β2 integrins differentially regulate γδ T cell subset thymic development and peripheral maintenance

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Classification

Biological Sciences; Immunology and Inflammation

Keywords

β_2 integrins, γδ T cells, immune homeostasis

Author Contributions

C.L.M. and V.L.M. designed the project. C.L.M., L.M. and V.L.M. performed the experiments and analyzed the data. J.C.R. developed the online platform for visualizing the sequencing data. T.D.O. analyzed the sequencing data. C.S.G. and A.H. provided valuable contribution to the direction of the project. C.L.M. and V.L.M. wrote the manuscript with input from all co-authors.

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Abstract

γδ T cells reside predominantly at barrier sites and play essential roles in immune protection against infection and cancer. Despite recent advances in the development of γδ T cell immunotherapy, our understanding of the basic biology of these cells, including how their numbers are regulated *in vivo*, remains poor. This is particularly true for tissue-resident γδ T cells. We have identified the β_2 family of integrins as novel regulators of γδ T cells. β_2 integrin-deficient mice displayed a striking increase in numbers of IL-17-producing Vγ6Vδ1$^+$ γδ T cells in the lungs, uterus and circulation. Thymic development of this population was normal. However, single cell RNA sequencing revealed the enrichment of genes associated with T cell survival and proliferation specifically in β_2 integrin-deficient IL-17$^+$ cells compared to their WT counterparts. Indeed, β_2 integrin-deficient Vγ6$^+$ cells from the lungs showed reduced apoptosis *ex vivo*, suggesting that increased survival contributes to the accumulation of these cells in β_2 integrin-deficient tissues. Furthermore, our data revealed an unexpected role for β_2 integrins in promoting the thymic development of the IFNγ-producing CD27$^+$ Vγ4$^+$ γδ T cell subset. Together, our data reveal that β_2 integrins are important regulators of γδ T cell homeostasis, inhibiting the survival of IL-17-producing Vγ6Vδ1$^+$ cells and promoting the thymic development of the IFNγ-producing Vγ4$^+$ subset. Our study indicates new and unprecedented mechanisms of control for γδ T cell subsets.

Significance Statement

γδ T cells reside in barrier tissues and provide immune protection against infection and cancer. Their anti-tumor potential has led to recent advances in the development of γδ T cell immunotherapy. However, our understanding of the basic biology of these cells, including what molecules and pathways control their maintenance within barrier tissues, remains poor. We demonstrate that β_2 integrin adhesion molecules play a major role in regulating γδ T cell subset numbers during homeostasis: the loss of β_2 integrin expression results in a striking increase in IL-17-producing γδ T cells in the lungs and uterus due to enhanced survival. These findings illustrate a novel mechanism of γδ T cell regulation that may have significant implications for immunotherapy development.

Main Text

Introduction

γδ T cells are innate-like cells that play essential roles in immune surveillance, neonatal immunity and responses to infection and tumors. Tissue-resident γδ T cells localize within barrier sites such as the skin, gut, lungs, genital tract and oral mucosa, enabling rapid responses against infectious challenge without the need for clonal expansion. Upon recognition of foreign antigens, host stress-induced markers or innate-derived cytokines (1-3), γδ T cells mediate immune protection via direct killing of target cells and production of pro-inflammatory cytokines, IFNγ and IL-17.

γδ T cells are the major innate source of IL-17 during the early phase of an immune response (4). Mouse models have demonstrated protective roles for IL-17-producing γδ T cells in a range of mucosal infections (5-7). In humans, IL-17-producing γδ T cells have been reported in cord blood (8) and can be expanded from PBMCs *in vitro* (9, 10). Although their contribution to infection-driven immune responses remains poorly described, IL-17-producing γδ T cell numbers are increased in patients with bacterial meningitis (10), suggesting an important role.

We set out to explore the regulatory roles of β_2 integrins in γδ T cell populations that reside in barrier tissues. β_2 integrins are leukocyte-specific adhesion molecules that consist of the β_2 chain (CD18) coupled to CD11a (α_L), CD11b (α_M), CD11c (α_X) or CD11d (α_D). It is well characterized that Leukocyte Function-associated Antigen-1 (LFA-1; CD11a/CD18) is essential for many aspects of αβ T cell function (11): by binding intercellular adhesion molecules (ICAMs) on endothelial cells LFA-1 mediates T cell recruitment to lymph nodes (LN) and sites of inflammation; LFA-1 localizes to the immunological synapse between interacting T and antigen-presenting cells, stabilizing contacts and lowering the threshold for T cell activation; T cell LFA-1 mediates interactions with target cells (e.g. B cells or cancerous/infected cells) facilitating effector T cell functions. However, the involvement of β_2 integrins in γδ T cell recruitment, interactions and function is less well defined (reviewed in (12)). Whilst some reports demonstrate a role for β_2 integrins in mediating γδ T cell interactions with endothelial cells (13), γδ T cell trafficking to sites of inflammation can occur independently of β_2 integrins, as shown in multiple sclerosis (14) and psoriasis (15) models.

Evidence suggests that β_2 integrins control γδ T cell numbers and/or localization: under conditions of skin and periodontal inflammation in CD18-deficient ($CD18^{hypo}$ PL/J) and CD11a knockout (KO) mice, respectively, increased numbers of γδ T cells were found at inflamed sites (15, 16). Even in the absence of overt inflammation, increased proportions and/or numbers of γδ T cells have been reported in the cervical LNs (17), spleen and mesenteric LNs (18) of CD18 KO mice. Based on this evidence, we hypothesized that β_2 integrins regulate γδ T cell numbers during homeostasis and inflammation. However, these studies have been limited in the tissues analyzed, making it unclear if the alterations in cell number are due to expansion or redistribution. Additionally, γδ T cell numbers, subset profiles and phenotype at barrier sites (e.g. lungs, small intestine, uterus, gingiva), where these cells have major immune protective roles, have not been analyzed in β_2 integrin-deficient mice.

In this study we investigated the impact of β_2 integrin-deficiency on γδ T cell subset numbers, localization and phenotype. We demonstrate that loss of β_2 integrin expression leads to a tissue-restricted increase in IL-17-producing Vγ6Vδ1$^+$ γδ T cells that is prominent in the lungs, uterus and spleen. We also reveal a novel role for β_2 integrins in promoting thymic development of IFNγ-producing CD27$^+$ Vγ4$^+$ γδ T cells.

Results

β_2 integrin-deficient mice have a tissue-restricted increase in γδ T cells

γδ T cell numbers were quantified in a range of lymphoid and mucosal tissues from β_2 integrin-deficient (CD18 KO) mice. αβ T cells were analyzed for comparison. γδ T cell numbers were increased in the lungs, uterus, spleen and blood of CD18 KO mice, whereas numbers in the skin (includes both dermis and epidermis), oral mucosa, gut and other lymphoid tissues were unaffected by β_2 integrin loss (Fig. 1; gating shown in SI Appendix, Fig. S1). As anticipated, αβ T cell numbers were reduced in the LNs, Peyer's patches and bone marrow, as a result of defective homing (19, 20). Importantly, we saw no reduction in γδ T cell numbers in any of the tissues analyzed, indicating

that the increase in γδ T cells in CD18 KO mice is not due to redistribution but, instead, a tissue-specific expansion.

β_2 integrin loss leads to an increase in IL-17-producing γδ T cells

γδ T cells are broadly divided into two mutually exclusive phenotypes based on cytokine profile: IFNγ-producing cells and IL-17-producing cells. Splenic γδ T cells from CD18 KO mice produced significantly higher levels of IL-17 as both proportion and absolute numbers compared to WT cells (Fig. 2A-B). Increased IL-17 was observed across the tissues where γδ T cell numbers were increased (Fig. 2B). The enhanced numbers of IL-17-producing γδ T cells in CD18 KO mice contributed to an increase in serum IL-17 levels (SI Appendix, Fig. S2A-B). Despite the apparent defect in the percentage of IFNγ-producing CD18 KO γδ T cells (Fig. 2A), the number of IFNγ-producing γδ T cells was equivalent (Fig. 2B).

