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Critical role for Slam/SAP signaling in the thymic developmental programming of IL-17- and IFN- γ -producing $\gamma\delta$ T cells

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Abstract

During thymic development, mouse $\gamma\delta$ T cells commit to either an IFN- γ - or an IL-17-producing phenotype through mechanisms that remain unclear. Here, we investigated the extent to which the SLAM/SAP signaling pathway regulates the functional programming of $\gamma\delta$ T cells.

Characterization of SLAM family receptor expression revealed that thymic $\gamma\delta$ T cell subsets were each marked by distinct co-expression profiles of SLAMF1, SLAMF4, and SLAMF6. In the thymus, V γ 1 and V γ 4 T cells that exhibited a SLAMF1⁺SLAMF6⁺ double positive (DP) phenotype were largely contained within immature CD24⁺CD73⁻ and CD24⁺CD73⁺ subsets, while SLAMF1 single positive (SP), SLAMF6 SP, or SLAMF1SLAMF6 double negative (DN) cells were found within mature CD24⁻CD73⁺ and CD24⁻CD73⁻ subsets. In the periphery, SLAMF1 and SLAMF6 expression distinguished IL-17- and IFN- γ -producing $\gamma\delta$ T cells, respectively. Disruption of SLAM family receptor signaling through deletion of SAP resulted in impaired thymic V γ 1 and V γ 4 T cell maturation at the CD24⁺CD73⁻SLAMF1⁺SLAMF6⁺ DP stage that was associated with a decreased frequency of CD44⁺ROR γ t⁺ $\gamma\delta$ T cells. Impaired development was in turn associated with decreased $\gamma\delta$ T cell IL-17 and IFN- γ production in the thymus as well as in peripheral tissues. The role for SAP was subset-specific, as V γ 1V δ 6.3, V γ 4, V γ 5, but not V γ 6 subsets were SAP-dependent. Together, these data suggest that the SLAM/SAP signaling pathway plays a larger role in $\gamma\delta$ T cell development than previously appreciated, and represents a critical checkpoint in the functional programming of both IL-17 and IFN- γ -producing $\gamma\delta$ T cell subsets.

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Introduction:

Innate-like $\gamma\delta$ T cells are unusual T cells that are highly enriched in skin and mucosal tissues where they constitute a prominent source of cytokines and chemokines (1). In mice, most $\gamma\delta$ T cells produce either IL-17 or IFN- γ , and IL-17 production is primarily restricted to the CD27⁻CCR6⁺ V γ 4 and V γ 6 subsets (Heilig and Tonegawa nomenclature (2)), whereas IFN- γ production is observed in CD27⁺CCR6⁻ V γ 1, V γ 4, V γ 5, and V γ 7 subsets (3-6). Accumulating data suggest that the relative balance of these IL-17 or IFN- γ -producing subsets can have significant consequences on disease outcome. IFN- γ -producing $\gamma\delta$ T cells, for example, contribute to anti-tumor immunity (7) and inflammation in a model of cerebral malaria (3, 8). IL-17-producing $\gamma\delta$ T cells, in contrast, have been associated with protection against bacterial and fungal infections (9-11), and promotion of tumor metastasis (12, 13) and immunopathologies in settings of autoimmunity (14-19).

Like their innate-like T cell counterparts, iNKT cells and MAIT cells, the functional programming of innate-like $\gamma\delta$ T cells occurs during thymic development. Unlike iNKT and MAIT cells, however, a significant portion of $\gamma\delta$ T cell developmental programming is restricted to a discrete developmental window (20), as it has been demonstrated that most natural IL-17-producing $\gamma\delta$ T cells ($\gamma\delta$ T17) are generated only during the embryonic/early post-natal stage (21). Although the mechanisms that regulate the differentiation of $\gamma\delta$ T cells into IFN- γ or IL-17-producing subsets remain unclear, a significant body of data supports a model in which strong or weak TCR signals skew the developing $\gamma\delta$ T cell toward IFN- γ (strong signal) or IL-17 (weak signal) production (22-26). In contrast, there is also evidence supporting a TCR-independent model in which the development of some $\gamma\delta$ T17 subsets is dependent on pre-existing hardwired transcriptional programs (27-29). Exactly how these seemingly independent mechanisms converge to regulate the developmental programming of innate-like $\gamma\delta$ T cell subsets remains an open question.

Slam receptors comprise a family of nine cell surface receptors, SLAMF1 (SLAM; CD150), SLAMF2 (CD48), SLAMF3 (Ly9; CD229), SLAMF4 (2B4; CD244), SLAMF5 (CD84), SLAMF6 (Ly108; CD352), SLAMF7 (CRACC; CD319), SLAMF8 (BLAME), and SLAMF9 (SF2001; CD84H), that are expressed primarily on hematopoietic cells and which play diverse roles in immune development and function (30). Most SLAM family receptors interact in a homophilic manner and therefore serve as self-ligands, the exception to this rule being the heterophilic interaction between SLAMF4 and SLAMF2. SLAM receptor signals can be both activating or inhibitory, depending on balanced recruitment of the cytosolic signaling adapter proteins SAP (31) and EAT-2 (32), and inhibitory SHP-1 or SHIP phosphatases to immunoreceptor tyrosine switch motifs in the SLAM family receptor cytoplasmic domain.

Inactivation of the gene encoding SAP (*Sh2d1a*) disrupts the signaling pathway of the majority of SLAM family receptors and results in the development of the primary immunodeficiency X-linked lymphoproliferative disease (XLP) which is characterized by broad immunological defects, among which is a failure in iNKT (33, 34) and MAIT (35) cell development. Investigations into the identity of the relevant SLAM family receptors involved in NKT cell development have revealed a complex interplay of individual

receptors, including SLAMF1, SLAMF3, and SLAMF6 (34, 36-40). Interestingly, within the $\gamma\delta$ T cell compartment, it was previously demonstrated that development of the V γ 1V δ 6.3 ($\gamma\delta$ NKT) T cell subset is dependent on SAP (41), indicating that the requirement for SLAM/SAP signaling during development is shared by NKT cells, MAIT cells, and at least one $\gamma\delta$ T cell subset. Yet, very little is known regarding SLAM family receptor expression on $\gamma\delta$ T cells in either the periphery or the thymus, or the extent to which this receptor system is involved in $\gamma\delta$ T cell development. In this study, therefore, we explored the extent to which the SLAM/SAP signaling pathway regulates the developmental programming of thymic $\gamma\delta$ T cells that determines their effector function.

Materials and Methods

Mice

C57BL/6J, BALB/cJ, and B6.*Sh2d1a*^{-/-} (*SAP*^{-/-}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed and bred in a specific pathogen-free barrier room at the AAALAC-approved animal facility of the University of Vermont. Age and sex matched mice between 8 and 12 weeks of age were used for experiments involving adult naïve mice as indicated in the figure legends. To analyze $\gamma\delta$ T cells in embryonic thymus, timed-pregnant matings were used. The presence of a vaginal plug indicated day 0.5 of pregnancy. No attempt of sex matching was done for fetuses and newborn mice. All procedures involving animals were approved by the University of Vermont Institutional Animal Care and Use Committee.

Cell preparation and stimulation

Single cell suspensions of splenocytes, lymph nodes, and thymocytes were obtained by gently pressing tissue through nylon mesh. RBCs were lysed using Gey's solution. Lung cell preparations were conducted as described (42). Lungs were dissected away from the trachea and finely minced in enzymatic digestion buffer (Dulbecco's modified Eagle's medium (DMEM), 1 mg/ml collagenase type IV (Invitrogen), 0.2 mg/ml DNase (Sigma)). Lung tissue was resuspended in additional enzymatic digestion buffer and shaken at 200 rpm at 37°C for 20 min. The samples were triturated with a 16-gauge needle and allowed to digest for an additional 20 min, after which they were triturated one last time. After RBC lysis using Gey's solution, the digested tissue was passed through a 70- μ m filter and washed in PBS/2% FCS.

Single cell suspensions from skin were prepared as described (43). Briefly, depilated skin was digested in Trypsin/GNK buffer (0.3% bovine pancreatic trypsin (Type XI) (Sigma), 5 mM KCl, 150 mM NaCl, and 0.1% glucose, pH 7.6) for 2 h at 37 °C., after which the epidermis was separated from the dermis. Epidermis was then placed in Trypsin/GNK buffer containing 0.5% DNase I and incubated for 15 min at 37 °C. After incubation, samples were passed through 70 μ m mesh, washed with DMEM + 10% FBS, and resuspended in RPMI 1640 supplemented with 2 mM l-glutamine, 50 μ M 2-mercaptoethanol, 50 U/ml penicillin, 50 ug/ml streptomycin + 10 U/ml IL-2 and incubated overnight at 37 °C in a 5% CO₂ incubator prior to staining.

Liver intrahepatic leukocytes (IHLs) were isolated as previously described (44). Briefly, livers were perfused through the portal vein with PBS, subsequently removed, minced, and gently pressed through nylon mesh. The resulting cell suspension was washed twice and then resuspended in isotonic 33.8% Percoll (GE Healthcare). After centrifugation, the IHL cell pellet was resuspended, washed with PBS, and red blood cells were lysed using Gey's solution. Single cell suspensions were resuspended in flow cytometry staining buffer.

Intestinal Intraepithelial Lymphocytes (IEL) were isolated as previously described (45). Briefly, mouse small intestines were flushed with cold CMF buffer (1 mM HEPES, 2.5 mM NaHCO₃, 2% FBS in Ca²⁺/Mg²⁺-free HBSS pH 7.2). Surrounding fat tissue was removed, intestines opened with a longitudinal incision, and excess mucous removed by gentle scraping. Tissue was cut into small pieces, washed 3 times with CMF, followed by shaking in CMF+10% FBS/1 mM dithioerythritol (DTE) for 20 minutes at 37 °C. This step was repeated and the cells in the supernatants of both extractions were combined. Cells were incubated for 20 minutes at 4 °C, before transferring cell supernatants into a fresh tube. Cells were centrifuged, washed once with RPMI, before resuspending in 44% Percoll solution. Cells were overlaid on a 67% Percoll solution, spun for 20 minutes at room temperature. Cells at the interface were collected, washed twice with RPMI, prior to counting and further analysis.

