonstrate a requirement for CD200R1 for efficient acquisition of effector function (RORγt expression and IL-17 production) in γδ17 T cells, which is observed from the very early stages of development. These findings show CD200R1 is required to support the development of murine γδ17 T cells.

4. Discussion

γδ T cells are critical immune cells, being able to rapidly respond in both innate and adaptive manners, producing large amounts of IFN-γ or IL-17. Here we show that mice deficient for CD200R1 have fewer γδ17 T cells (Fig. 1) due to a requirement for CD200R1 for efficient development of γδ17 T cells and for their acquisition of effector function. This was observed most clearly in FTOCs in which CD200R1 promoted RORγt expression and IL-17 production (Fig. 4).

CD200R1 may affect γδ17 T-cell development via indirect effects involving other cell types. This would most likely be via thymic DCs, which have high expression levels of CD200R1 (Fig. 3C and G); however, a clear role for these cells in γδ T-cell development is yet to be defined. cTECs are crucial for Vγ4 T-cell activity[37] and support αβ T-cell development by providing NOTCH

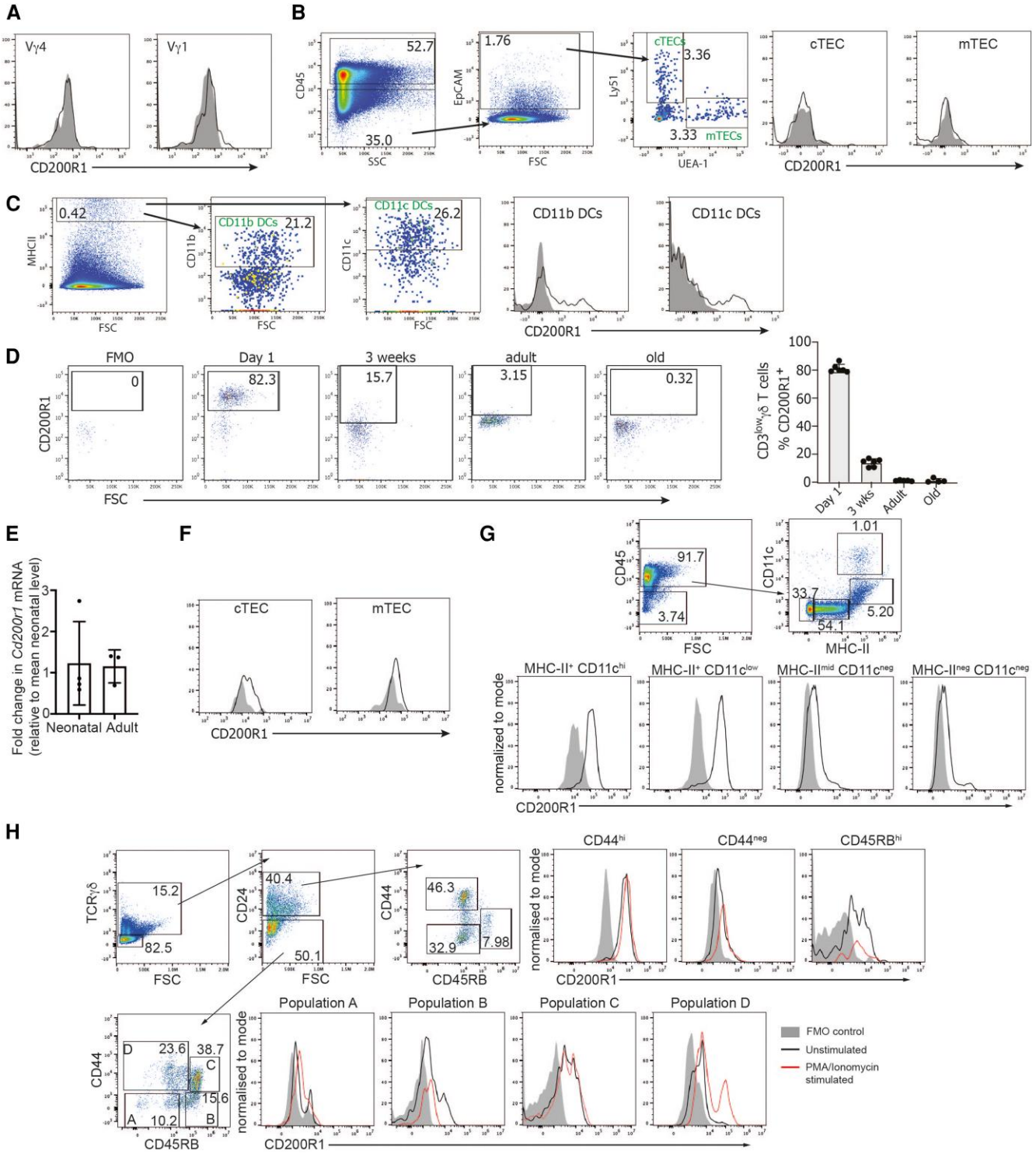

Fig. 3. CD200R1 is not expressed by murine adult γδ cells but is expressed by developing γδ cells and by thymic DCs. Flow cytometry was used to determine cell types expressing CD200R1 in skin or thymus from mice of different ages. Gray histograms depict fluorescence minus 1 (FMO) controls for CD200R1 staining, black lines depict CD200R1 staining on unstimulated cells, and red lines depict CD200R1 staining after 3-h stimulation with PMA and ionomycin. A) Expression of CD200R1 on cutaneous $V\gamma4^+$ and $V\gamma1^+$ T cells. B) Representative (adult mouse thymus) flow cytometric gating on cTEC ($CD45^-$ $EpCAM^+$ Ly-51^+) and mTEC ($CD45^-$ $EpCAM^+$ UEA-1^+) and CD200R1 expression on these populations. C) Representative (adult mouse thymus) flow cytometric gating on DC populations ($CD45^+$ $MHCII^{hi}$ $CD11b^+$ or $CD11c^+$) and CD200R1 expression on these populations. D) CD200R1 expression in cutaneous γδ T cells in 1-day-old, 3-week-old, adult (8 to 12 wk), or old (10 mo) mice. E) Dermal $CD3^{mid}$ γδ T cells were flow cytometrically sorted from adult dorsal skin and 2-day-old neonatal trunk skin. qPCR was performed to detect *Cd200r1* and *Hprt* (housekeeping control gene). Data shown are fold change in expression relative to the mean of the neonatal data. F) Representative CD200R1 expression on FTOC cTEC and mTEC populations. G) Thymic DC gating in FTOCs and CD200R1 expression on each subset. H) CD200R1 expression on populations of developing γδ T cells in FTOCs. $CD24^+$ cells are less mature, whereas $CD24^{neg}$ are more mature and can be split into subsets capable of producing IFN-γ (populations B and C), and IL-17 (populations A and D). Plots shown are representative of at least 2 independent experiments and contain data from at least 3 mice.

Fig. 4. CD200R1 is required for optimal development of IL-17$^+$ and RORγt$^+$ γδ T cells. WT and KO FTOCs were set up and cultured for 8 d. Cells were stimulated with PMA and ionomycin for 3 h, and cell populations were examined by flow cytometry. Data are normalized to the average of the WT data for each individual experiment. Proportions and cell numbers of populations within the total γδ T-cell population (A), CD24$^+$ γδ T-cell population (B), and CD24^{neg} γδ T-cell population (C). Data points indicate individual FTOC data. Data are pooled from 2 independent experiments. $n = 9$ for WT, $n = 13$ for KO. Student's t test was used to determine statistically significant differences, with Welch's correction for unequal variance applied where required: number CD44^{hi}, %CD44^{low}, number CD44^{low}, %RORγt$^+$ in (B); and %IL-17$^+$, number RORγt$^+$ in (C). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