The expanded γδ T cell population in β_2 integrin-deficient mice express the Vγ6Vδ1 TCR

Next, we questioned whether the expanded γδ T cell population was due to an increase in one or more γδ T cell subsets in β_2 integrin-deficient mice. Murine γδ T cell subsets express different Vγ chains, numbered Vγ1-7 (Heilig and Tonegawa nomenclature (21)), which localize to specific tissues and have different capacity for cytokine production (22). We predicted that CD18 KO mice would have an increase in Vγ4$^+$ and/or Vγ6$^+$ γδ T cells, as these subsets are the predominant IL-17-producers (23). Vγ chain expression was initially analyzed using antibodies against Vγ1, Vγ4 and Vγ5. Despite proportional differences in multiple subsets (SI Appendix, Fig. S3A), calculation of absolute numbers revealed a significant increase only in the Vγ1$^-$Vγ4$^-$Vγ5$^-$ γδ T cells in CD18 KO mice (Fig. 2C). This was true for all tissues where an increase was previously found in total γδ T cell numbers (Fig. 1 and Fig. 2C). PCR analysis of the 'unknown' Vγ1$^-$Vγ4$^-$ population in CD18 KO mice revealed expression of the Vγ6 and Vδ1 TCR chains (Fig. 2D; SI Appendix, Fig. S3B). Note that although Vγ2 is expressed at the mRNA level, protein expression does not correlate with in-

frame transcript expression, and high RNA level may reflect poor allelic exclusion (24). Extensive flow cytometric analysis confirmed that CD18 KO splenic γδ T cells displayed a marker phenotype consistent with IL-17-producing cells: they were predominantly CD62L^{lo}, CD44^{hi}, CD27^{-}, CD45RB^{-} and Rorγt^{+} (SI Appendix, Fig. S3C), suggestive of IL-17 production (25, 26); and they expressed higher levels of CD3 and TCRγδ than their WT counterparts (SI Appendix, Fig. S3C), a phenotype that implies a Vγ6-expressing phenotype (27). Together, these data show that the absence of β_2 integrin expression leads to an increase in IL-17-producing γδ T cells that express the Vγ6Vδ1 TCR.

The increase in IL-17-producing γδ T cells is due to loss of LFA-1

β_2 integrins are a family of four receptors, the loss of one or more of which could cause the γδ T cell expansion seen in CD18 KO mice. Analysis of CD11 chain KO mice revealed that the increase in γδ T cell numbers was due to the specific loss of LFA-1, as increased numbers of γδ T cells were detected in the spleen and lungs of CD11a KO mice but not in mice lacking CD11b, CD11c or CD11d (SI Appendix, Fig. S4A). We confirmed that the enhanced capacity to produce IL-17 was also observed in the expanded γδ T cell population in CD11a KO mice (SI Appendix, Fig. S4B).

Thymic development of Vγ6^{+} γδ T cells is unaffected by β_2 integrin loss, whilst CD27^{+}Vγ4^{+} γδ T cell development is impaired

We next sought to investigate the mechanism of expansion of Vγ6^{+} IL-17-producing γδ T cells in β_2 integrin-deficient mice. Vγ6^{+} γδ T cells egress from the thymus from embryonic 16.5 (E16.5) to a few days after birth and are, thereafter, maintained in the periphery by self-renewal (28). Analysis of the thymus showed equivalent numbers of Vγ6^{+} γδ T cells in WT and CD18 KO mice in embryonic, neonatal (1 and 3 day old), and adult (4 week old) mice (Fig. 3A). As thymic output of Vγ6^{+} cells both at the peak of development and in adult mice was normal, we conclude that the development of Vγ6^{+} cells is not influenced by β_2 integrin deficiency.

Surprisingly, whilst Vγ1$^+$ and Vγ5$^+$ γδ T cell development was also equivalent in WT and CD18 KO mice, we found a reduction in the number of thymic Vγ4$^+$ γδ T cells in embryonic and neonatal mice (Fig. 3A). Further analysis to differentiate between immature and mature populations revealed that the reduced number of total Vγ4$^+$ cells was due to a loss of immature CD24$^+$ cells at E18.5, and then mature CD24$^-$ Vγ4$^+$ cells in 4 week old mice (Fig. 3B). This suggests that the loss of β_2 integrin expression impairs Vγ4$^+$ γδ T cell development from the early, immature stage. The number of Vγ6$^+$ cells, regardless of maturation status, was similar in the thymus of WT and CD18 KO mice, further confirming that Vγ6$^+$ γδ T cell development is unaffected by β_2 integrin loss.

IL-17- and IFNγ-producing γδ T cells can be distinguished by expression of CD27, which is present only on IFNγ-producers (25). Further analysis revealed that numbers of CD27$^+$ Vγ4$^+$ cells in the thymus were reduced in CD18 KO mice across all ages of mice analyzed (Fig. 3C). No differences were found in CD27$^-$ Vγ4$^+$ cells. These data suggest that β_2 integrin loss causes a specific defect in the development of CD27$^+$ Vγ4$^+$ γδ T cells that have the potential to produce IFNγ.

To determine whether this impaired development impacts on the numbers of Vγ4$^+$ cells in the periphery, tissues were analyzed in adult mice. We previously showed that the number of Vγ4$^+$ cells was equivalent in CD18 KO mice at sites where the expanded Vγ6$^+$ cells were found (lungs, uterus, spleen and blood) (Fig. 2C). However, as Vγ4$^+$ cells commonly reside within lymphoid tissues, we performed a more thorough examination. The number of Vγ4$^+$ cells was significantly decreased in the peripheral LNs (inguinal, brachial and axillary pooled). A similar trend of reduced Vγ4$^+$ cell numbers was observed in the mediastinal LN and bone marrow, but this did not reach statistical significance (Fig. 3D). These findings indicate that the impaired development of Vγ4$^+$ γδ T cells in CD18 KO mice results in reduced numbers of these cells specifically in the peripheral LNs.

Single cell sequencing reveals evidence of enhanced survival of β_2 integrin-deficient Vγ6$^+$ cells

Next, single cell RNA sequencing (scRNAseq) was used to investigate the possible mechanism(s) by which β_2 integrins control γδ T cell numbers in the periphery. Principal component analysis of lung γδ T cells revealed 5 biologically distinct clusters: the *Il17a*+ cluster which contains both Vγ6$^+$ (*Cd163l1*$^+$ *Trdv4*$^+$) and Vγ4$^+$ (*5830411N06Rik*$^+$) cells, two *Cd27*$^+$ clusters namely *Ccr7*$^+$ and *Ly6c2*$^+$, and much smaller clusters of killer cells (*Gzma*$^+$) and dividing cells (*Cdk6*$^+$) (Fig. 4A; SI Appendix, Fig. S5A-C). As expected, the CD18 KO cells were over-represented in the *Il17a*+ cluster (Fig. 4B). High expression of the Vγ6 (*Tcrg-V6*) and Vδ1 (*Trdv4*) TCR subunits as well as Scart1 (*Cd163l1*), confirmed the expanded CD18 KO population as Vγ6$^+$ cells (SI Appendix, Fig. S5D). The number of differentially expressed (DE) genes when comparing WT to KO was much greater in the *Il17a*$^+$ cluster (1439 genes) than the *Ccr7*$^+$ (74 genes) or *Ly6c2*$^+$ (32 genes) clusters (Fig. 4C; Dataset S1A-C). We determined the DE genes that were unique to the IL-17-producing population (Fig. 4C; Dataset S2), excluding DE genes dependent on the loss of the β_2 integrin which were common to 2 or 3 of the dominant γδ T cell populations. Pathway analysis revealed enrichment of genes associated with T cell activation, survival and proliferation in CD18 KO cells from the IL-17-producing cluster (Fig. 4D; SI Appendix, Fig. S5E; Dataset S3). Specifically, the pro-survival genes *Bcl2a1a* and *Bcl2a1d*, known to support the survival of skin-resident Vγ6$^+$ cells (29), were uniquely enriched in CD18 KO *Il17a*$^+$ γδ T cell cluster (Fig. 4E; SI Appendix, Fig. S5E). Other genes reported to enhance survival in T cells (TCR subunits and signaling molecules, the IL-2 receptor, *Cd44* and *Icos*) as well as in cancer cells (*Crip1*) (30) were also enriched in the CD18 KO *Il17a*$^+$ cluster (Fig. 4E; SI Appendix, Fig. S5E). Together, scRNAseq analysis revealed the dysregulation of pathways involved in cell survival and proliferation specifically in the CD18 KO IL-17-producing γδ T cell population.