For all tissues, cell numbers were determined on a MACSQuant VYB (Miltenyi) using propidium iodide (1 µg/ml in PBS) for dead cell exclusion. Cell stimulations were conducted in Click's medium (Sigma) supplemented with 2 mM l-glutamine, 50 µM 2-mercaptoethanol, 50 µg/ml gentamycin, 50 U/ml penicillin, 50 µg/ml streptomycin, and 5% FBS with 25 ng/ml PMA and 500 ng/ml ionomycin for 4 hours at 37 °C and 5% CO₂. After 2 hours, 2 µM monensin was added to the culture.

Flow Cytometry

Cells were stained with Live Dead Fixable Blue staining reagent (ThermoFisher, Grand Island, NY) at 4°C in PBS for 30 min, after which they were washed and resuspended in PBS/2% FCS containing 0.1% sodium azide and Fc-Block (Biolegend) to block non-specific antibody binding prior to the addition of antibodies. Brilliant Violet Staining buffer (BD Biosciences) was used in experiments containing more than one Brilliant Violet or Brilliant Ultraviolet fluorophore conjugated antibody. To identify the Vγ6 subset, the staining procedure described in Roark et al. was followed (46).

Cells were washed twice with PBS/2% FCS/0.1% sodium azide solution and fixed in PBS/1% PFA buffer. For intracellular cytokine staining, cells were permeabilized with PBS/1% FCS/0.1% sodium azide/0.1% saponin after surface staining and fixation. 25 µg/ml total rat IgG were added to reduce unspecific binding prior to cytokine staining with fluorescent-labeled antibodies. Nuclear transcription factors were analyzed using the Foxp3 transcription factor fixation/permeabilization reagent (ThermoFisher). Briefly, after staining for surface markers cells were resuspended in the fixation/permeabilization reagent and incubated overnight at 4 °C. Cells were washed, incubated with 25 µg/ml total rat IgG prior to adding antibodies against transcription factors. After staining at 4 °C, cells were washed and resuspended in PBS/2% FCS/0.1% sodium azide. Flow cytometry data were collected

on a LSRII flow cytometer (BD Bioscience) and analyzed with FlowJo software (Treestar). Representative gating schemes used to identify $\gamma\delta$ T cell subsets in thymus and lung are shown in the supplement (Suppl. Fig. 1).

Antibodies used in these experiments were: CD11b (M1/70), CD19 (1D3), CD24 (M1/69), CD27 (LG.3A10), CD44 (IM7), CD45 (30-F11), CD48 (HM48-1), CD73 (TY/11.8), CD84 (mCD84.7), CD150 (TC15-12F12.2), CD229 (Ly9ab3), CD352 (330-AJ), IFN- γ (XMG1.2), IL-17A (TC11-18H10.1), TCR β (H57-597), TCR δ (GL3), V γ 1 (2.11), V γ 5 (536), V δ 6.3 (C504.17c) from Biolegend; CD196 (140706), CD244 (2B4), ROR γ t (Q31-378) from BD Biosciences; CD244.1 (C9.1), CD352 (13G3-19D), Eomes (Dan11mag), V γ 4 (UC3-10A6) from eBiosciences

t-SNE analysis

Dimensionality reduction of flow cytometry data was conducted using t-stochastic neighbor embedding (t-SNE) in FlowJo. First, traditional gating was used to identify $\gamma\delta$ T cells, after which the $\gamma\delta$ T cell node data from 5 B6 and 5 BALB/c mice was concatenated for analysis. t-SNE was conducted on the SLAMF1, SLAMF4, SLAMF6, V γ 1, V γ 4, V γ 6, CD44, and CD27 parameters on the concatenated B6/BALB/c data using 1000 iterations, perplexity = 30, learning rate = 957, after which the B6 and BALB/c data were extracted for independent visualization.

Transcriptional profiling

Leukocytes from lung cell preparations were isolated using anti-CD45 Microbeads and LS columns according to the manufacturer's instruction (Miltenyi). Cells were stained with Fixable Viability Dye eFluor780 (eBioscience), washed, and surface stained with Abs against SLAMF1, SLAMF6, NK1.1, TCR δ , TCR-V γ 1, TCR-V γ 4, TCR β , CD11b, CD19, and CD45 in sterile PBS/2% FBS. Cells were sorted on a FACS Aria III (BD Bioscience) into culture medium, pelleted, washed, and snap frozen until later use. RNA was isolated using a RNeasy Micro kit (Qiagen). RNA quality was assessed using an Agilent Bioanalyzer 2100, and RNA concentration was calculated using a Qubit fluorometer (ThermoFisher Scientific). Amplified cDNA was prepared using the Ovation Pico WTA System V2 (Nugen), and biotinylated using the Encore biotin module (Nugen). Hybridization with GeneChip Mouse Gene 2.0 ST arrays (Affymetrix) was conducted overnight at 45 °C. at 60 rpm. CEL files were analyzed using Transcriptome Analysis Console (ThermoFisher) software. Genes that demonstrated a greater than or less than 2-fold difference in expression and a false-discovery rate of <0.2 were identified. This resulted in 316 SLAMF1-enriched genes and 229 SLAMF6-enriched genes. These gene lists were filtered by removing unannotated transcripts, predicted transcripts, and by removing all but 1 of the 28 multiple transcript isoforms of *Syne1*, yielding 244 SLAMF1-enriched and 181 SLAMF6-enriched transcripts. All differentially expressed genes in this list exhibited a p-value < 0.004. The raw data files for this analysis were deposited in NCBI GEO (GSE128544), <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE128544>.

Data were analyzed for gene ontological and pathway enrichment using NIH DAVID analysis and IPA (filtered to include only those with a p-value and FDR <0.05). Heatmaps

were generated using prettyheatmap with row-scaling. Upstream regulator prediction using IPA analysis was conducted on the 425 differentially expressed genes based on fold-change (SLAMF1⁺SLAMF6⁻/SLAMF1⁻SLAMF6⁺). The result was filtered to include only those predicted regulators with p-value of overlap $< 1 \times 10^{-5}$, ranked according to activation z-score, after which the ten highest (predicted to be active in SLAMF1⁺SLAMF6⁻) and lowest (predicted to be active in SLAMF1⁻SLAMF6⁺) were chosen for display.

Statistical analysis

Statistical analysis was performed using Prism (GraphPad Software, San Diego, CA). Unpaired Student t-tests, one-way ANOVA, or two-way ANOVA was used where appropriate. For ANOVA posttest comparisons, the Dunnett, Tukey, or Sidak multiple comparison tests were used where appropriate. In all cases, tests were considered significant when $p < 0.05$. Unless otherwise indicated, error bars represent standard deviation.

Results:

Thymic $\gamma\delta$ T cell developmental stages are associated with distinct SLAM family receptor expression profiles.

$\gamma\delta$ T cell effector functions are largely programmed during thymic development, and IL-17 functional programming is thought to occur primarily during embryonic and neonatal thymic development (21). Therefore, we examined the co-expression of SLAMF1 through SLAMF6 receptors on $\gamma\delta$ T cell subsets in C57BL/6 embryonic day 17 (E.17) thymus. This analysis revealed that SLAMF5 was undetectable on $\gamma\delta$ T cells and that the expression of SLAMF2 and SLAMF3 was relatively uniform among $\gamma\delta$ T cell subsets (Suppl. Fig. 2). In contrast, SLAMF1, SLAMF4, and SLAMF6 exhibited a surprising degree of heterogeneity in their co-expression (Fig. 1A). For example, a significant fraction of E.17 thymic $\gamma\delta$ T cells were SLAMF1⁻SLAMF4⁺SLAMF6⁻ and this population consisted primarily of V γ 5 T cells (Fig. 1B). In the skin, mature V γ 5 T cells exhibit the same SLAMF1⁻SLAMF4⁺SLAMF6⁻ phenotype (Suppl. Fig. 2), suggesting that the V γ 5 SLAM family receptor expression profile is established during embryonic thymic development (47). Similarly, we observed that V γ 6 T cells were predominantly SLAMF1⁺SLAMF4⁻SLAMF6⁻ in the E.17 thymus (Fig. 1A), similar to the SLAM receptor expression profile that they exhibit in the adult lung (see below).

In contrast to the V γ 5 and V γ 6 populations, E.17 V γ 1 and V γ 4 T cells showed broad expression of SLAMF1 and SLAMF6 and they rarely expressed SLAMF4 (Fig. 1A). We identified prominent SLAMF1 and SLAMF6 single positive (SP) V γ 1 and V γ 4 T cell populations that represented the predominant phenotypes detectable in peripheral tissues (Fig. 1A). Consistent with a previous report that IL-17-producing $\gamma\delta$ T cells preferentially express SLAMF1 (48), ROR γ t⁺ E.17 $\gamma\delta$ T cells were largely found within a SLAMF1^{hi}SLAMF6^{low} $\gamma\delta$ T cell subset (Fig. 1C) and the majority of E.17 IL-17-producing $\gamma\delta$ T cells were SLAMF1⁺, while the majority of IFN- γ -producing $\gamma\delta$ T cells were SLAMF1⁻ (Fig. 1D).

