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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 foetal thymi in temporally controlled waves where, unlike αβ T cells, many γδ T cells adopt their effector fate, becoming either IFNγ 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 foetal thymus where it supports the development of γδ17 T cells, enhancing IL-17-producing and RORγt$^+$ γδ T cell numbers in foetal 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.

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

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 have also been shown to drive inflammatory disease (6). γδ T cells are potent and rapid producers of inflammatory cytokines, being activated in either a T cell receptor (TCR)-dependent, or independent manner. 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 MHC restriction. Instead, γδ TCRs recognise 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 defence 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 anti-tumour 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 IFNγ 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 IFNγ 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 IFNγ-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), but *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 (DETC)) 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 node (LN) (16), then perinatally, Vγ7 (intra-epithelial 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 (DN) cells receiving strong TCR signals commit to the γδ lineage and strong TCR signalling is key for IFNγ-producing γδ T cell development (19, 20). However, weaker TCR signals are required for γδ17 T cell development (16, 19-22), where distinct TCR-signalling pathways are engaged, specifying γδ17 T cell fate (23). Negative regulators of TCR signalling 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 cMAF, 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 *γδtcr* recombination (26-30). IL-7 signalling particularly impacts γδ17 T cells, as high levels of IL-7/IL-7Rα signalling favour IL-17 production (31, 32) and IL-7Rα is required for the homeostasis of γδ17 T cells (33). Lymphotoxin (LT) signalling is key for the development of both IFNγ-producing and γδ17 T cells, where LTβR drives the NF-κB family member, RELB, which in turn maintains RORγt expression (34). TGFβ 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, as 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 (both αβ and γδ) T cells, NK cells, antigen presenting cells, subsets of granulocytes, and innate lymphoid cells (ILC) in human skin (39), and on mast cells, ILCs macrophages, monocytes and 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, 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 anti-fungal immune responses (45-47), the role of CD200R1 in regulating murine γδ T cell IL-17 production was investigated. We show that CD200R1-deficient mice have impaired IL-17 production by γδ T cells, due to reduced γδ17 T cells, with IFNγ-producing cells not 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 time-period demonstrating a role for CD200R1 in supporting the development of γδ17 T cells. Therefore, here we identify for the first time CD200R1 as a key factor required for the development of murine γδ17 T cells, shaping immunity at barrier sites.

Materials and Methods

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 weeks old at the start of procedures, unless otherwise stated. Where neonatal tissues and foetal thymi were used, sex was not determined.

Skin cell isolation

Ears were split in half and floated on 0.8% w/v 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 TM (Roche) at 37 °C for 1 hr. The fat was removed from dorsal skin before floating on Trypsin as above. Tissue was chopped and digested with 1 mg/ml Dispase II (Roche) at 37 °C for 1 hr. Digested skin tissue suspensions were passed through 70 µm cell strainers, washed and counted.

Lymph node, thymus and spleen cell isolation

Lymph nodes (LN, inguinal, axillary and brachial), 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.

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 labelled antibodies. Cells were fixed with FOXP3/Transcription Factor Buffer Staining Set (eBioscience) for between 30 min and 16 hr at 4°C.

For cytokine analysis, 10 µM Brefeldin A was added to cell cultures for 4 hr prior to staining for cell surface markers as described above. After overnight fixation, cells were

permeabilized with FOXP3/Transcription Factor Buffer Staining Set (eBioscience) and were stained with antibodies against intracellular markers or cytokines. Cells were analysed on a Fortessa, LSRII (both BD Biosciences) or Cytoflex (Beckman Coulter) flow cytometer. Data were analysed using FlowJo (TreeStar). Antibodies are detailed in Supplementary Table 1.

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. 7AAD (Biolegend) was added and cells were analysed within 2 hours.

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

For pERK staining, skin cells were isolated and cultured for 30 mins to acclimatise. Cells were then stimulated for 10 min with either 50 ng/ml 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).

Post fixation, cells were permeabilized at 4°C in Phosflow Perm Buffer III (BD Biosciences) for 30 minutes, 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 minutes, before washing and analysing by flow cytometry as detailed above.