Cell survival, rather than proliferation, is the dominant driver of Vγ6$^+$ γδ T cell accumulation in β_2 integrin-deficient mice

Assessing proliferation *in vivo*, we detected a small but significant increase in Ki67 expression in Vγ6$^+$ cells from both the spleen and lungs of CD18 KO mice compared to WT (Fig. 5A). However, we found similar incorporation of BrdU by WT or CD18 KO Vγ6$^+$ or Vγ4$^+$ cells in either tissue over the 7-day period of analysis (Fig. 5B), suggesting that β_2 integrin-deficient Vγ6$^+$ cells do not show enhanced proliferation in adult mice. This data is supported by the scRNAseq analysis which shows that CD18 KO cells are not enriched in the dividing *Cdk6*$^+$ cluster (Fig. 4B), and by GO enrichment analysis where pathways associated with apoptosis dominated (Fig. 4D).

Next, we sought to determine whether cell survival is influenced by β_2 integrin deficiency. Detailed analysis of tissues from CD18 KO mice of various ages revealed that Vγ6$^+$ γδ T cell numbers in the lungs and spleen were equivalent to WT mice at E18.5, when this population begins to egress the thymus (Fig. 6A; SI Appendix, Fig. S6A). However, at 1-day post-birth, CD18 KO mice show increased numbers of Vγ6$^+$ γδ T cells in the lungs, and from 3-day old mice onwards, the numbers in the spleen were also elevated. Interestingly, whilst Vγ6$^+$ γδ T cell numbers in the lungs reach a plateau from day 3, numbers in the spleen of CD18 KO mice continue to rise as the mice age, increasing to 40-fold more than WT at 4 weeks (Fig. 6A). Surface marker analysis confirmed that the expanded γδ T cell population displayed the expected CD45RB$^-$ CD44$^+$ CD27$^-$ phenotype consistent with IL-17-producing potential (SI Appendix, Fig. S6B). These data indicate that the increased number of Vγ6$^+$ γδ T cells initially arise in the lungs and then the spleen. The accumulation of cells over time is consistent with enhanced Vγ6$^+$ cell survival in CD18 KO mice.

IL-7 is known to drive T cell survival through Bcl2 (31). As *Il7r* was expressed at higher levels in CD18 KO cells compared to WT in the *Il17a*$^+$ cluster (SI Appendix, Fig. S5D) and “cellular response to IL-7” was a hit in pathway analysis (Dataset S3), we assessed expression of IL-7R and Bcl2 in lung γδ T cells. Expression of IL-7R was higher in Vγ6$^+$ cells compared to Vγ4$^+$ cells but was equivalent between WT and CD18 KO cells (SI Appendix, Fig. S7A). Bcl2 expression was also equivalent in WT and CD18 KO γδ T cells (SI Appendix, Fig. S7B). Thus, signaling through the IL-7R-Bcl2 axis is not elevated in β_2 integrin-deficient γδ T cells.

Next, we investigated the effect of β_2 integrin loss on γδ T cell survival by direct *ex vivo* analysis. Vγ6$^+$ γδ T cells from the lungs of CD18 KO mice displayed reduced apoptosis compared to WT, with increased proportion of live cells and reduced proportion of late apoptotic cells (Fig. 6B). This effect was specific to the Vγ6$^+$ cells, as the proportions of live and apoptotic cells in the Vγ4$^+$ population were equivalent. Furthermore, the enhanced survival of CD18 KO cells was specific to the lungs, as cells in the spleen showed similar levels of apoptosis regardless of genotype or subset (Fig. 6B). Of note, this enhanced survival was most apparent in 4-week-old mice (Fig. 6C) as well as adult mice (Fig. 6B), whereas viability of Vγ6$^+$ cells in neonatal mice was equivalent in WT and CD18 KO mice (Fig. 6C). Thus, the enhanced survival of lung Vγ6$^+$ cells, particularly in adult mice, likely contributes to the accumulation of this γδ T cell subset in CD18 KO mice.

LFA-1 expression is higher in Vγ6$^+$ cells compared to Vγ4$^+$ cells, particularly in the lungs, in WT mice

Finally, we questioned why β_2 integrin loss led to the selective expansion of the Vγ6$^+$ subset. Assessment of β_2 integrin expression in γδ T cells revealed only CD11a (LFA-1) and not CD11b or CD11c expression (SI Appendix, Fig. S8). In the lungs, expression of CD11a was highest in the Vγ6$^+$ population compared to Vγ1$^+$ and Vγ4$^+$ cells, whereas γδ T cell populations expressed equivalent, lower levels of CD11a in the spleen (Fig. 6C). CD11a expression was equivalent across γδ T cell populations in the pLN (SI Appendix, Fig. S8) where Vγ6$^+$ cell numbers are unaffected by CD18 deficiency (Fig. 3D). These data suggest that Vγ6$^+$ γδ T cells in the lungs may be most affected by β_2 integrin loss as this population express the highest levels of LFA-1.

Discussion

In this study we reveal that the absence CD18 expression in mice leads to a striking increase in Vγ6Vδ1$^+$ IL-17-producing γδ T cell numbers in the lungs and uterus, as well as the blood and

spleen. Vγ6$^+$ cells showed no difference in thymic development or proliferation but displayed a transcriptional profile consistent with enhanced survival and reduced levels of apoptosis *ex vivo.* Surprisingly, we also found that β_2 integrin loss impaired the development of IFNγ-producing (CD27$^+$) Vγ4$^+$ γδ T cells, leading to reduced numbers of this subset in peripheral LNs. Together, our findings highlight novel roles for β_2 integrins in promoting Vγ4$^+$ γδ T cell development and in negatively regulating Vγ6$^+$ γδ T cell numbers in the periphery via apoptosis. In doing so, β_2 integrins alter the balance between IL-17- and IFNγ-producing γδ T cells at mucosal and lymphoid sites.

Importantly, we found no defect in γδ T cell numbers in any tissue, showing conclusively that the increase in γδ T cell numbers reported here and in previous studies in integrin-deficient mice (17, 18) is not a redistribution effect but a tissue-specific increase in γδ T cells that occurs in the absence of LFA-1. In agreement with previous studies (14, 15), our findings indicate that homing of γδ T cells to an extensive range of mucosal and lymphoid tissues is not dependent on β_2 integrins.

It was unsurprising that the increase in γδ T cell numbers was most apparent in the lungs and uterus (in addition to the spleen), as Vγ6$^+$ cells preferentially localize to these tissues (26, 32). However, Vγ6$^+$ cells also reside in the dermis (33) and gingiva (34), where no differences were found. Analysis of whole skin (epidermis and dermis) may have masked any differences exclusive to the dermis. In addition, there may be stronger competition by other skin-resident T cells, precluding accumulation of Vγ6$^+$ cells in the dermis. Alternatively, the expanded Vγ6$^+$ γδ T cell population in CD18 KO mice may be truly tissue specific. Vγ6$^+$ cells are not described to preferentially home to the spleen, although they have been shown to reside there (26, 27). We propose that the increasing number of Vγ6$^+$ cells exceeds the capacity within their resident tissues (lungs and uterus), which forces the γδ T cells into the circulation and causes accumulation in the spleen. The enhanced number of γδ T cells in the blood and appearance of increased numbers in the lungs prior to the spleen in newborn mice support this.

Analysis of CD18^{hypo} mice (CD18 expression at 2-16% of WT) revealed the expanded γδ population in the skin to have a CD27$^-$ IL-17$^+$ phenotype (15). The authors reported expression of Vγ4 rather than Vγ6 in the majority (~50%) of cells, although markers for Vγ6$^+$ cells were not assessed. The

potential difference in Vγ chain expression in CD18 KO mice versus $CD18^{hypo}$ mice could be due to genetic differences (C57BL/6J versus PL/J backgrounds), the level of CD18 expression (absent versus low), or changes induced by inflammation (no overt inflammation in our CD18 KO mice; severe dermatitis in the $CD18^{hypo}$ mice (15)).

Although thymic development of Vγ6$^+$ cells was unaffected by β_2 integrin loss, we found an unexpected defect in Vγ4$^+$ cell development. The defect was specific to the IFNγ-producing CD27$^+$ Vγ4$^+$ cells. The loss of CD18 impacts early in Vγ4$^+$ γδ T cell development (fewer immature Vγ4$^+$ cells at E18.5) but is not absolutely necessary, as the Vγ4$^+$ cells are reduced in number but not completely ablated. Interestingly, the numbers of Vγ4-expressing γδ T cells were only reduced in the peripheral LNs of adult CD18 KO mice and not the spleen or lungs where this population also resides. This may indicate that migration of Vγ4$^+$ cells to peripheral LNs is partially dependent on β_2 integrins or that factors present in the spleen and lungs but absent in peripheral LNs preferentially expand or retain Vγ4$^+$ cells. The functional consequences of the reduced numbers of Vγ4$^+$ cells in peripheral LNs of CD18 KO mice remains to be determined. As IFNγ production by Vγ4$^+$ cells is protective in models of melanoma (35, 36) and viral infection (37), reduced numbers of Vγ4$^+$ cells may enhance pathology in these models.