In addition to the SLAMF1 and SLAMF6 SP V γ 1 and V γ 4 T cell populations, we detected prominent SLAMF1SLAMF6 double positive (DP) V γ 1 and V γ 4 T cell populations (Fig. 1A) that were rarely observed in the periphery (see below). To characterize this SLAMF1SLAMF6 DP population in more detail, we evaluated neonate thymus V γ 1 and V γ 4 SLAMF1 and SLAMF6 expression in the context of CD24 and CD73 expression which can be used to track $\gamma\delta$ T cell development (49, 50). Recently, two distinct pathways of V γ 1 and V γ 4 maturation have been defined using these markers (51). In pathway 1, utilized by both V γ 1 and V γ 4 T cells, immature CD24⁺CD73⁻ $\gamma\delta$ T cells upregulate CD73 (CD24⁺CD73⁺) prior to CD24 downregulation and maturation (CD24⁻CD73⁺). In pathway 2, utilized by V γ 4 and V γ 6 T cells, immature CD24⁺CD73⁻ $\gamma\delta$ T cells downregulate CD24 (CD24⁻CD73⁻) prior to CD73 upregulation. Consistent with a previous report (51), we found that day 7 thymus V γ 1 and V γ 4 T cells exhibited distinct patterns of CD24 and CD73 expression and that V γ 1 cells exhibited prominent immature CD24⁺CD73⁻ and mature CD24⁻CD73⁺ populations, while V γ 4 cells were primarily CD24⁺CD73⁻ and CD24⁻CD73⁻ (Fig. 1E).

Examination of SLAMF1 and SLAMF6 expression on these subsets revealed that in both V γ 1 and V γ 4 T cells, the SLAMF1SLAMF6 DP populations largely resided within the immature CD24⁺CD73⁻ population, while SLAMF1 SP and SLAMF6 SP and SLAMF1SLAMF6 DN cells predominated in the mature CD24⁻CD73⁺ and CD24⁻CD73⁻ populations (Fig. 1F). In V γ 1 cells, which develop using pathway 1, there was a significant decrease in the frequency of SLAMF1SLAMF6 DP cells as cells progressed through the CD24⁺CD73⁻ to CD24⁺CD73⁺ to CD24⁻CD73⁺ stages (Fig. 1G), and the prominent mature CD24⁻CD73⁺ V γ 1 population was largely dominated by SLAMF6 SP V γ 1 cells (Fig. 1F). In V γ 4 cells, which develop primarily using pathway 2 during neonatal development (51), there was a significant decrease in the frequency of SLAMF1SLAMF6 DP cells as they progressed from CD24⁺CD73⁻ to CD24⁻CD73⁻ (Fig. 1G). Interestingly, mature CD24⁻CD73⁻ V γ 4 cells were predominantly SLAMF1 SP, while the small number of mature CD24⁻CD73⁺ V γ 4 cells were predominantly SLAMF6 SP and SLAMF1SLAMF6 DN (Fig. 1F). Similar results were observed in adult thymus, with the exception that in V γ 4 cells, which develop primarily using pathway 1 during adult development (51), there was a decrease in the frequency of SLAMF1SLAMF6 DP cells as they progressed from CD24⁺CD73⁻ to CD24⁺CD73⁺ (Suppl. Fig. 2). In both V γ 1 and V γ 4 subsets, we noted the presence of SLAMF1 SP and/or SLAMF6 SP $\gamma\delta$ T cells in the immature CD24⁺CD73⁻ populations (Fig. 1F), suggesting that the alterations in SLAM family receptor expression are already occurring at this developmental stage. Taken together, these data indicated that the co-expression of SLAMF1, SLAMF4, and SLAMF6 distinguished 3 groups of $\gamma\delta$ T cell subsets in the thymus: V γ 1/V γ 4, V γ 5, and V γ 6. Our findings were consistent with a developmental scheme in which immature CD24⁺CD73⁻ thymic V γ 1 and V γ 4 T cells initially possess a SLAMF1SLAMF6 DP profile that resolves into either mature SLAMF1, SLAMF6 SP, or SLAMF1SLAMF6 DN phenotypes upon maturation and acquisition of function.

Impaired $\gamma\delta$ T development in SAP-deficient mice

SLAMF1, SLAMF4, and SLAMF6 transduce signals by utilizing the signaling adapter proteins SAP and EAT-2, which bind to immunoreceptor tyrosine switch motifs (ITSMs) located in the cytoplasmic tails of SLAM family receptors (30). *Sh2d1a* (encoding SAP) is the most widely expressed of these adapter proteins in $\gamma\delta$ T cells and SAP-deficient mice have previously been reported to be deficient in the IFN- γ -producing V γ 1V δ 6.3 ($\gamma\delta$ NKT) subset (52). To determine whether the SLAM/SAP signaling pathway played any additional roles in the functional programming that $\gamma\delta$ T cells undergo during thymic development, we examined $\gamma\delta$ T cell development in SAP-deficient mice during embryonic and neonatal stages of development.

A comparison of thymic $\gamma\delta$ T cells between B6 and B6.SAP-deficient mice revealed no significant change in the overall frequencies of V γ 4, V γ 5, or V γ 6 T cell subsets, although we did confirm the critical role of SAP in the development of the V γ 1V δ 6.3 subset on day 5 (52)(Suppl. Fig. 3). An examination of the SLAM family receptor expression profiles on E.17 $\gamma\delta$ T cells revealed a significant decrease in the frequency of SLAMF1 SP V γ 1 and V γ 4 T cells (Fig. 2A). The reduction in the SLAMF1 SP subset coincided with a significant increase in the frequency of SLAMF1SLAMF6 DP subsets in both E.17 (Fig. 2A) and neonate (Suppl. Fig. 3) B6.SAP^{-/-} thymus. In accordance with our observations that SLAMF1SLAMF6 DP largely resided within the CD24⁺CD73⁻ population, while SLAMF1 and SLAMF6 SP cells largely resided within the CD24⁻CD73⁻ and CD24⁻CD73⁺ populations (Fig. 1F), we observed a significant increase in the frequency of CD24⁺CD73⁻ thymic $\gamma\delta$ T cells in SAP-deficient mice (Fig. 2B). Concomitant with this increase was a significant decrease in CD24⁺CD73⁺, CD24⁻CD73⁺ and CD24⁻CD73⁻ $\gamma\delta$ T cells (Fig. 2B).

Examination of the individual $\gamma\delta$ T cell subsets revealed that among V γ 1 T cells, SAP deficiency resulted in increased CD24⁺CD73⁻ and CD24⁺CD73⁺ populations and a decreased frequency of CD24⁻CD73⁺ cells (Fig. 2C). This was due in large part to the near complete absence of the V γ 1V δ 6.3⁺ subset, which was primarily CD24⁺CD73⁺ and CD24⁻CD73⁺ (Suppl. Fig. 3), as we observed no significant SAP-dependent changes in maturation among the V γ 1V δ 6.3⁻ T cells (Fig. 2C). Therefore, among neonate V γ 1 T cells, the major effect of SAP deletion was on the V γ 1V δ 6.3⁺ subset. Surprisingly, however, we also observed that among V γ 4 T cells, SAP deficiency resulted in significantly more CD24⁺CD73⁻, and significantly fewer CD24⁻CD73⁻ V γ 4 cells, suggesting that SAP was critical in the CD24⁺CD73⁻ to CD24⁻CD73⁻ (i.e., Pathway 2) transition of a V γ 4 subset (Fig. 2D). No effect of SAP deficiency on the CD24 expression of the V γ 6 subset was observed (not shown). Together, these data suggested that SAP-mediated signaling is integral to the developmental progression of V γ 4 as well as V γ 1 $\gamma\delta$ T cells during thymic development and that it functions to promote $\gamma\delta$ T cell maturation.

Next, we assessed whether SAP-deficient thymic $\gamma\delta$ T cells exhibited any functional defects. Examination of the E.17 CD44⁺ROR γ t⁺ $\gamma\delta$ T cell population revealed a significant decrease in the frequency of CD44⁺ROR γ t⁺ $\gamma\delta$ T cell subsets in SAP-deficient mice (Fig. 3A). Significant decreases were observed in the V γ 1, V γ 4, and V γ 1⁻4⁻5⁻ subsets, but not in V γ 5, very few of which were CD44⁺ROR γ t⁺ (Fig. 3A). Examination of neonatal day 5 thymocytes revealed that the SAP-dependent decrease in CD44⁺ROR γ t⁺ $\gamma\delta$ T cells was not

restricted to embryonic thymic development, as there was a significant decrease in the frequency of CD44⁺ROR γ t⁺ V γ 4 T cells, which comprise the majority of thymic $\gamma\delta$ T cells at this stage of development (Fig. 3B). However, the SAP-dependent decrease in CD44⁺ROR γ t⁺ V γ 1 and V γ 1⁻4⁻5⁻ populations was not observed in the neonatal thymus (Fig. 3B).

Next, we investigated whether SAP played a role in the acquisition of $\gamma\delta$ T cell function during thymic development. A comparison of E.17 $\gamma\delta$ T cell cytokine production between B6 and B6.SAP^{-/-} mice revealed a significant decrease in both IL-17 and IFN- γ production in SAP-deficient mice (Fig. 3C). Subset analysis revealed that the reduction in IFN- γ production was due primarily to an effect on V γ 5 $\gamma\delta$ T cells (Fig. 3D), while the reduction in IL-17 production was instead observed in both V γ 1 and V γ 4 $\gamma\delta$ T cell subsets (Fig. 3E). No significant decrease in the IL-17-producing V γ 1⁻V γ 4⁻V γ 5⁻ population was observed at this time, consistent with SAP-independent development of the V γ 6 population (data not shown).