***In vitro* γδ T cell activation**

Mouse dorsal skin cells were cultured in complete RPMI (RPMI 1640 supplemented with 10% heat inactivated FBS, 1% penicillin streptomycin solution, 2 mM L-glutamine, 1 mM sodium pyruvate, 20 mM HEPES, 1X non- essential amino acid solution, 25 nM 2-mercaptoethanol (Sigma Aldrich)) and stimulated with 40 ng/mL IL-23 (Biolegend) for 16 hr. Cells were cultured with 10 µM Brefeldin A (Sigma Aldrich) for 4 hr 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 hr before staining for flow cytometric analysis.

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

QPCR to detect *Cd200r1* in neonatal and adult γδ T cells

Two-day old neonatal body skin and adult dorsal skin was removed from euthanised C57BL/6 mice and cells were isolated as detailed above. Cells were stained with a dead cell dye and antibodies as described above, 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 1000-6000 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. QPCR was carried out in duplicate using the cDNA equivalent of 3.6-14 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 sec, 55 °C for 45 sec, 60 °C for 1 min 30 sec on a CFX Opus machine (Bio-Rad). Primers used for *Cd200r1* 5'-TGTGAGACAGTAACACCTGAAGG -3' and 5'- TGCCATTGCCTCACACACTGCA -3' and for *Hprt*, 5'- GCTGACCTGCTGGATTACATTAA -3' and 5'-TGATCATTACAGTAGTCTTTCAGTCRGA -3'. Melt curves were checked for single peaks at the correct melting temperature, and relative gene expression was calculated using the $2^{-\Delta\Delta C(t)}$ method.

Foetal thymic organ cultures (FTOCs)

FTOCs were set up and analysed as described previously (16). Briefly, E15-15.5 embryos were obtained from WT or CD200R1KO timed matings. Foetal thymic lobes were dissected and cultured on nucleopore membrane filters (Whatman) (4-5 per filter) in complete RPMI (as above) for 8 days. 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 hrs. Thymic lobes were

homogenised using a syringe plunger and were stained and analysed by flow cytometry as described above. To count cells, Precision count beads (Biolegend) were added to each FTOC sample prior to analysis.

Statistical analysis

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

Results

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 (Linley, 2023). Given the importance of γδ T cells for IL-17A production in these models, we measured IL-17A production by CD200R1-deficient cutaneous γδ T cells (Figure 1A), specifically examining the TCR $\gamma\delta^{int}$ dermal population containing IL-17-producing cells, in contrast to TCR $\gamma\delta^{hi}$ dendritic epidermal T cells (DETC) 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 (Figure 1B).

CD200R1 suppresses cytokine production in myeloid cells, therefore we hypothesized that CD200R1-deficient cultures may contain an overactive cell type which inhibits γδ T cell IL-17 production. To test this, CD200R1-deficient murine dorsal skin cells were fluorescently labelled and co-cultured with unlabelled WT cells before stimulation. CD200R1-deficient γδ T cells, retained an impairment in IL-17A production when co-cultured (Figure 1C), demonstrating that CD200R1-deficient 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 CD200R1-deficient 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, lymph node (LN) and thymus were stimulated with PMA and Ionomycin and IL-17A and IFNγ production by γδ T cells was measured by flow cytometry. WT and CD200R1-deficient γδ T cells had a similar ability to produce IFNγ, but CD200R1-deficient γδ T cells were impaired in IL-17A production in each tissue examined (Figure 1D), showing that CD200R1 promotes IL-17A production by γδ T cells, but does not affect IFNγ production.

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 IFNγ-producers respectively (21). In all these tissues, the proportion of $CD44^{hi}$ γδ T cells is reduced in CD200R1-deficient mice (Figure 2A), demonstrating that CD200R1 is required to maintain the balance between IL-17A and IFNγ-producing γδ T cell populations. Examining γδ T cell numbers shows reduced γδ T cells in lymph nodes (LN) and dermis in CD200R1KO mice, but similar overall numbers in thymus and spleen (Figure 2B). γδ T cells take on their effector fate during development in the thymus, when they rearrange their *Tcr* loci and express specific Vγ and δ TCR chains with $V\gamma4^{+}$ cells being the major IL-17A-producing γδ T cell subset in skin. To determine if CD200R1 affects specific populations of γδ T cells, inguinal LN cells were gated into $V\gamma4^{+}$, $V\gamma1^{+}$ and non-Vγ4 or Vγ1 expressing populations, demonstrating that the $V\gamma4^{+}$ subset specifically is reduced in the absence of CD200R1 (Figure 2C). Similarly, the number of $V\gamma4^{+}$ cells is reduced in number in CD200R1-deficient skin (Figure 2D). Therefore, CD200R1 promotes either the generation, or maintenance of $V\gamma4^{+}$ cells.