Our study highlights survival, rather than proliferation, as the dominant mechanism of Vγ6$^+$ cell accumulation in adult CD18 KO mice. We did find a slight but significant increase in Ki67 expression in Vγ6$^+$ cells from CD18 KO mice, potentially indicating longer time spent in active cell cycle (38), whilst not progressing to proliferation. Indeed, by BrdU incorporation we found little evidence of active γδ T cell proliferation regardless of genotype, which fits with published reports showing low turnover of Vγ6$^+$ cells in adult mice (33, 39). Although it is possible that CD18 KO cells may be undergoing increased proliferation compared to WT at a slow rate not detected, the scRNAseq analysis shows a very small population of dividing (*Cdk6*$^+$) γδ T cells, the frequency of which is unaltered in CD18 KO mice. Whilst it remains possible that proliferation contributes to an increase in Vγ6$^+$ cells in neonatal mice, our data illustrate that survival is the major contributor to Vγ6$^+$ population expansion in β_2 integrin-deficient mice from 4 weeks of age and in adult mice.

scRNAseq analysis of lung γδ T cells revealed a striking enrichment of genes associated with survival, specifically in the β_2 integrin-deficient Vγ6$^+$ γδ T cells. Importantly, *ex vivo* analysis confirmed that Vγ6$^+$ cells from the lungs of CD18 KO mice displayed decreased apoptosis compared to WT. Several TCR components as well as molecules downstream of the TCR signaling pathway (*Cd44, Icos, Lat, Nr4a1* (Nur77)) were increased in CD18 KO Vγ6$^+$ cells, suggesting that TCR signaling may contribute to the pro-survival phenotype. Although ligands that drive the expansion and retention of Vγ5$^+$ and Vγ7$^+$ γδ T cells have been characterized, namely Skint1 (40) and Btnl1 (41) respectively, a ligand specific for Vγ6$^+$ cells has not yet been identified. If such a ligand exists, an upregulation in CD18 KO lungs and uterus may contribute to the expansion phenotype seen in this study. Furthermore, two members of the Bcl2a1 family (*Bcl2a1a, Bcl2a1d*), that have been reported to support the survival of skin-resident Vγ6$^+$ cells (29), were also enhanced in our CD18 KO lung Vγ6$^+$ cells. Therefore, it is likely that a combination of signaling pathways that promote cell survival contribute to the accumulation of Vγ6$^+$ cells in β_2 integrin-deficient mice.

One remaining question is why the increase in γδ T cells is restricted to the Vγ6-expressing subset. β_2 integrins expressed by γδ T cells may contribute directly to the induction of apoptosis (42). As we found LFA-1 expression to be highest in Vγ6$^+$ cells in the lungs, this may explain the specificity of survival of the Vγ6$^+$ subset in LFA-1-deficient mice.

Although not definitive from our study, a cell-intrinsic role for LFA-1 in suppressing γδ T cell apoptosis is supported by the following evidence: (i) γδ T cell expansion was dependent on the loss of LFA-1 but no other β_2 integrin family members; (ii) in our hands, γδ T cells highly expressed LFA-1 but no other CD11 subunits; (iii) the expansion of γδ T cells was apparent in the tissues of CD18 KO mice from 1 day post-birth when other lymphocyte populations are still developing (e.g. very few αβ T cells are present); and (iv) the increase in γδ T cells was first observed in the lungs where stromal populations (negative for LFA-1) rather than leukocytes predominate. We, therefore, propose a model where LFA-1, potentially in conjunction with TCR and/or death receptor signaling, contributes to the induction of apoptosis in Vγ6$^+$ γδ T cells.

The functional consequences of the expanded IL-17-producing γδ T cell population in β_2 integrin-deficient mice remain to be determined. IL-17-producing γδ T cells are known to be required for effective pathogen clearance, particularly in the lungs and uterus (5-7, 43), and yet contribute to pathology in models of autoimmune disorders (44, 45) and cancer (46). We would predict that the expanded $V\gamma6^+$ cell population will alter outcome in these immune challenge models in an IL-17-dependent manner.

Although a subset equivalent to the $V\gamma6^+$ γδ T cells identified in mice does not exist in humans, future analysis of Vδ1- and Vδ2-expressing subsets, both of which have been shown to produce IL-17, may reveal a potential role for β_2 integrins in regulating specific subsets of human γδ T cells. The targeting of IL-17-producing γδ T cells has been identified as a therapeutic strategy for patients with spondyloarthritis (47), therefore further work to elucidate the role of β_2 integrins in the regulation human γδ T cells may reveal novel targets for therapy.

In summary, our findings reveal unanticipated roles for the β_2 integrin, LFA-1, in regulating the balance between IL-17- and IFNγ-producing γδ T cell subsets: β_2 integrins promote the development of IFNγ-producing $CD27^+$ $V\gamma4^+$ cells in the thymus, whilst suppressing the survival of IL-17-producing $CD27^-$ $V\gamma6^+$ cells in the periphery. The striking expansion of $V\gamma6^+$ cells in β_2 integrin-deficient mice demonstrates the central role this integrin plays in regulating this elusive γδ T cell population. The impact of these and other adhesion molecules on the survival and phenotype of γδ T cells should be carefully considered when developing cancer therapeutics to target the immuno-protective functions of this lymphocyte population.

Materials and Methods

Mice

CD18 (#003329) KO mice were purchased from The Jackson Laboratory. Age and sex-matched mice were used in all experiments. C57BL/6J mice (Envigo) or transgenic negative littermates were

used as controls. Mice were housed at the University of Glasgow. All protocols were conducted under licenses issued by the U.K. Home Office under the Animals (Scientific Procedures) Act of 1986 and approved by the local ethics committee.

Tissue Processing

Single cell suspensions were prepared from spleen, thymus, mesenteric LNs, mediastinal LNs, peripheral LNs (inguinal, brachial and axillary pooled) and Peyer's patches by mashing between 45µm NITEX pieces (Cadisch Precision Meshes Ltd.). Cells were isolated from bone marrow by flushing using a 26G needle. Spleen, blood and bone marrow cell suspensions were lysed with RBC lysis buffer (eBioscience). Uterus' were digested using Multi Dissociation Kit 1 (Miltenyi Biotec) according to the manufacturer's instructions. Ear skin was digested in 2mg/ml Collagenase IV, 2mg/ml hyaluronidase (both Sigma-Aldrich), and 100 U/ml DNase I (Invitrogen) at 37°C for 40 min at 180 RPM in a rotating incubator, then dissociated using a gentlemacs (Miltenyi Biotec). The following tissues were digested as described: lungs(48), small intestine(49), gingiva(50). Cells were counted by trypan blue exclusion.

Flow cytometry

Details of flow cytometry antibodies can be found in the SI Appendix. Cells were stained with fixable viability dye eFluor506 (eBioscience) for 30mins at 4°C. Extracellular staining was performed in Fc block (supernatant from 2.4G2 cell line) for 30mins at 4°C. For intracellular cytokine staining, cells were stimulated with 10ng/ml PMA (Sigma Aldrich), 500ng/ml ionomycin (Sigma Aldrich) and 1:1000 GolgiPlug (BD Biosciences) for 4h at 37°C. Following extracellular staining, cells were fixed using Cytofix/Cytoperm and stained in Perm/Wash (both BD Biosciences). For transcription factor staining, cells were fixed using FoxP3 Transcription Factor Fixation/Permeabilisation buffer and stained in permeabilization buffer (both eBioscience). All intracellular staining was for 1hr at room temperature (RT). For apoptotic cell staining, cells were stained with AnnexinV (Biolegend) and 7AAD (BD Biosciences) in AnnexinV staining buffer (Biolegend) for 15mins at RT. All cells were re-

suspended in FACS buffer (PBS 2% FBS 2mM EDTA) and acquired on LSRII or LSR Fortessa (BD Biosciences). Data was analyzed using Flowjo software (Treestar).