Analysis of later stages of development revealed that the effect of SAP deficiency was dependent on both the subset and developmental stage. A significant effect of SAP deficiency was observed on thymic V γ 4 IL-17 production at all stages examined except for day 0. Conversely, we found that V γ 4 IFN- γ production was significantly decreased in SAP-deficient mice on day 0, but not any of the other timepoints examined. Similarly, we noted decreased thymic V γ 1 IL-17 in B6.SAP^{-/-} mice only at E.17, and decreased thymic V γ 1 IFN- γ only on days 5 and 7 (Fig. 3E). Together, these data suggested that the SLAM/SAP signaling pathway played a significant role in the developmental programming of thymic V γ 1, V γ 4, and V γ 5 IL-17 and IFN- γ production during embryonic and neonatal thymic development and that SAP coordinately regulated $\gamma\delta$ T cell maturation and the acquisition of $\gamma\delta$ T cell function.

Tissue-specific heterogeneity in SLAM receptor expression profiles on $\gamma\delta$ T cell subsets in the periphery.

Next, we examined the extent to which SAP-dependent thymic $\gamma\delta$ T cell development regulated $\gamma\delta$ T cell function in peripheral tissues. We first assessed the co-expression of SLAMF1 through SLAMF6 on $\gamma\delta$ T cell subsets from different tissues. Similar to our results in the thymus, we found little evidence of SLAMF5 expression, and we found uniform expression SLAMF2 and SLAMF3 on $\gamma\delta$ T cells and from the lung, spleen, and lymph node (Suppl. Fig. 4). In contrast, our analysis revealed significant heterogeneity in SLAMF1, SLAMF4, and SLAMF6 co-expression on $\gamma\delta$ T cell subsets obtained from different tissues (Fig. 4A). In the lung, we found two distinct populations of V γ 1 and V γ 4 $\gamma\delta$ T cells which were either SLAMF1 SP or SLAMF6 SP. In contrast, V γ 6 $\gamma\delta$ T cells were uniformly SLAMF1 SP with no detectable SLAMF6 expression (Fig. 4A). A similar SLAM receptor expression pattern was observed in the spleen although the frequency of the SLAMF1 SP V γ 4 T cell population was markedly reduced compared with the lung and essentially absent in the V γ 1 subset (Fig. 4A). In contrast to lung and spleen, a significant proportion of SLAMF4-expressing $\gamma\delta$ T cell subsets were detected in the liver, while gut IEL $\gamma\delta$ T cells only expressed SLAMF4, and not SLAMF1 or SLAMF6 (Fig. 4A).

Heterogeneity in SLAM family receptor co-expression was also observed in BALB/cJ $\gamma\delta$ T cells, which possess a different *Slam* haplotype (53) (Fig. 4B). We did note significant differences between the strains indicating that SLAM receptor co-expression profiles were influenced by genetic background. Notably, we observed a significantly greater proportion of SLAMF1⁺SLAMF6⁻ lung and spleen V γ 4 T cells in BALB/c compared to their B6 counterparts (Fig. 4B).

Given the association of SLAMF1 expression with $\gamma\delta$ T cell IL-17 production in the thymus (Fig. 1D), we assessed whether distinct SLAM family receptor expression profiles coincided with CD44 and CD27 expression which are canonical markers of IL-17 and IFN- γ -producing $\gamma\delta$ T cells, respectively. We focused our attention on lung $\gamma\delta$ T cells, since they exhibited a diversity of SLAM family receptor expression profiles. This analysis revealed that in both B6 and BALB/c mice, SLAMF1⁺SLAMF6⁻ $\gamma\delta$ T cells were primarily CD44⁺CD27⁻ consistent with an IL-17-producing phenotype (48), while SLAMF1⁻SLAMF6⁺ $\gamma\delta$ T cells were primarily CD44⁻CD27⁺ suggestive of an IFN- γ -producing phenotype (Fig. 4C). SLAMF4-expressing $\gamma\delta$ T cells, in contrast were distributed among both the CD44⁺CD27⁻ and CD44⁻CD27⁺ populations (Fig. 4C). Together, these data indicated that the co-expression of SLAM family receptors on $\gamma\delta$ T cells constituted unique SLAM receptor expression profiles that varied in both a tissue-dependent and V γ subset-dependent manner. The data also suggested that the SLAM receptor expression profiles of $\gamma\delta$ T cell subsets in the periphery largely reflected those established upon maturation during thymic development, the exception being SLAMF4 whose expression on some $\gamma\delta$ T cell subsets appeared to be modulated in the periphery. Finally, the data indicated that these SLAM receptor expression profiles were present in strains representative of *Slam* haplotypes 1 and 2, and that in each haplotype SLAMF1 and SLAMF6 expression exhibited a strong association with canonical markers of $\gamma\delta$ T cell function.

SLAM receptor expression profiles distinguish $\gamma\delta$ T cell functional subsets in the periphery.

To determine whether SLAM receptor expression profiles marked functional subsets of $\gamma\delta$ T cells, we assessed lung $\gamma\delta$ T cell cytokine production after stimulation with PMA/ionomycin. These data revealed that lung $\gamma\delta$ T cell IFN- γ production was associated with SLAMF1⁻SLAMF6⁺ $\gamma\delta$ T cells, while IL-17 production was associated with SLAMF1⁺SLAMF6⁻ $\gamma\delta$ T cells (Fig. 5A). Similarly, stimulation of both spleen (Fig. 5B) and lymph node (Fig. 5C) $\gamma\delta$ T cells revealed a strong association of SLAMF1 and SLAMF6 expression with $\gamma\delta$ T cell IL-17 and IFN- γ production, respectively. Together, these data suggested that the mutually exclusive expression of SLAMF1 and SLAMF6 marked IL-17- and IFN- γ -producing V γ 1, V γ 4, and V γ 6 $\gamma\delta$ T cell subsets in the periphery.

To explore this association further, we compared the *ex vivo* transcriptional profiles of sorted lung SLAMF1⁺SLAMF6⁻ and SLAMF1⁻SLAMF6⁺ lung V γ 4 from naïve C57BL/6J mice. This analysis revealed the differential expression of 244 genes in SLAMF1⁺SLAMF6⁻ and 181 genes in SLAMF1⁻SLAMF6⁺ lung V γ 4 (Fig. 6A). In accordance with our functional analysis, we found that SLAMF1⁺SLAMF6⁻ lung V γ 4 $\gamma\delta$ T cells exhibited a transcriptional profile highly suggestive of IL-17 production. We observed differential expression of *Il23r*,

Ill7a, Ill7f, Illr1, Blk, Rorc, Sox13, Maf, Ccr6, Ccr2, and Scart2 all of which have been previously associated with a $\gamma\delta$ T17 phenotype (27, 28, 54) (Fig. 6A). In contrast, *Ifng, Eomes, IL12rb, and Crtam* were preferentially expressed in SLAMF1⁻SLAMF6⁺ V γ 4 $\gamma\delta$ T cells, suggestive of an IFN- γ -producing phenotype (Fig. 6A). These findings were consistent with a recent report demonstrating that EOMES expression marks IFN- γ -producing $\gamma\delta$ T cells (55).

Pathway analysis (IPA) of differentially expressed genes revealed that transcripts enriched in SLAMF1⁺SLAMF6⁻ lung V γ 4 cells exhibited significant overlap with genes regulated by ZBTB16 (PLZF), IL23R, ROR γ T, and IL-1B. In contrast, transcripts enriched in SLAMF1⁻SLAMF6⁺ lung V γ 4 exhibited significant overlap with genes regulated by IL-12, TBX21 (T-bet), IFN- γ , IL-27, and TCF3 (E2A) (Fig. 6B). These transcriptional profiles were confirmed using flow cytometry which revealed that ROR γ T expression was largely confined to the SLAMF1⁺SLAMF6⁻ $\gamma\delta$ T cells in both lung and lymph node, while EOMES expression was found in the SLAMF1⁻SLAMF6⁺ $\gamma\delta$ T cell subsets (Fig. 6C). Together, these data suggested that lung SLAMF1⁺SLAMF6⁻ and SLAMF1⁻SLAMF6⁺ V γ 4 $\gamma\delta$ T cells possessed transcriptional profiles consistent with IL-17 and IFN- γ production, respectively.

Impaired $\gamma\delta$ T function in SAP-deficient mice

Finally, we examined the extent to which impaired thymic $\gamma\delta$ T cell developmental programming in SAP-deficient mice affected $\gamma\delta$ T cell function in peripheral lung and lymphoid organs. A comparison of neonate lung $\gamma\delta$ T cells between B6 and B6.SAP^{-/-} mice revealed that there was a significant decrease in lung V γ 4 IL-17, but not IFN- γ , production (Fig. 7A) and this decrease corresponded to a significant decrease in SLAMF1⁺IL-17⁺ V γ 4 cells (Fig. 7B). No difference was observed in either lung V γ 1 IL-17 or IFN- γ production (Fig. 7A), nor did we observe any SAP-dependent effects on V γ 6 IL-17 production (data not shown). Interestingly, the SAP-dependent decrease in V γ 4 T cell IL-17 production did not persist in the adult lung. Instead, a comparison of adult lung $\gamma\delta$ T cell cytokine production between B6 and B6.SAP^{-/-} mice revealed a significant decrease in both lung V γ 1 and V γ 4 IFN- γ production (Fig. 7C). We detected no SAP-dependent effect on V γ 6 IL-17 production (data not shown). A comparison of adult spleen V γ 1 and V γ 4 cytokine production between B6 and B6.SAP^{-/-} mice also revealed significantly less IFN- γ in SAP-deficient V γ 1 and V γ 4 T cells than in their B6 counterparts, indicating that the SAP-dependent decrease was not tissue-specific (Fig. 7D). Together, these data demonstrated that $\gamma\delta$ T cell IL-17 and IFN- γ production in the periphery was significantly impaired in SAP-deficient mice. In addition, the data indicated that the SAP-dependent impairment in $\gamma\delta$ T cell function in the periphery was both age and subset-dependent.