No increase in the proportion of apoptotic $V\gamma4^{+}$ T cells, or the proliferation of these cells was observed in the absence of CD200R1 (Supplementary Figure 1), suggesting that CD200R1 affects the generation of $V\gamma4^{+}$ 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 (Figure 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, signalling downstream of activation was examined. Lymph node cells were stimulated with IL-23 and the resulting phosphorylation of STAT3 within γδ T cells was examined by phosflow, demonstrating that in the absence of CD200R1, γδ T cells are less able to signal downstream of IL-23 stimulation (Figure 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 (Figure 2G), or indeed in the CD44hi γδ T cell subset (Figure 2H). Together this demonstrates that CD200R1 promotes signalling in response to IL-23 or TCR activation in IL-17-producing subsets of γδ T cells.

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 (Figure 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 (Figure 1D), and the maintenance of Vγ4$^+$ T cells, measured by apoptosis and proliferation frequency, is not affected by CD200R1 (Supplementary Figure 1). 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 dendritic cells (Figure 3B) but, is absent from both cortical and medullary thymic epithelial cells (Figure 3C), suggesting that effects of CD200R1 on adult thymus may be via thymic dendritic cells.

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 foetal 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 (Figure 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 (Figure 3E), suggesting that regulation of CD200R1 levels may be post-transcriptional.

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

CD200R1 promotes foetal γδT17 cell development

The impact of CD200R1 on foetal γδ T cell development was examined using FTOC. Total numbers of γδ T cells were not affected by CD200R1 deficiency (Supplementary Figure 2A), but the overall number of IL-17-producing γδ T cells and both proportions and numbers of RORγt-expressing γδ T cells are reduced in CD200R1-deficient FTOC (Figure 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. The less mature $CD24^{+}$ populations were examined, showing distinct populations based on CD44 expression (Figure 4 and Supplementary Figure 2). In CD200R1-deficient FTOC, the $CD24^{+}$ $CD44^{hi}$ population is reduced in both proportion and number, and CD200R1-deficient FTOCs also have reduced proportions and numbers of RORγt-expressing cells and reduced numbers of IL-17-producing $CD24^{+}$ γδ T cells (Figure 4B), demonstrating that CD200R1 supports the early development of γδT17 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 (Supplementary Figure 2C), CD200R1 promotes IL-17 production by these cells (Figure 4C). Overall, these data demonstrate a requirement for CD200R1 for efficient acquisition of effector function (RORγt-expression and IL-17-production) in γδT17 cells, which is observed from the very early stages of development.

Together this shows that CD200R1 is required to support the development of murine γδT17 cells.

Discussion

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

CD200R1 may affect γδ17 T cell development via indirect effects involving other cell types. This would most likely be via thymic dendritic cells which have high expression levels of CD200R1 (Figure 3C, 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 signals and IL-7 (36), which could also be important for supporting γδ17 T cell development. However, CD200R1 expression is low on these cells (Figure 3B, F) suggesting 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 signalling, or via

effects on other signalling pathways. CD200R1 is part of the immunoglobulin superfamily and is an 'immune checkpoint' pathway being therapeutically blocked in cancer to promote immune responses and allow the immune system to target the tumour (50). However, the evidence for CD200R1 specifically affecting T cells or TCR signalling is weak. Cytotoxic T cell function is inhibited by CD200R1 signalling (51), and blocking CD200R1 signalling promotes TCR-driven $CD4^+$ T cell proliferation in Lupus patients, but there was no effect on healthy T cells (52). Therefore, a direct effect of CD200R1 on T cell activation or TCR signalling may be likely but, remains to be proven. Signalling 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, is reduced in the absence of CD200R1 (Figure 2F-H), demonstrating that CD200R1 is required for efficient signalling in response to activation in γδ cells. However, as CD200R1 is expressed at very low levels on adult skin γδ T cells (Figure 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 inCD200R1 expression was observed on activation of developing population D ($CD24^-$ $CD44^{hi}$, IL-17-producing subset) γδ T cells in FTOC (Figure 3H). The relevance of this upregulation is not known, but activation of mature adult γδ T cells did not result in up-regulation of CD200R1 (data not shown), suggesting that 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 which 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 $CD24^+$, as CD200R1 is particularly highly expressed on the $CD44^{hi}$ $CD24^+$ γδ T cell population (Figure 3H). However, details of the developmental processes occurring during this stage are lacking.