signals and IL-7,[36] which could also be important for supporting γδ17 T-cell development. However, CD200R1 expression is low on these cells (Fig. 3B and F), which suggests that any effects of CD200R1 on these cells would not be direct.

The mechanism by which CD200R1 promotes γδ17 T-cell development is therefore likely to be via a cell-intrinsic mechanism, perhaps via suppression of TCR signaling or via effects on other signaling pathways. CD200R1 is part of the immunoglobulin superfamily and is an immune checkpoint pathway being blocked therapeutically in cancer to promote immune responses and allow the immune system to target the tumor.[50] However, the evidence for CD200R1 specifically affecting T cells or TCR signaling is weak. Cytotoxic T-cell function is inhibited by CD200R1 signaling,[51] and blocking CD200R1 signaling promotes TCR-driven CD4$^+$ T-cell proliferation in patients with lupus, but there was no effect on healthy T cells.[52] Therefore, a direct effect of CD200R1 on T-cell activation or TCR signaling may be likely but remains to be proven. Signaling in response to γδ T-cell activation, including phosphorylation of STAT3 in response to IL-23 stimulation and phosphorylation of ERK in response to PMA or TCR γδ activation, was reduced in the absence of CD200R1 (Fig. 2F–H), demonstrating that CD200R1 is required for efficient signaling in response to activation in γδ cells. However, because CD200R1 was expressed at very low levels on adult skin γδ T cells (Fig. 3A), it seems likely that these effects are due to a role for CD200R1 in the earlier development or differentiation of these cells, and further research will be necessary to fully elucidate the mechanism involved.

An increase in CD200R1 expression was observed on activation of developing population D (the CD24$^-$ CD44^{hi}, IL-17–producing subset) γδ T cells in FTOCs (Fig. 3H). The relevance of this

upregulation is not known, but activation of mature adult γδ T cells did not result in upregulation of CD200R1 (data not shown), suggesting this may be developmental-stage and population specific and, therefore, may be important during development. However, it remains unknown if there are endogenous activation signals in vivo during development that would also be capable of inducing this peak in CD200R1 expression. Alternatively, CD200R1 may have effects during the early stages of γδ T-cell development when the cells are $CD24^+$, because CD200R1 is particularly highly expressed on the $CD44^{hi}$ $CD24^+$ γδ T-cell population (Fig. 3H). However, details of the developmental processes occurring during this stage are lacking.

These findings demonstrate an important role for CD200R1 in driving early-life γδ17 T-cell acquisition of effector function without affecting the development of IFN-γ–producing subsets. The precise mechanism by which CD200R1 drives γδ17 T-cell development and what regulates CD200R1 expression remains to be identified. It will also be important to determine if CD200R1 plays a similar role in human γδ17 T-cell development; however, this research is technically difficult to undertake. Understanding the development of γδ17 T cells and their acquisition of effector function is important because these cells have crucial functions in immune responses, tumor surveillance, and wound healing, but they also contribute to inflammatory disease. Therefore, a better understanding of the factors involved in the development and function of these important cells has the potential to improve multiple areas of health.

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Supplementary material

Supplementary material is available at *Journal of Leukocyte Biology* online.

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Conflicts of interest. The authors declare no conflict of interests.

Data availability

Most data are evident in the article text. Any data that support the findings of this study that are not evident are available from the corresponding author upon reasonable request.

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