Fluorescence-activated cell sorting

γδ T cells from CD18 KO spleens were pre-enriched using EasySep™ Mouse T cell Isolation Kit (Stemcell Technologies). Live, $CD3^+TCR\gamma\delta^+$, $V\gamma1^-V\gamma4^-$ cells were then sorted using a FACS Aria IIU (BD Biosciences).

RNA isolation, cDNA and PCR

RNA was purified using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA synthesis was carried out using High-Capacity RNA-to-cDNA Kit (Applied Biosystems). Vγ and Vδ chain sequences were amplified using 0.02U/µl, 1X Phusion HF buffer, 3% DMSO, 2mM $MgCl_2$ (Vγ6 reaction only) (all Thermo Fisher), 0.2mM dNTPs (Invitrogen) and 0.3µM primers (IDT Technologies). Primer sequences for Vγ1, Vγ4, Vγ5, Vγ6 and Vγ7 were kindly provided by Dr Pierre Vantourout (King's College London) and for Vγ2 and Vδ have been described(51). DNA fragments were separated on 1% agarose (Invitrogen) gels and visualized using Gel Doc™ XR+ system (BioRad).

Single cell RNA sequencing

γδ T cells were isolated from the lungs of 2 WT mice and 1 CD18 KO mouse by FACS-sorting for $CD3^+$ $TCR\gamma\delta^+$ cells. Single cell suspensions were loaded into a Chromium Controller (10X Genomics). Subsequently, cDNA was generated from polyadenylated RNA, amplified and converted to Illumina compatible libraries according to standard protocol published in Single Cell 3' Reagent Kits v3 User Guide (10X Genomics). These were sequenced on an Illumina NextSeq500 sequencer with a sequencing depth of approximately 50,000 read pairs per cell. The read 1 and 2 were 28bp and 91bp long, respectively. scRNAseq reads were mapped in Cellranger (version 3.1.0) (52) against the current *Mus musculus* reference (mm10-3.0.0). The obtained readcount matrixes were analyzed in Seurat (53), version 3.1.2 in R version 3.5.3. We followed the analysis as described in (54). To clean the data of doublets and dying cells, we filtered cells for a

mitochondria amount less than 18% and applied the nFeature_RNA a cut-off of 2200, 3200 and 2500 cells for WT1, WT2 and KO, respectively. To remove technical variation, we used the SCtransform protocol in Seurat. WT1 and KO samples were integrated first, and then WT2 added which was down sampled to 4000 cells. The number of PCA transformation dimensions was 15; and the "findcluster" resolution was 0.15. Marker genes were selected for cluster annotation, non-T cell clusters removed, and clustering then repeated. The default Seurat values (FindMarkers function) were used to determine differentially expressed (DE) genes, with an adjusted p-value cut-off of 0.01. Venny 2.1 (bioinfogp.cnb.csic.es/tools/venny/) was used to compare DE genes in different clusters. GO enrichment pathway analysis was performed in Panther (pantherdb.org/). The reads and read counts are available in ArrayExpress under the accession number E-MTAB-8732. Furthermore, we designed a cell atlas to query the data, which can be accessed via this link: http://cellatlas.mvls.gla.ac.uk/gdT-cell/ This cell atlas was programmed in R-shiny using the Seurat pipeline as describe above.

Statistical analysis

Student's *t* test or two-way ANOVA (Graphpad Prism) were used to calculate statistical significance. Sidak's multiple comparisons test was used to correct for multiple comparison. P values shown as ns not significant, *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001.

Data Availability Statement

The reads and read counts from the single cell RNA sequencing are available in ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) under the accession number E-MTAB-8732. All other data are included in the main text or *SI Appendix*. Further information about data and reagents used is available by request to the corresponding author.

Acknowledgments

We thank Drs Seth Coffelt and Sarah Edwards for critical reading of the manuscript; Prof Christie Ballantyne (Baylor College of Medicine, Texas, USA) for providing CD11c KO mice; Dr Pierre Vantarout (King's College London) for providing primer sequences; the Flow Cytometry Core Facility (University of Glasgow) for assistance with cell sorting; and the Central Research Facility (University of Glasgow) for assistance with timed matings; and Dr Pawel Herzyk and Julie Galbraith at Glasgow Polyomics research facility (University of Glasgow) for performing the single cell RNA sequencing. Portions of this paper were developed from C.L.M.'s PhD thesis. This work was supported by Versus Arthritis (to V.L.M.; 20848), The Carnegie Trust (to V.L.M.; RIG007542), Wellcome (to V.L.M.; 204820/Z/16/Z), 10X Genomics (to V.L.M.), and the European Union's Horizon 2020 research and innovation program (Marie Skłodowska-Curie grant to L.M.; 792383).

Competing Interest Statement

The authors declare no competing interest.

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Figure Legends

Fig. 1. CD18 KO mice have increased numbers of γδ T cells in the lungs, uterus, spleen and blood. Cells were isolated from various lymphoid and mucosal tissues of WT (open circles) and CD18 KO (closed circles) mice then analyzed by flow cytometry. Skin includes both dermis and epidermis. Cells were gated on $CD3^+$ then $TCR\beta^+$ or $TCR\gamma\delta^+$ as shown in SI Appendix, Fig. S1. Absolute number of αβ and γδ T cells per tissue is shown. For blood, numbers were calculated per 100μl. SI = small intestine; mLN = mesenteric LNs; pLN = peripheral LNs (brachial, axillary and inguinal pooled). n=10, data pooled from a minimum of 3 independent experiments; each symbol

represents and individual mouse. Statistical differences were determined using a two-way ANOVA *p<0.05, **p<0.01, ****p<0.0001.

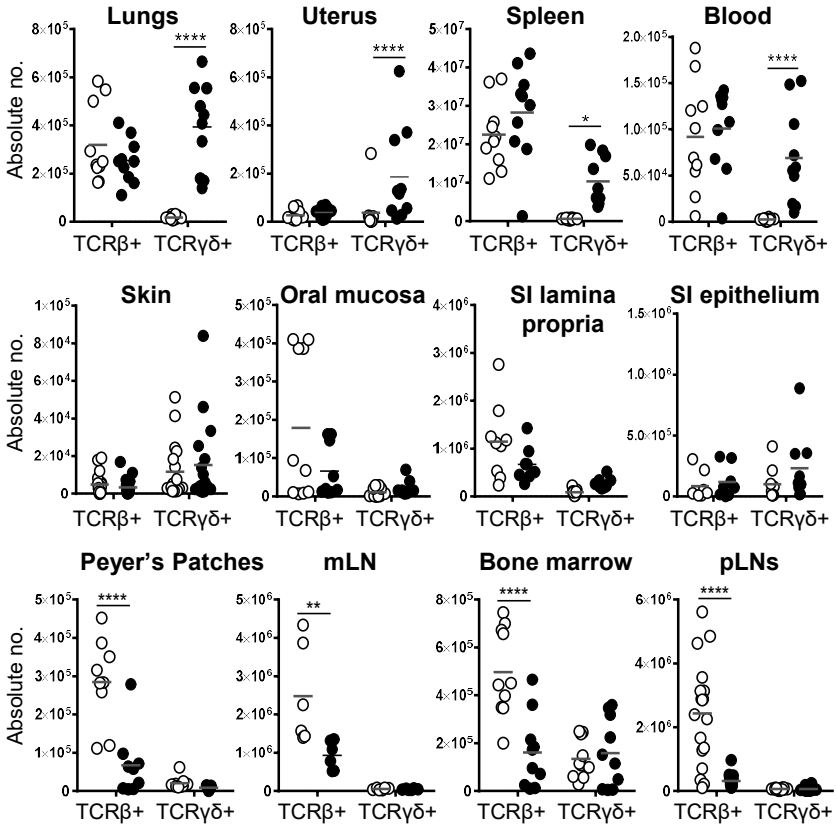
Fig. 2. IL-17-producing Vγ6Vδ1$^+$ γδ T cells are increased in CD18 KO mice. (A) Representative plot showing percentage of IL-17$^+$ and IFNγ$^+$ γδ T cells from WT and CD18 KO spleen following *in vitro* stimulation with PMA/ionomycin/BfA for 4h. (B) Number of IL-17$^+$ and IFNγ$^+$ γδ T cells from the lungs, uterus and spleen of WT and CD18 KO mice following *in vitro* stimulation with PMA/ionomycin/BfA for 4h. n=9 pooled from 3 independent experiments. (C) Cells isolated from WT and CD18 KO tissues were analyzed for Vγ chain expression by flow cytometry. Samples were gated on CD3$^+$TCRγδ$^+$ then Vγ1$^+$, Vγ4$^+$, Vγ5$^+$ or Vγ1$^-$Vγ4$^-$Vγ5$^-$. Absolute number of each Vγ subset. n=10 pooled from 4 independent experiments. (D) CD18 KO splenocytes were sorted by FACS for CD3$^+$ TCRγδ$^+$ Vγ1$^-$ Vγ4$^-$ cells then RNA isolated and RT-PCR performed. Representative gels showing expression of Vγ and Vδ genes. Representative of n=4. Positive controls for each Vγ and Vδ chain are shown in SI Appendix, Fig. S3C. Statistical differences were determined using a two-way ANOVA. **p<0.01, ****p<0.0001. Each symbol represents an individual mouse with mean shown.