Together this demonstrates an important role for CD200R1 in driving early life γδ17 T cell acquisition of effector function, without affecting the development of IFNγ-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 as these cells have crucial functions in immune responses, tumour surveillance and wound healing, but they also contribute to inflammatory disease. Therefore, a better understanding 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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Data availability

Most data are evident in the results section. Any data that support the findings of this study that are not evident, are available from the corresponding author, [AS], upon reasonable request.

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Figure Legends

Figure 1: γδ T cells from CD200R1-deficient mice have an impaired ability to produce IL-17A

Cells were isolated from WT or CD200R1-deficient (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 hrs. IL-17A production within the TCRγδ^{low} cells was analysed 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, CD200R1-deficient (KO) cells were labelled with a fluorescent dye, then mixed with unlabelled WT cells. n=7 per group. **D.** Cells were isolated from thymus, spleen and lymph nodes and were stimulated with 50 ng/ml PMA and 500 ng/ml Ionomycin for 4 hrs, before flow cytometric analysis for IL-17A and IFNγ production by γδ T cells. For thymus WT n=6, KO n=7. 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 two independent experiments. Students' t-tests were used to determine statistically significant differences except where data are not normally distributed, where Mann Whitney U tests were used (**D.** IL-17 and IFNγ production in spleen and IL-17 production in LN). * signifies $p<0.05$, ** signifies $p<0.01$, *** signifies $p<0.001$.

Figure 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 CD200R1-deficient (KO) mice and were analysed by flow cytometry. **A.** Proportion of γδ T cells expressing high levels of CD44 (γδ17 T cells) or expressing CD27 (cells with potential for IFNγ production). Thymus n=7, spleen and LN n=3 for CD27^{+} population, n=7 for CD44^{hi}. n= 8 for dermis. **B.** Numbers of γδ T cells in thymus, spleen and LN, or TCRγδ^{low} T cells in skin. n=6 per group. **C.** No. of γδ T cells within each Vγ subset in one inguinal LN. n=6 per group. **D.** The numbers of Vγ4^{+} T cells in skin. WT n=6,

KO n=10. **E.** Proportion of Vγ4$^+$ 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 analysed by flow cytometry for pSTAT3 within the γδ T cell gate. n=4-6 per group as indicated by data points. **G-H.** Skin cells were stimulated with either PMA or anti-TCR γδ antibodies for 10 min and analysed 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 performed, except for G-H which is one experiment. Numbers on flow plots show percentages of cells in each gate. Students' t-tests were used to determine statistically significant differences except where data are not normally distributed, where Mann Whitney U tests were used (**A.** CD44^{hi} data in spleen and LN. **C.** Vγ1/ Vγ4$^-$ population). Where variances were significantly different, Welch correction was used (**A.** Thymus CD44^{hi}. **E.** skin data). For F, where there are 3 data groups, a Browne and Forsythe and Welch Anova was used. * signifies $p<0.05$, ** signifies $p<0.01$, *** signifies $p<0.001$.

Figure 3: CD200R1 is not expressed by murine adult γδ cells, but is expressed by developing γδ cells and by thymic dendritic cells

Flow cytometry was used to determine cell types expressing CD200R1 in skin or thymus from mice of different ages. Grey histograms depict Fluorescence minus one (FMO) controls for CD200R1 staining, black lines depict CD200R1 staining on unstimulated cells, red lines depict CD200R1 staining after a 3 hour stimulation with PMA and Ionomycin. **A.** Expression of CD200R1 on cutaneous Vγ4$^+$ and Vγ1$^+$ 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 dendritic cell (DC) populations (CD45$^+$ MHCII^{hi} CD11b$^+$ or CD11c$^+$) and CD200R1 expression on these populations. **D.** CD200R1 expression in cutaneous γδ T cells in one-day-old, three-week-old, adult (8-12 weeks), or old (10 months) mice. **E.** Dermal CD3^{mid} γδ T cells were