Discussion

Here, we identified the SLAM/SAP signaling pathway as a critical regulator of the functional programming that generates both IL-17 and IFN- γ -producing $\gamma\delta$ T cells during thymic development. We found that maturation of thymic V γ 1 and V γ 4 was associated with the transition from a SLAMF1⁺SLAMF6⁻ DP phenotype to either SLAMF1⁺ or SLAMF6⁺ SP

or SLAMF1⁺SLAMF6⁻ DN phenotypes, and that this transition was concomitant with the acquisition of $\gamma\delta$ T cell effector function in the thymus. In the periphery, SLAMF1 and SLAMF6 marked IL-17⁻ and IFN- γ -producing $\gamma\delta$ T cells, respectively. Importantly, our findings indicated that in addition to its role in V γ 1V δ 6.3 T cell development, SAP also regulated V γ 4 T cell development, and that SAP-deficient V γ 4 T cells exhibited impaired developmental progression in the thymus, and impaired effector function in both the thymus and the periphery. Taken together, these findings suggest that the SAP-dependent signaling through SLAM receptors has a greater influence on $\gamma\delta$ T cell development than previously appreciated and that this signaling pathway is a major contributor to the developmental programming that governs $\gamma\delta$ T cell effector function.

The exact developmental stage(s) at which thymic $\gamma\delta$ T cell functional programming occurs remains an open question. It has previously been shown that the CD24⁻CD44⁻CD45RB⁻ $\gamma\delta$ T cell population can give rise to both IFN- γ and IL-17 producers (24), but it is unclear whether this population is composed of uncommitted precursors or a mixture of already committed $\gamma\delta$ T cells consisting of several functional subsets. Our findings that immature CD24⁺ thymic $\gamma\delta$ T cells are SLAMF1⁺SLAMF6⁻ DP and that mature CD24⁻ $\gamma\delta$ T cells are enriched in SLAMF1 and SLAMF6 SP cells suggest that the CD24⁻CD44⁻CD45RB⁻ $\gamma\delta$ T cell population could be a heterogeneous mixture of SLAMF1 and SLAMF6 SP $\gamma\delta$ T cells that have already committed to either IL-17 or IFN- γ production. Our results are consistent with a model in which $\gamma\delta$ T cell functional programming occurs within the immature CD24⁺ population since both ROR γ t⁺ and ROR γ t⁻ $\gamma\delta$ T cells are found within the CD24⁺CD73⁻ population (51), as are SLAMF1 SP $\gamma\delta$ T cells which constitute the major subset of IL-17-producing $\gamma\delta$ T cells.

A number of studies support a model in which high-affinity binding of $\gamma\delta$ TCR during development promotes differentiation toward IFN- γ producing cells, while low-affinity interactions favor programming for IL-17 production (22-26, 56). In light of our findings indicating that both IL-17 and IFN- γ production are altered in SAP-deficient mice, it is interesting to note that SLAM receptor signaling has previously been shown to modulate TCR signaling thresholds in conventional $\alpha\beta$ T cells (57). In addition, recent reports indicate that NKT cell differentiation is regulated by TCR signal strength (58, 59), and that the net effect of SLAM receptor signaling is to reduce NKT TCR signal strength (36). Indeed, the co-expression of both SLAMF1 and SLAMF6 on immature CD24⁺CD73⁻ $\gamma\delta$ T cells puts them in an optimal position to influence TCR signal strength and differentiation. Since CD73 expression has been reported to increase as a result of TCR signaling (49), SLAM/SAP-mediated regulation of TCR signal strength might be expected to influence the CD24⁺CD73⁻ to CD24⁺CD73⁺ transition. However, some of our findings are not entirely consistent with such a model. First, SAP deletion did not affect neonatal V γ 4 CD73 expression. Instead, it appeared to block the transition from CD24⁺CD73⁻ to CD24⁻CD73⁻ (Pathway 2). Second, we found that the frequency and function of V γ 6 T cells, whose development is regulated by TCR signal strength (25), did not appear to be dependent on SAP. Additional investigation will be required to determine whether the SAP regulates $\gamma\delta$ TCR signaling, or a TCR-independent process.

Indeed, accumulating evidence suggests that the development of at least some IL-17-producing $\gamma\delta$ T cells can be regulated by TCR-independent mechanisms. $\gamma\delta$ T cell development is dependent on a characteristic subset of transcription factors including SOX4, SOX13, Blk, c-maf, HEB, and PLZF, among others (27, 28, 51, 54, 60, 61). HEB was recently demonstrated to be critical for the progression of neonatal IL-17-producing V γ 4 T cells through Pathway 2 (51). Therefore, our finding that SAP also regulates the progression of V γ 4 cells through Pathway 2 provides a point of intersection between HEB and SLAM/SAP signaling that warrants future examination. In addition, it was recently reported that at least some $\gamma\delta$ T17 programming results from a SOX13-dominated transcriptional hard-wiring of early thymocyte precursors (29). These SOX13 progenitors appear to give rise to IL-17-producing V γ 4, but not V γ 6 cells. Our observation that V γ 4, but not V γ 6 subsets are SAP-dependent are therefore consistent with a role for SLAM receptors and SAP in the initiation of a transcriptional program critical for the acquisition of function. Indeed, because SLAM receptors are expressed on hematopoietic stem cells (62-64), they are in a position to influence $\gamma\delta$ T cell programming at the earliest stages of development. Still, we note that because littermate controls were not used in these studies, we cannot exclude the possibility that the SAP-dependent effects on $\gamma\delta$ T cell development and function observed here are due to strain-specific differences in microbiota or to developmental differences among the different litters. A future assessment of the role that SLAM/SAP signaling plays in $\gamma\delta$ T cell precursor-product relationships using fetal thymic organ culture will be helpful in confirming our observations here, and in elucidating the mechanism(s) through which this pathway regulates $\gamma\delta$ development.

Our findings demonstrate the presence of distinct SLAM family receptor expression profiles on $\gamma\delta$ T cell subsets in the periphery that are tightly associated with function. These findings not only confirm a previous report that SLAMF1 marks $\gamma\delta$ T17 cells (48), they reveal the presence of a mutually exclusive expression pattern of SLAMF1 and SLAMF6 on $\gamma\delta$ T cells that is tightly associated with distinct transcriptional programs and with IL-17 and IFN- γ production, respectively. Heterogeneous SLAM family receptor co-expression was observed among both B6 and BALB/c $\gamma\delta$ T cells which possess different *Slam* haplotypes (53), although we did note significant strain-dependent differences. Specifically, there was a greater proportion of SLAMF1SP V γ 4 in BALB/c lung, liver, and spleen, which would suggest an increased frequency of IL-17-producing V γ 4. Although we have yet to confirm this prediction, it is interesting to note that NKT cell subsets are also differentially distributed in a strain-dependent manner, and that IL-17-producing NKT cells are also especially prevalent in BALB/c mice (65). The finding that certain SLAM family receptor expression patterns are established during thymic development, and that they are already associated with function as early as embryonic day 17, suggests that they result from a developmental program.

In support of this notion, significant changes in SLAMF1, SLAMF4, SLAMF6, and SLAMF7 expression have been described on B cells as they progress through developmental stages (66), and SLAM receptor expression profiles on hematopoietic stem cell and multipotent progenitor cell populations (62) have recently been demonstrated to mark functionally distinct progenitor populations (64). Together, our findings suggest the possibility that the coordinate signaling of distinct arrays of SLAM family receptors plays a

role in the development of $\gamma\delta$ T cells in the thymus. This would be consistent with reports indicating that multiple SLAM receptors are involved in NKT cell development (34, 36, 38, 39), and may explain the inability to observe $\gamma\delta$ T17 defects in mice lacking only a single SLAM family receptor (48).

Our observations indicated that SAP deletion did not completely abolish the development of IL-17-producing V γ 4, but instead reduced their numbers in thymus and neonatal lung by approximately 40%. This finding likely suggests the existence of one or more specific SAP-dependent V γ 4 subsets, as is the case with the V γ 1 T cell population that is composed of the SAP-dependent V γ 1V δ 6.3 subset and other SAP-independent subsets. Recent data demonstrating that the TCR repertoire of IL-17-producing V γ 4 T cells is highly restricted (67, 68), suggests the possibility that the partial decrease in V γ 4 IL-17 production observed here may be due to a complete failure in the development of a specific IL-17-producing V γ 4 subset. Such a model is also in line with the observation that the SAP-dependent decrease in lung $\gamma\delta$ T cell IL-17 production observed in neonates was not consistently observed in adult mice. We speculate that the restricted development of IL-17-producing V γ 4 T subsets in embryonic and neonatal thymus (21) results in decreased SAP-dependent V γ 4 T subsets in the neonatal lung, and that over time, homeostatic proliferation of SAP-independent V γ 4 T subsets results in a repopulation of the adult lung. A future analysis of the $\gamma\delta$ TCR repertoire in SAP-deficient mice should shed light on this issue.

In summary, our findings demonstrate that the SLAM/SAP signaling pathway plays an important role in shaping the balance of IL-17- and IFN- γ -producing $\gamma\delta$ T cell commitment during thymic development. The findings here not only extend the influence of this signaling pathway to $\gamma\delta$ T cell subsets other than the V γ 1V δ 6.3 $\gamma\delta$ NKT cell subset, they also establish a framework of SLAM family receptor co-expression that might be useful in dissecting and understanding $\gamma\delta$ T cell development and function. Future studies aimed at understanding the specific mechanisms of SLAM family receptor signaling in this process will be required to integrate this pathway into existing models, and should provide insight into the central mechanisms that regulate $\gamma\delta$ T cell functional programming.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key Points:

$\gamma\delta$ T cell subsets possess distinct SLAM family receptor co-expression profiles.