Fig. 3. CD18 KO mice show no difference in Vγ6$^+$ thymic development but have impaired development of Vγ4$^+$ γδ T cells. (A-C) Thymocytes were analyzed from E18.5, 1 day, 3 day and 4 week old WT and CD18 KO mice by flow cytometry. Samples were gated on CD3$^+$TCRγδ$^+$ then Vγ1$^+$, Vγ4$^+$, Vγ5$^+$ or Vγ1$^-$Vγ4$^-$Vγ5$^-$ (Vγ6$^+$). (A) Absolute number of each Vγ subset. (B) Absolute number of Vγ4$^+$ and Vγ6$^+$ cells gated on either immature CD24$^+$ (left) or mature CD24$^-$ (right). (C) Absolute number of mature CD24$^-$ Vγ4$^+$ γδ T cells gated on either CD27$^+$ or CD27$^-$. (D) Absolute number of Vγ4$^+$ and Vγ6$^+$ γδ T cells from lymphoid tissues of adult mice. pLN = peripheral lymph nodes (inguinal, brachial and axillary pooled); medLN = mediastinal lymph node; mLN = mesenteric lymph nodes. (A-C) n=6-11 pooled from a minimum of 3 independent experiments. (D) pLN, mLN and bone marrow n=9, data pooled from 3 independent experiments. medLN n=6, data pooled from 2 independent experiments. Each symbol represents an individual mouse. Statistical differences were determined using a two-way ANOVA. *p<0.05, **p<0.01, and ***p<0.001.

Fig. 4. Single cell RNA sequencing reveals enhanced expression of genes known to negatively regulate apoptosis, specifically in Vγ6$^+$ cells from CD18 KO mice. γδ T cells were purified from the lungs of 2 WT and 1 CD18 KO mice by FACS. scRNAseq was performed and data analyzed as described in detail in the Materials and Methods section. The number of cells sequenced were: WT1, 918 cells; WT2, 6315 cells; KO, 4034 cells. (A) Principal component analysis plot showing the 5 identified clusters. (B) Frequency of cells in each of the identified clusters. (C) Venn diagram showing the shared and unique differentially expressed genes between WT and KO in the 3 major clusters, namely *Il17a*$^+$*, Ccr7*$^+$ and *Ly6c2*$^+$. (D) The apoptosis pathways identified in GO enrichment analysis of the differentially expressed genes unique to the *Il17a*$^+$ cluster. Fold enrichment and p-value are shown for each pathway. +ve = positive; -ve = negative; reg. = regulation; PCD = programmed cell death. (E) Expression levels of selected genes identified by pathway analysis as associated with the negative regulation of leukocyte apoptosis.

Fig. 5. No evidence of enhanced proliferation in Vγ6$^+$ γδ T cells from CD18 KO mice. (A) Cells isolated from the spleen and lungs of WT and CD18 KO mice were stained for the expression of Ki67. Representative histograms are shown from the spleen. Pooled data shows n=6 from 2 independent experiments. (B) WT and CD18 KO mice were injected with 1mg BrdU i.p. then fed 0.8mg/ml BrdU in the drinking water for 7 days. Cells isolated from the spleen and lungs were analyzed by flow cytometry for expression of BrdU. Representative histograms are shown from the spleen. Pooled data shows n=6 from 2 independent experiments. +ve = positive control (thymus). Each symbol represents an individual mouse. Statistical differences were calculated using a two-way ANOVA, *p<0.05, **p<0.01.

Fig. 6. CD18 KO Vγ6$^+$ γδ T cells display enhanced survival. (A) Analysis of γδ T cells from the lungs and spleen of WT and CD18 KO mice at various ages. Cells were gated on CD3$^+$ TCRγδ$^+$ then Vγ1$^+$, Vγ4$^+$, Vγ5$^+$ or Vγ1$^-$Vγ4$^-$Vγ5$^-$ (Vγ6$^+$). Absolute number of Vγ6$^+$ (Vγ1$^-$Vγ4$^-$Vγ5$^-$) γδ T cells is shown. Where significant differences between WT and CD18 KO were found, the fold difference is shown. WT spleen & lungs at 3 day old n=6. CD18 KO spleen at 1 day old, WT/CD18 KO lungs, WT/CD18 KO spleen at 4 week old, WT lungs 4 week old n=7. WT spleen at 1 day old n=8. CD18 KO spleen at 3 day old, CD18 KO lungs 3 days/4 weeks old, WT spleen E18.5 n=10. CD18 KO spleen & lungs E18.5 n=11. Data pooled from 4-6 litters, with each litter run as an independent experiment, each symbol represents an individual mouse. Statistical differences were determined using a Student's *t* test with false discovery rate approach two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. (B) Cells isolated from adult WT and CD18 KO lungs and spleen were analyzed by flow cytometry for expression of apoptosis markers. Cells were gated on CD3$^+$ TCRγδ$^+$ then Vγ4$^+$ or Vγ1$^-$Vγ4$^-$ (Vγ6$^+$). Proportions of Vγ6$^+$ or Vγ4$^+$ γδ T cells that were live (Annexin V$^-$ 7AAD$^-$), early apoptotic (Annexin V$^+$ 7AAD$^-$) or late apoptotic (Annexin V$^+$ 7AAD$^+$) are shown. Graphs show n=9 pooled from 3 independent experiments; each symbol represents an individual mouse. (C) Viable cells (staining negative for viability dye) were calculated in each γδ T cell subset at various ages of mice. N is as described in (A). (D) CD11a expression in lung and spleen γδ T cell subsets was quantified by flow cytometry. n=8; 3 independent experiments. Statistical differences were determined using a two-way ANOVA. NS not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.