flow cytometrically sorted from adult dorsal skin and two-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 dendritic cell (DC) gating in foetal thymic organ cultures and CD200R1 expression on each subset. **H.** CD200R1 expression on populations of developing γδ T cells in foetal thymic organ cultures (FTOC). $CD24^{+}$ cells are less mature, whereas $CD24^{neg}$ are more mature and can be split into subsets capable of producing IFNγ (population B and population C), and IL-17 (population A and D). Plots shown are representative of at least 2 independent experiments, containing data from at least 3 mice.

Figure 4: CD200R1 is required for optimal development of IL-17+ and RORγt+ γδ T cells

WT and CD200R1-deficient (KO) foetal thymic organ cultures (FTOC) were set up and cultured for 8 days. Cells were stimulated with PMA and Ionomycin for 3 hrs, and cell populations were examined by flow cytometry. Data are normalised to the average of the WT data for each individual experiment. Proportions and cell numbers of populations within the **A.** total γδ T cell population. **B.** CD24+ γδ T cell population. **C.** $CD24^{neg}$ γδ T cell population. Data points indicate individual FTOC data. Data are pooled from two independent experiments. n=9 for WT, n=13 for KO. Students' t-tests were used to determine statistically significant differences with Welch's correction for unequal variance where required (**B.** no. $CD44^{hi}$, $\%CD44^{low}$, no. $CD44^{low}$, $\%ROR\gamma t^{+}$. **C.** $\%IL\text{-}17^{+}$, no. $ROR\gamma t^{+}$). * signifies $p<0.05$, ** signifies $p<0.01$, *** signifies $p<0.001$.