SLAMF1 and SLAMF6 identify IL-17- and IFN- γ -producing $\gamma\delta$ T cells, respectively.

SAP regulates the development of both IL-17- and IFN- γ -producing $\gamma\delta$ T cell subsets.

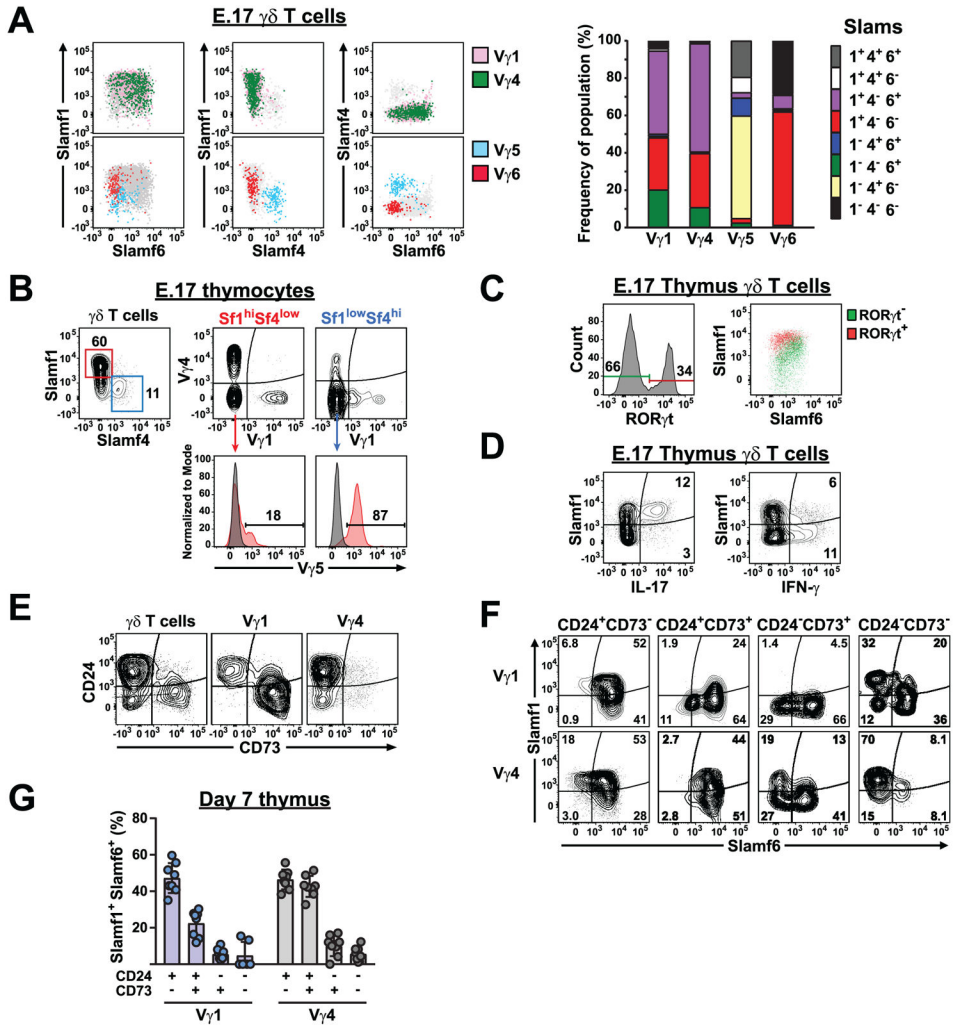


Fig. 1. Slam receptor expression profiles on different $\gamma\delta$ T cell subsets are established during thymic development.
 A) SLAMF1, SLAMF4, and SLAMF6 expression on B6 E.17 $\gamma\delta$ T cells subsets. The position of $V\gamma 1^+$ (pink) and $V\gamma 4^+$ (green) $\gamma\delta$ T cells on plots of SLAMF1, SLAMF4, and SLAMF6 is shown above. The position of $V\gamma 5^+$ (blue) and $V\gamma 6^+$ (red) $\gamma\delta$ T cells is shown below. Total $\gamma\delta$ T cells are indicated in grey. Right, cumulative frequencies of SLAM receptor expression profiles on the thymic $\gamma\delta$ T cell subsets are shown as bar graph. Data are representative of 2 independent experiments, 6 mice each, sex was not determined. B) SLAMF1 and SLAMF4 expression on E.17 thymic $V\gamma 1$, $V\gamma 4$, and $V\gamma 5$ subsets. Representative contour plots of SLAMF1 and SLAMF4 staining on thymic $\gamma\delta$ T cells is shown at left. SLAMF1^{hi}SLAMF4^{lo} and SLAMF1^{lo}SLAMF4^{hi} $\gamma\delta$ T cells are gated in red and blue, respectively. $V\gamma 1$, $V\gamma 4$, and $V\gamma 5$ staining on SLAMF1^{hi}SLAMF4^{lo} and SLAMF1^{lo}SLAMF4^{hi} subsets is shown at right. Data is representative of 2 independent experiments, 6-7 mice each, sex was not determined. C) SLAMF1 and SLAMF6 expression on ROR γ t⁺ and ROR γ t⁻ E.17 thymic $\gamma\delta$ T cells. The position of ROR γ t⁺ (red) and ROR γ t⁻ (green) cells are shown on a dot plot of SLAMF1 and SLAMF6 expression. Data are representative of 3 independent experiments with 3-8 mice each experiment, sex was not

determined. D) SLAMF1 expression on IL-17 and IFN- γ -producing E.17 thymic $\gamma\delta$ T cells. Representative contour plots are shown. Data are representative of 2 independent experiments with 4 and 6 mice each experiment, sex was not determined. E) CD24 and CD73 expression on day 7 thymus $\gamma\delta$ T cell subsets. Data shown are concatenated data from 8 male and female B6 mice and are representative of 2 independent experiments. F) SLAMF1 and SLAMF6 expression on day 7 thymus V γ 1 and V γ 4 T cell subsets at different stages of development. Data shown are concatenated data from 8 B6 mice and are representative of 2 independent experiments. G) Frequency of SLAMF1SLAMF6 DP at different stages of $\gamma\delta$ T cell maturation. Day 7 thymus V γ 1 and V γ 4 T cells were gated according to CD24 and CD73 expression, and the frequency of SLAMF1SLAMF6 DP cells was calculated. Each circle represents an individual B6 male or female mouse and are representative of 2 independent experiments.

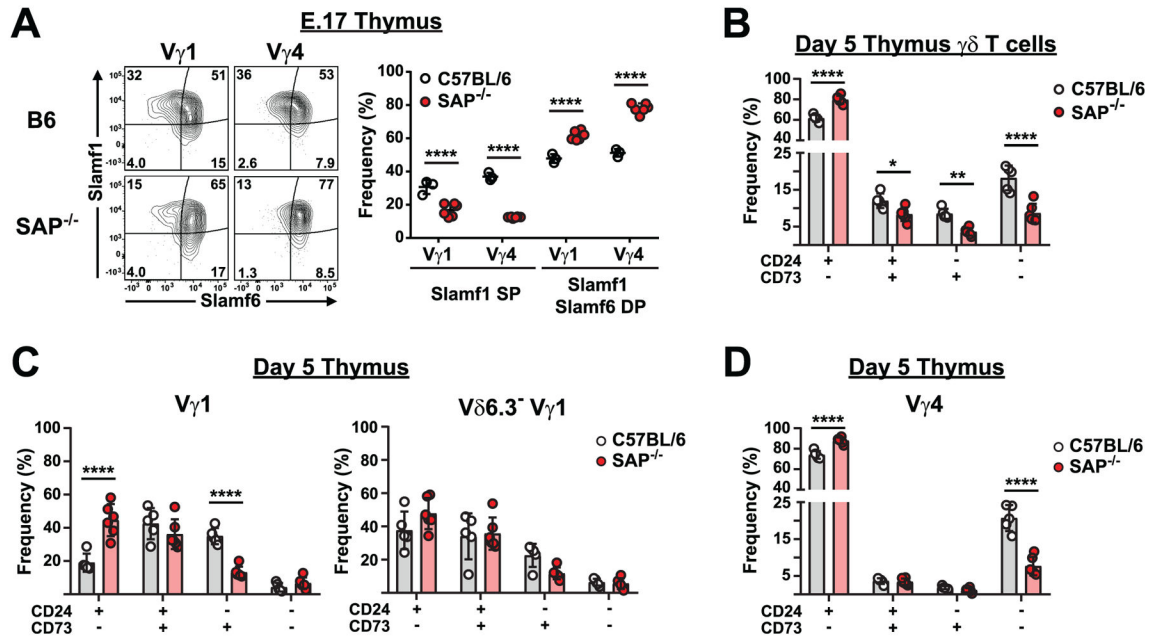


Fig. 2. Altered $\gamma\delta$ T cell developmental progression in SAP-deficient mice.
 A) SAP-dependent alteration in SLAMF1 SP and SLAMF1SLAMF6 DP $\gamma\delta$ T cells. Representative contour plots depicting SLAMF1 and SLAMF6 expression on E.17 and day 6 thymic V γ 4 T cells is shown at left. The frequencies of SLAMF1 and SLAMF6-expressing subsets is shown at right, ****p < 0.0001, as determined by two-way ANOVA followed by Sidak’s multiple comparisons test. Sex of the mice was not determined. B) The effect of SAP deficiency on the developmental progression of thymus $\gamma\delta$ T cell. Flow cytometry was used to calculate the frequency of CD24 and CD73-expressing $\gamma\delta$ T cells from B6 and B6.SAP^{-/-} day 5 thymus. Data are representative of 2 independent experiments, 8-9 male and female mice per group. *p < 0.05, **p < 0.01, ****p < 0.0001, as determined by t-tests corrected for multiple comparisons using the Holm-Sidak method. C- D) The effect of SAP deficiency on the developmental progression of thymus C) V γ 1 and D) V γ 4 T cells. Flow cytometry was used to calculate the frequency of CD24 and CD73-expressing $\gamma\delta$ T cells from B6 and B6.SAP^{-/-} day 5 thymus. Data are representative of 2 independent experiments, 8-9 male and female mice per group. ****p < 0.0001, as determined by t-tests corrected for multiple comparisons using the Holm-Sidak method.