Lungs
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TCRβ+
TCRγδ+
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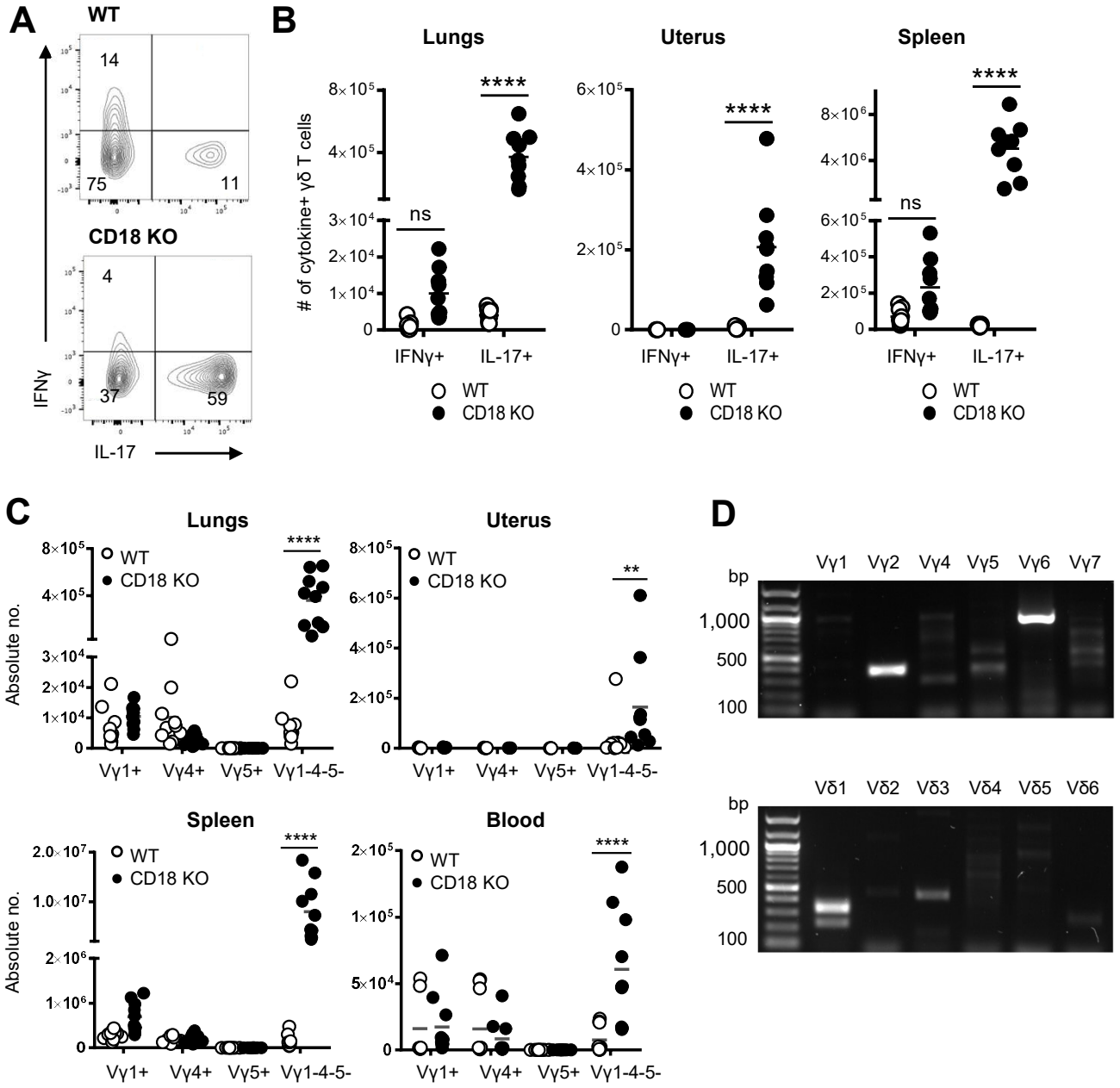
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C
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Uterus
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Absolute no.
WT
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Vγ1+
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Vγ5+
Vγ1-4-5-
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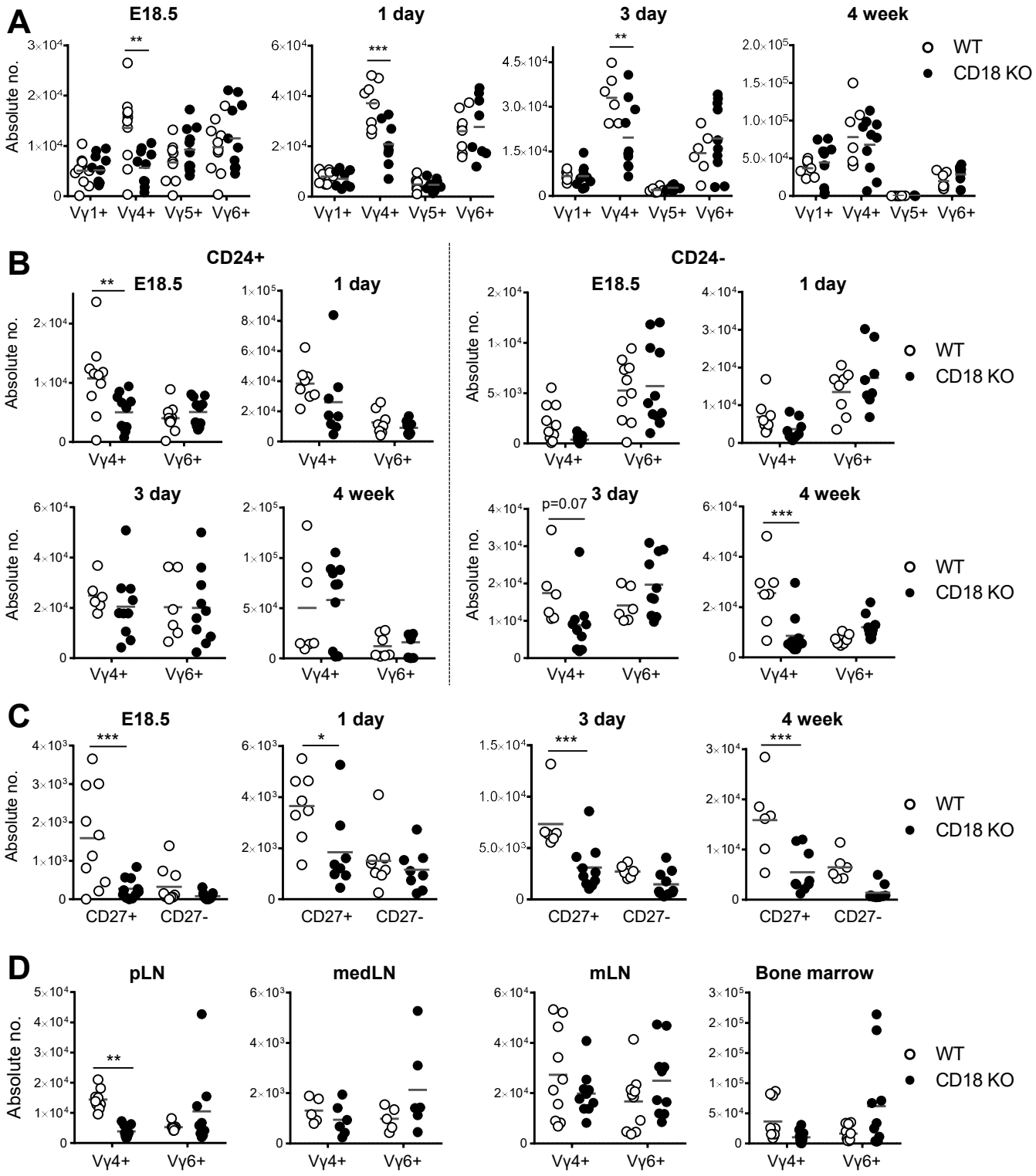
A
E18.5
1 day
3 day
4 week
Absolute no.
Vγ1+ Vγ4+ Vγ5+ Vγ6+
WT
CD18 KO
B
CD24+
CD24-
E18.5
1 day
3 day
4 week
Vγ4+ Vγ6+
p=0.07
C
E18.5
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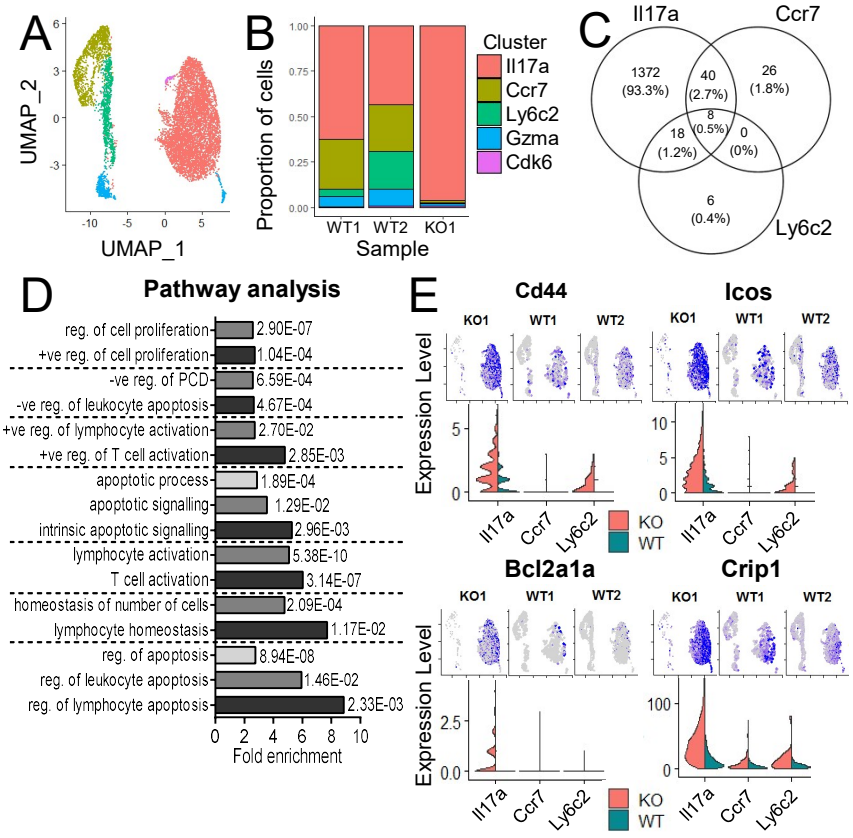
CD27+ CD27-
D
pLN
medLN
mLN
Bone marrow