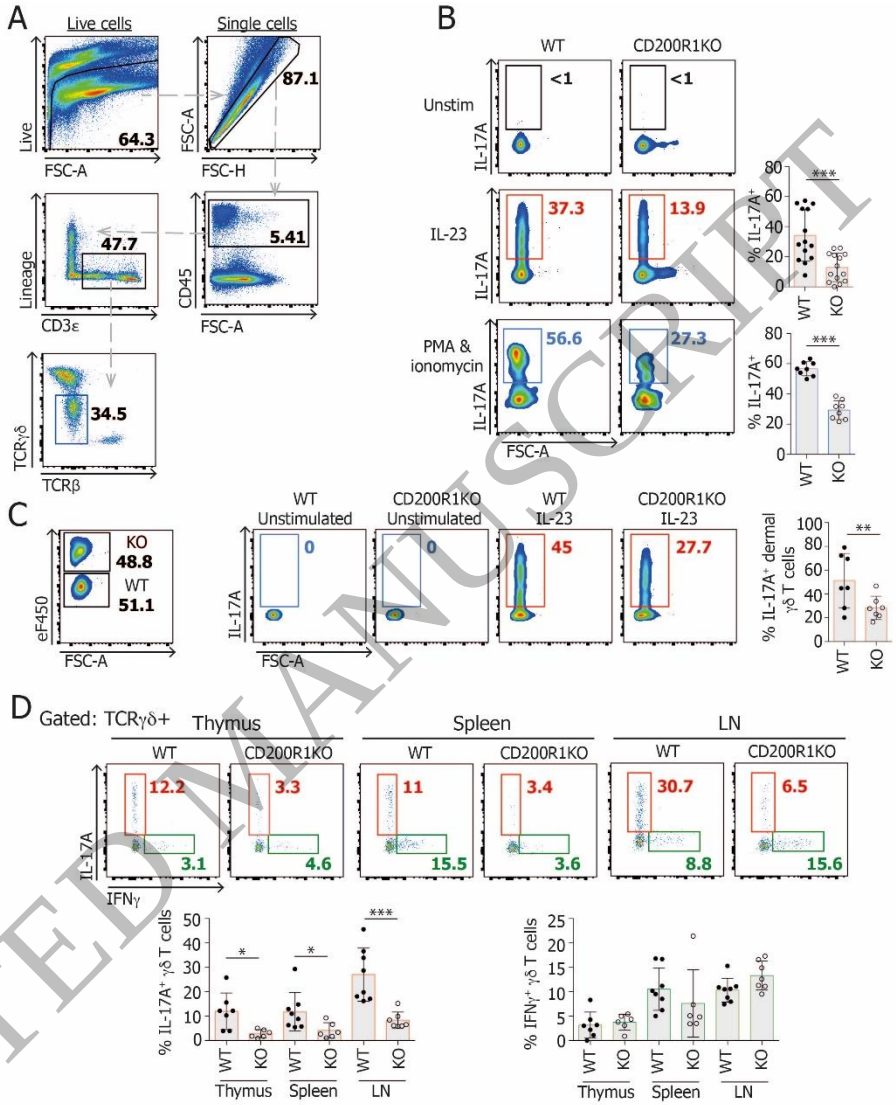

Figure 1
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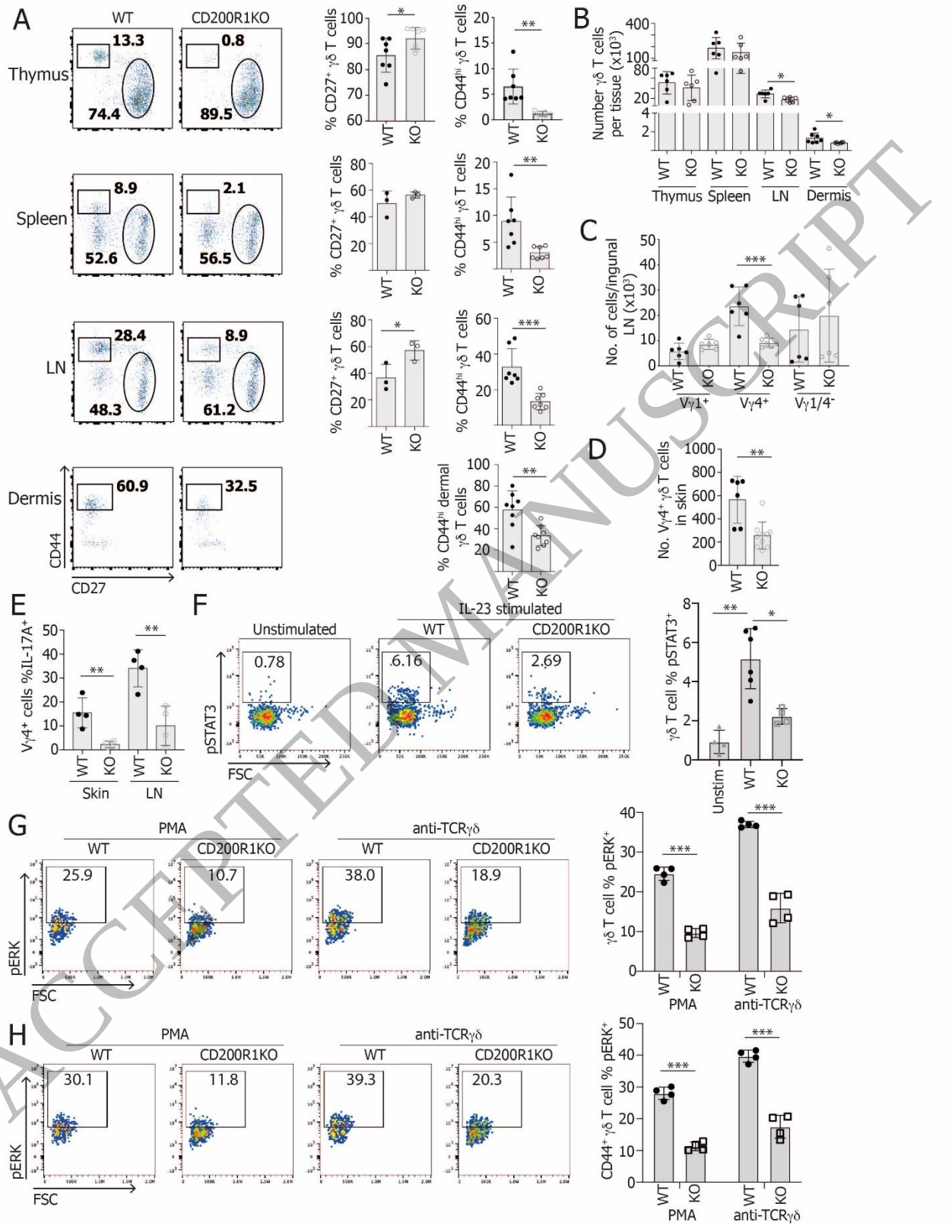