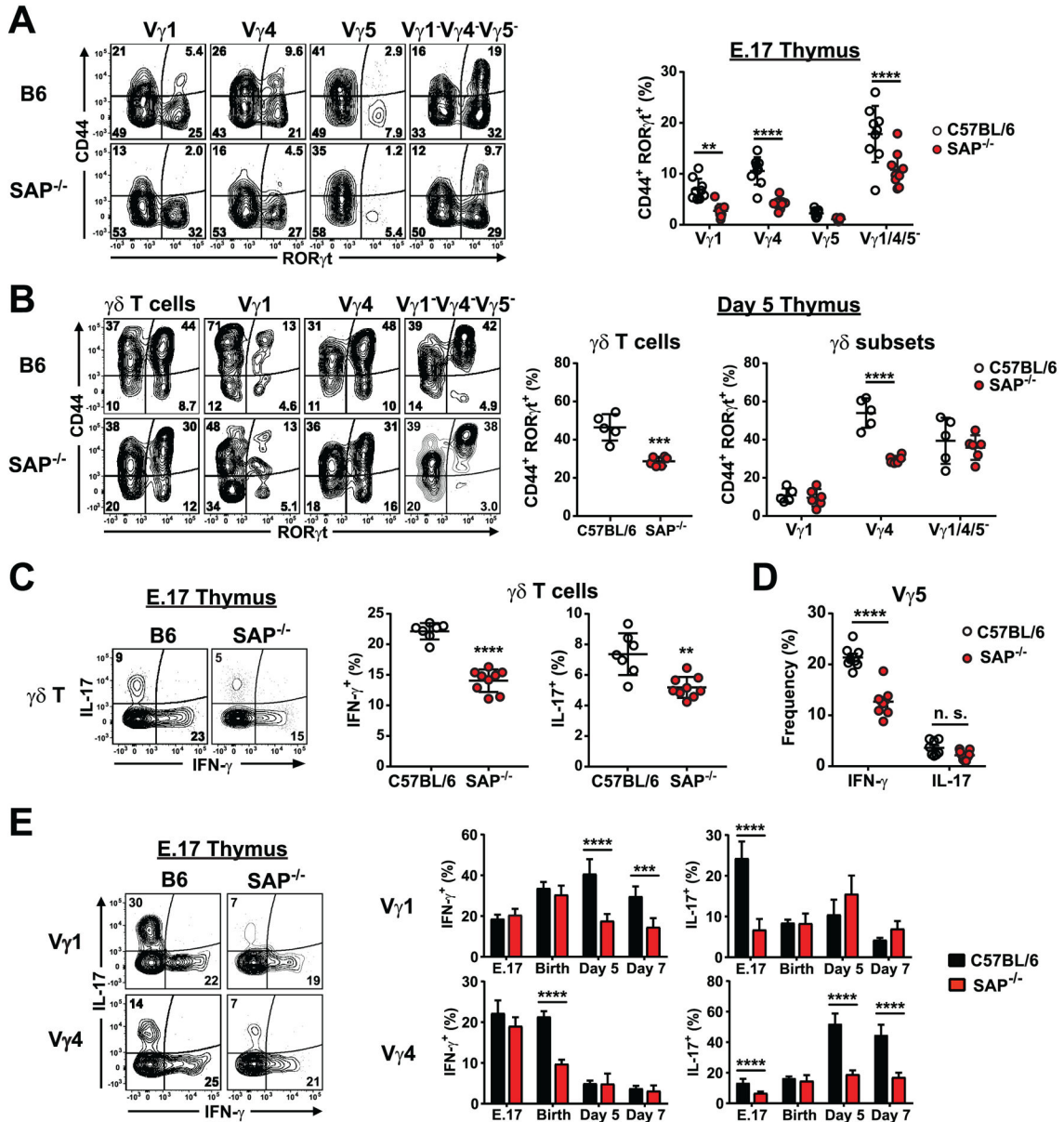


Fig. 3. Impaired $\gamma\delta$ T cell functional programming in SAP-deficient mice.

A) Significant decrease in CD44⁺RORγt⁺ thymic $\gamma\delta$ T cells in E.17 B6.SAP^{-/-} mice. Thymocytes from E.17 B6 and B6.SAP^{-/-} mice were stained for surface markers and nuclear staining was used to assess RORγt expression. Representative contour plots depicting CD44 and RORγt expression are shown at left. Cumulative frequencies of CD44⁺RORγt⁺ E.17 thymic $\gamma\delta$ T cells is shown at right, **p < 0.01, ****p < 0.0001, as determined by two-way ANOVA followed by Sidak's multiple comparisons test. Data are representative of 2 independent experiments, 7-9 mice per group, sex was not determined. B) SAP-dependent decrease in CD44⁺RORγt⁺ $\gamma\delta$ T cells in day 5 neonate thymus. Representative contour plots are shown at left. Cumulative frequencies of CD44⁺RORγt⁺ are shown at right, ***p < 0.001, ****p < 0.0001, as determined by two-way ANOVA

followed by Sidak's multiple comparisons test. Data are representative of 2 independent experiments, 5-6 mice per group, sex was not determined. C) Intracellular IFN- γ and IL-17 production in E.17 thymic $\gamma\delta$ T cells. Representative contour plots are shown at left. Numbers indicate the percentage of cells. Cumulative data are shown at right, ** $p < 0.01$, *** $p < 0.0001$ as determined by an unpaired two-tailed Student's t-test. Sex of the mice was not determined. D) Decreased E.17 V γ 5 IFN- γ production in SAP-deficient mice. *** $p < 0.0001$, as determined by Student's t-test, sex was not determined. E) IFN- γ and IL-17 production in thymic $\gamma\delta$ T cell subsets from B6 and B6.SAP^{-/-} mice at different developmental stages as indicated. Cumulative data represent the combined results of 4 independent experiments, 5 – 9 mice per group, sex was not determined. ** $p < 0.001$, *** $p < 0.0001$, as determined by two-way ANOVA followed by Sidak's multiple comparisons test.

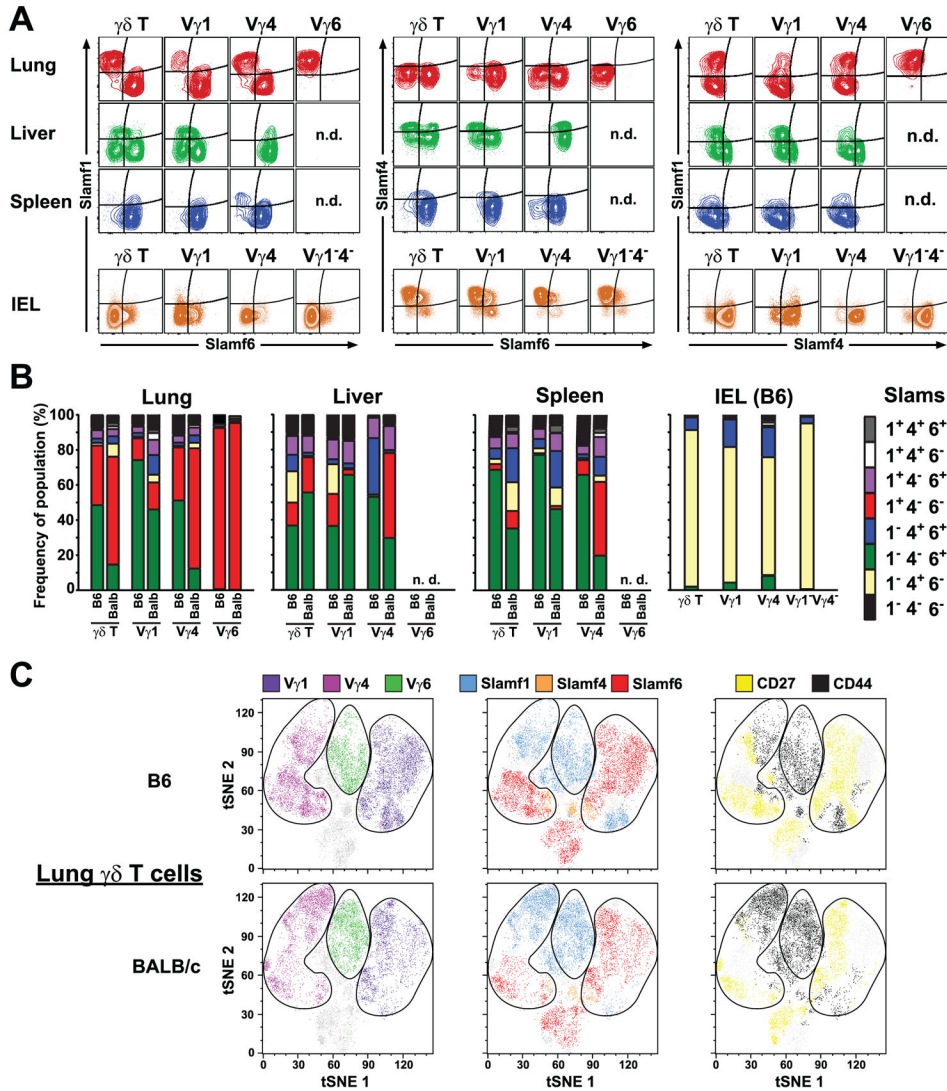


Figure 4. Tissue-dependent heterogeneity in $\gamma\delta$ T cell SLAM family receptor expression profiles. A) Representative contour plots of SLAMF1, SLAMF4, and SLAMF6 expression on lung, liver, spleen, and IEL $\gamma\delta$ T cells. Data represent concatenated files from 4 - 5 female B6 mice 11 weeks of age and are representative of 2-3 independent experiments. n.d., not detected. B) Cumulative frequencies of SLAM family receptor expression profiles on $\gamma\delta$ T cell subsets from B6 and BALB/c mice. Data represent groups of 4 - 5 B6 mice (as in A) and 4 female BALB/cJ mice, 12 weeks of age. IEL $\gamma\delta$ T cells from BALB/cJ mice were not analyzed. C) SLAM family receptor co-expression segregates with surrogate markers of $\gamma\delta$ T cell function. Flow cytometric data concatenated from 5 female C57BL/6J and 5 female BALB/cJ mice, all 10 weeks of age were analyzed using t-SNE. Clusters representing $V\gamma 1$, $V\gamma 4$ and $V\gamma 6$ subsets are circled, and SLAMF1, SLAMF4, and SLAMF6-expressing cells, as well as CD44- and CD27-expressing cells are colored as indicated.