A
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UMAP_1
B
Proportion of cells
Sample
WT1
WT2
KO1
Cluster
Il17a
Ccr7
Ly6c2
Gzma
Cdk6
C
Il17a
Ccr7
Ly6c2
1372 (93.3%)
40 (2.7%)
26 (1.8%)
8 (0.5%)
18 (1.2%)
0 (0%)
6 (0.4%)
D
Pathway analysis
reg. of cell proliferation
+ve reg. of cell proliferation
-ve reg. of PCD
-ve reg. of leukocyte apoptosis
+ve reg. of lymphocyte activation
+ve reg. of T cell activation
apoptotic process
apoptotic signalling
intrinsic apoptotic signalling
lymphocyte activation
T cell activation
homeostasis of number of cells
lymphocyte homeostasis
reg. of apoptosis
reg. of leukocyte apoptosis
reg. of lymphocyte apoptosis
2.90E-07
1.04E-04
6.59E-04
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2.70E-02
2.85E-03
1.89E-04
1.29E-02
2.96E-03
5.38E-10
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2.09E-04
1.17E-02
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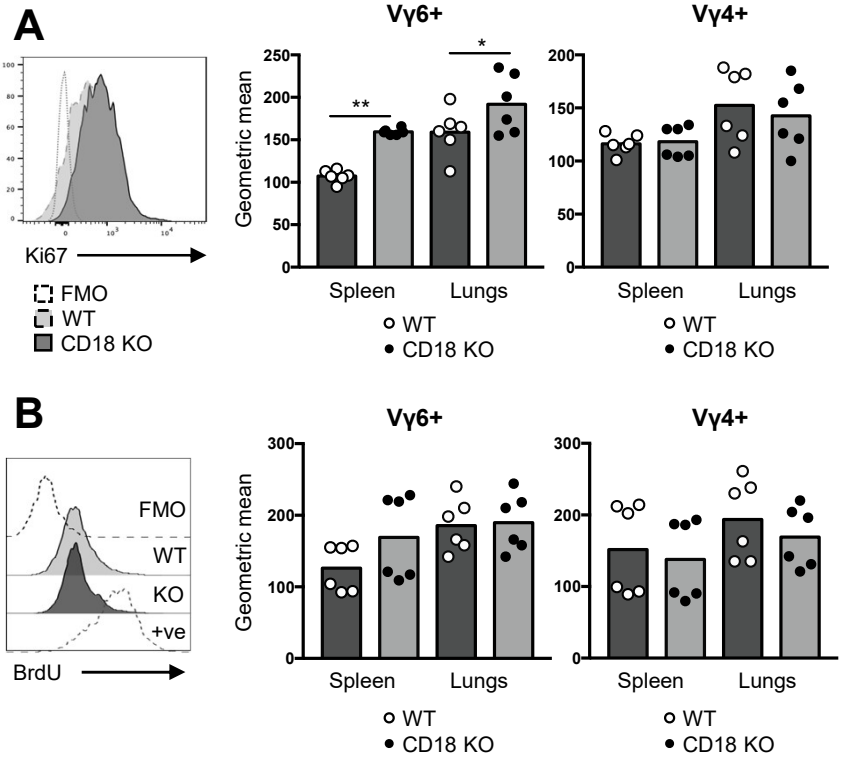
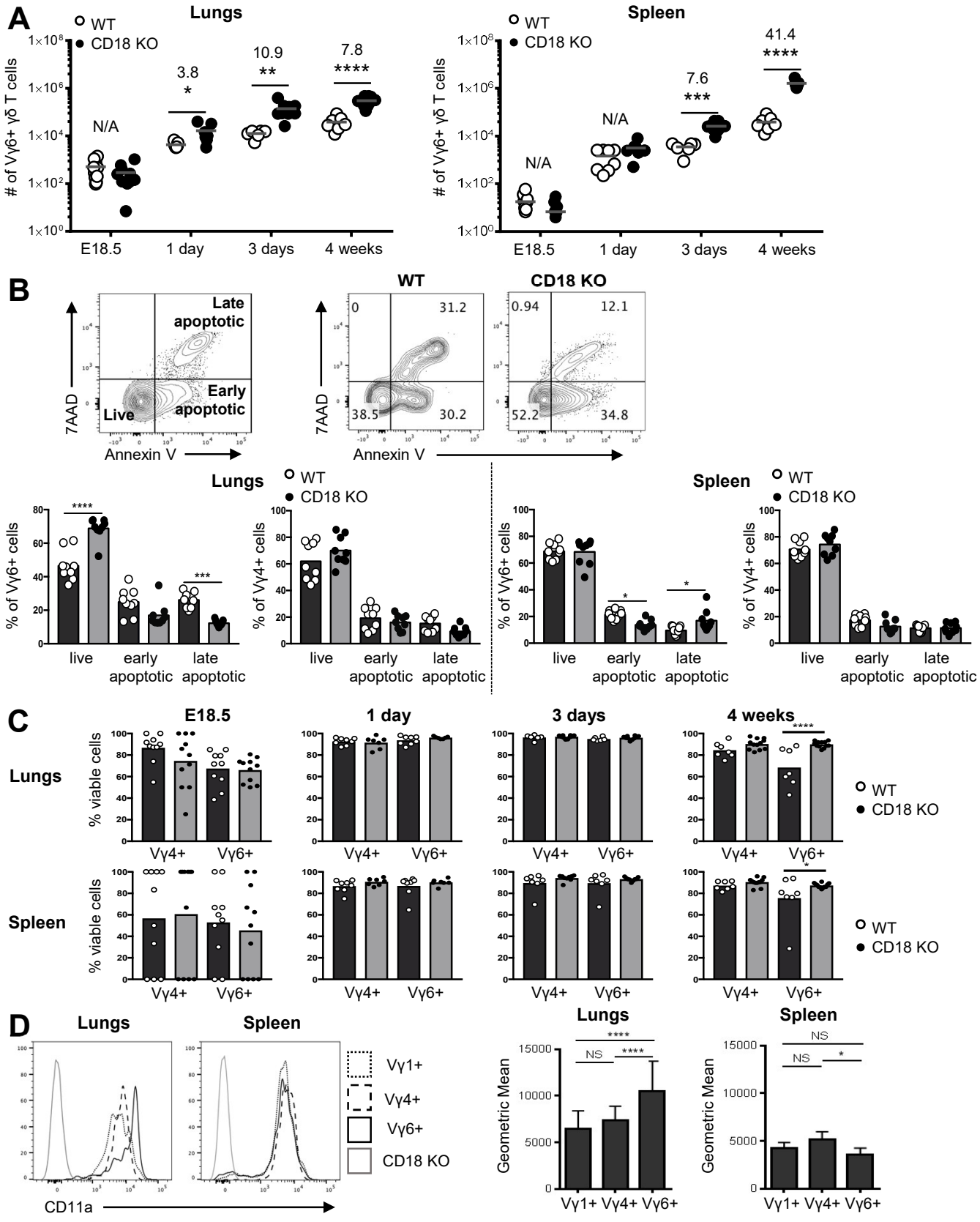
1.46E-02
2.33E-03
Fold enrichment
E
Cd44
Icos
Bcl2a1a
Crip1
Expression Level
KO1
WT1
WT2
KO
WT
Il17a
Ccr7
Ly6c2


A
Ki67
FMO
WT
CD18 KO
Vγ6+
Vγ4+
Geometric mean
**
*
Spleen
Lungs
WT
CD18 KO
B
FMO
WT
KO
+ve
BrdU


A
Lungs
Spleen
WT
CD18 KO
# of Vγ6+ γδ T cells
N/A
3.8
*
10.9
**
7.8
****
41.4
***
E18.5
1 day
3 days
4 weeks
B
WT
CD18 KO
Late apoptotic
Early apoptotic
Live
7AAD
Annexin V
0
31.2
38.5
30.2
0.94
12.1
52.2
34.8
Lungs
Spleen
% of Vγ6+ cells
% of Vγ4+ cells
live
early apoptotic
late apoptotic
C
E18.5
1 day
3 days
4 weeks
Lungs
Spleen
% viable cells
Vγ4+
Vγ6+
D
Lungs
Spleen
Vγ1+
Vγ4+
Vγ6+
CD18 KO
CD11a
Geometric Mean
NS