Figure 2
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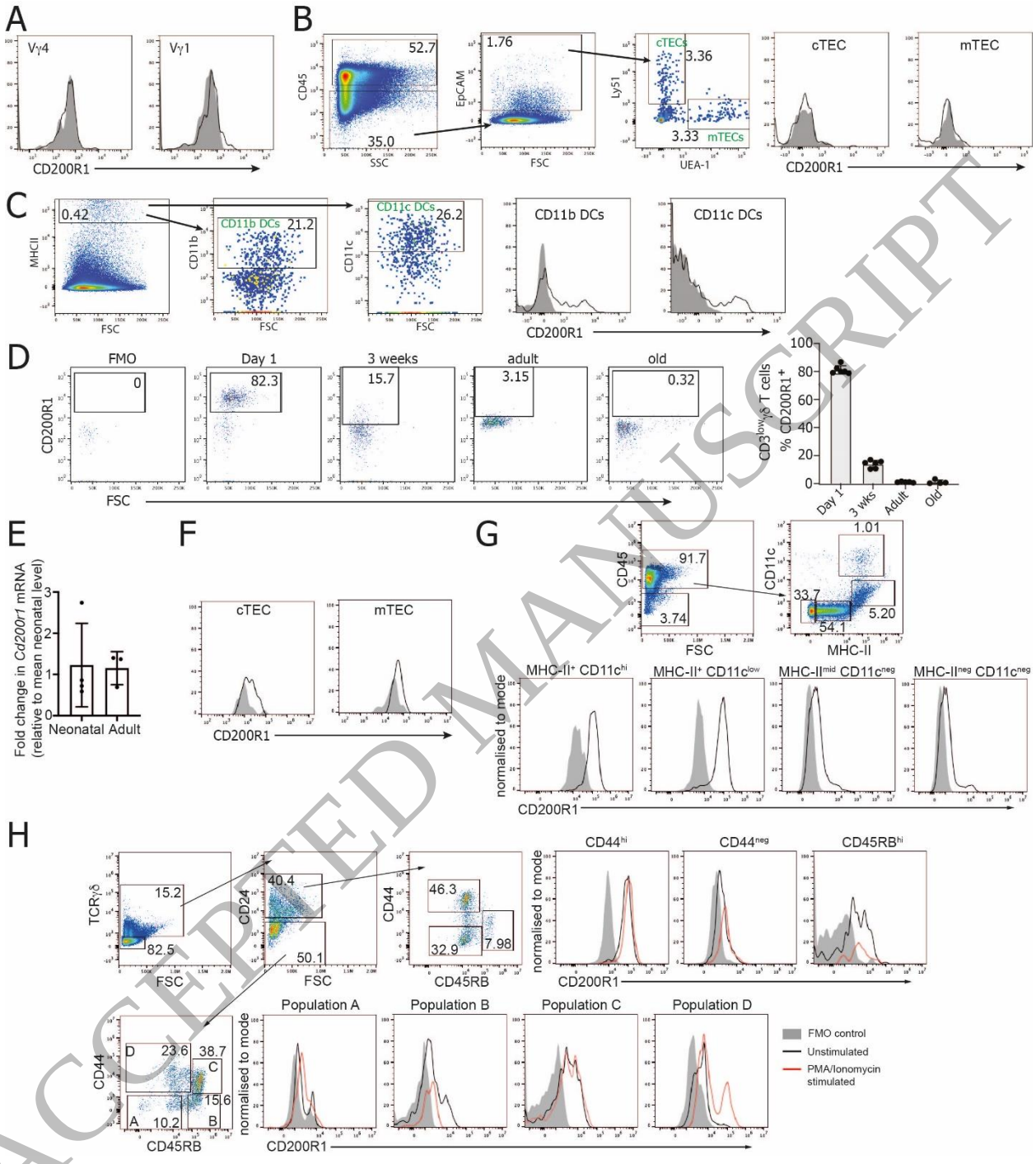

Figure 3
165x185 mm (x DPI)

Figure 4
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