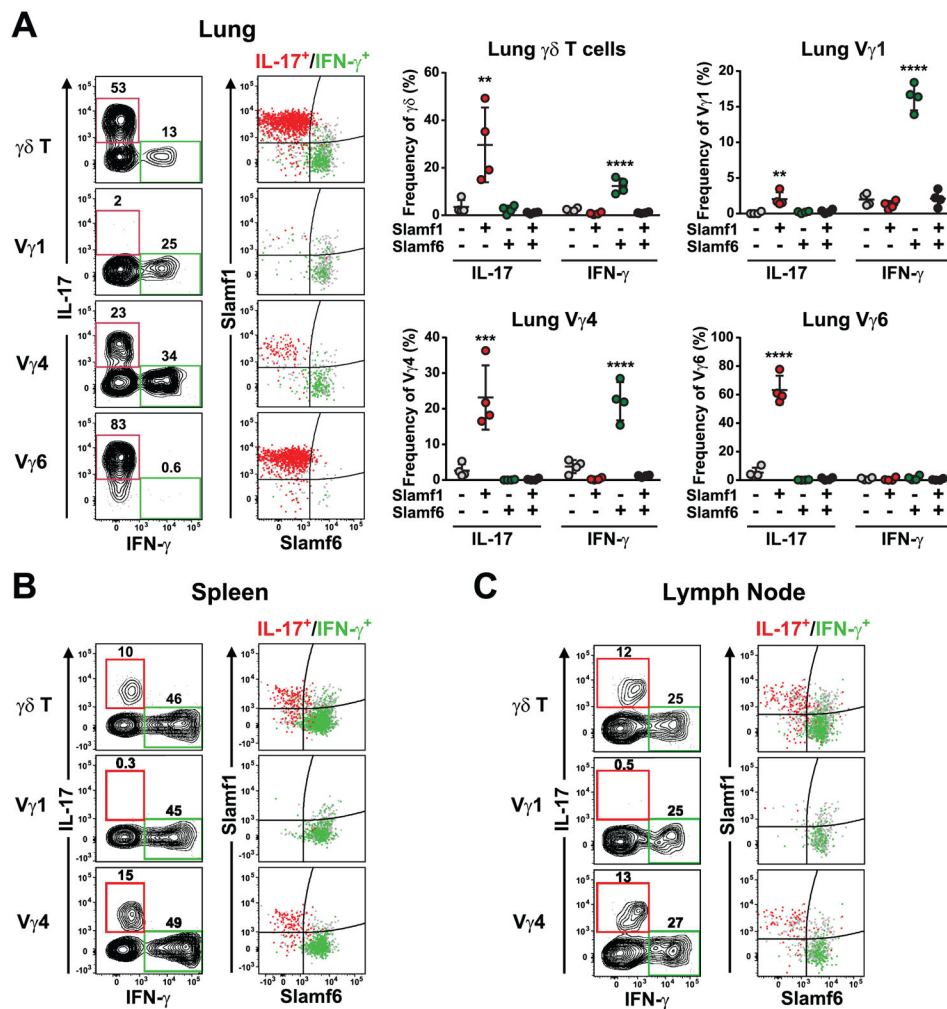


Figure 5. SLAM family receptor expression profiles distinguish distinct functional subsets of $\gamma\delta$ T cells in the periphery. SLAMF1 and SLAMF6 expression distinguish IL-17 and IFN- γ -producing $\gamma\delta$ T cells in lung (A), spleen (B), and lymph node (C). Cell suspensions were stimulated with PMA/ionomycin for 4 h at 37 °C. in the presence of monensin, after which they were stained for surface markers and intracellularly stained for IFN- γ and IL-17. Representative contour plots of $\gamma\delta$ T cell cytokine production after stimulation are shown at left. IL-17 and IFN- γ -producing cells are indicated by red and green gates, respectively. Dot plots depicting SLAMF1 and SLAMF6 expression are shown at right and the position of IL-17-gated and IFN- γ -gated cells are shown in red and green, respectively. All other cells are shown in grey. Cumulative data of lung $\gamma\delta$ T cell cytokine production is shown at far right. Data are representative of 3 independent experiments, 4 female C57BL/6J mice per group, 9.5 weeks of age, **p < 0.01, ***p < 0.001, ****p < 0.0001 as determined by one-way ANOVA followed by Tukey’s multiple comparison test.

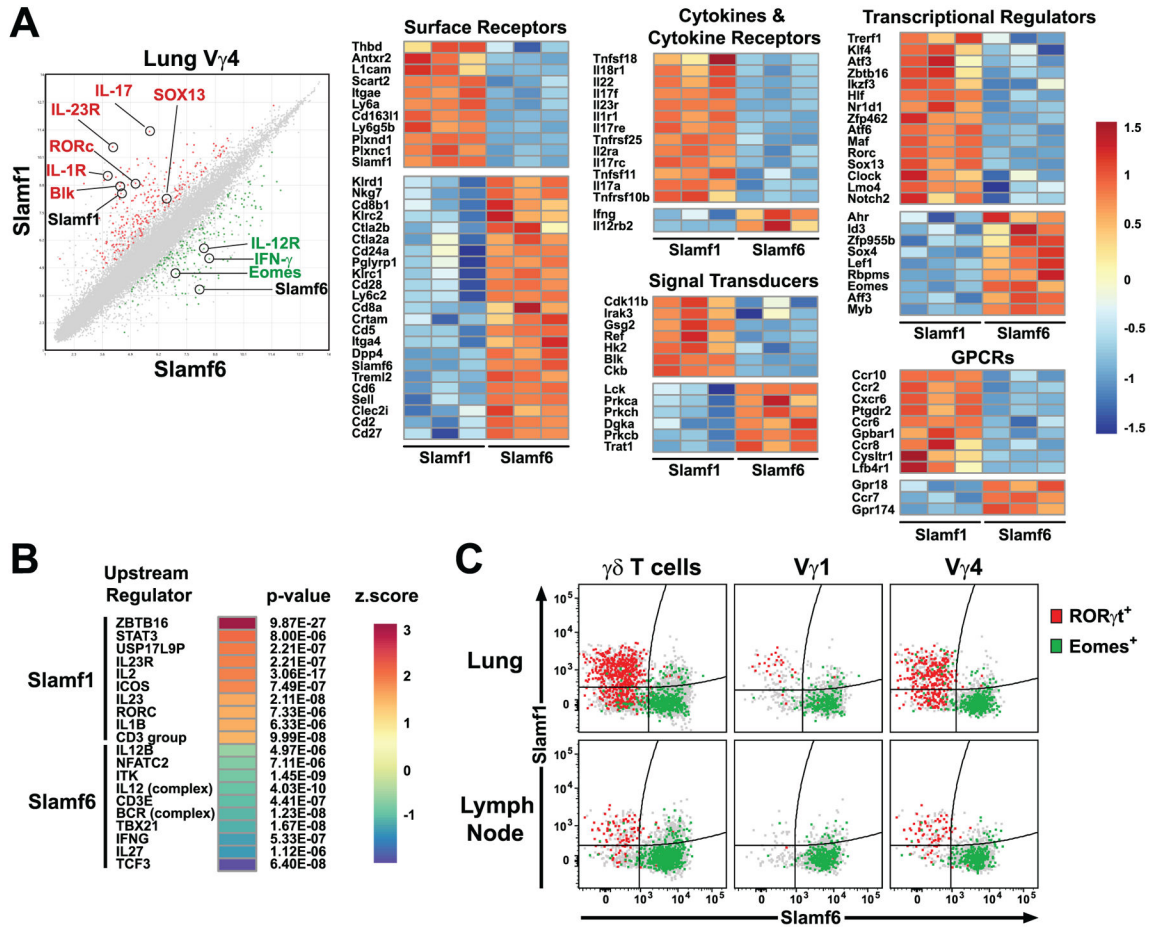


Figure 6. SLAMF1 and SLAMF6 expression distinguishes lung V γ 4 subsets with distinct transcriptional profiles.

SLAMF1⁺SLAMF6⁻ and SLAMF1⁻SLAMF6⁺ lung V γ 4 cells were FACS-sorted from lung cell suspensions pooled from 12 female C57BL/6J mice each (n = 3 pools). RNA from sorted cells was used in a microarray to assess differential gene expression. A) Scatter plot of average sample signals (log₂ transformed) observed in SLAMF1⁺SLAMF6⁻ and SLAMF1⁻SLAMF6⁺ cells is shown at left. Genes exhibiting a fold-change of greater or less than 2 and a false discovery rate < 0.2 are colored red (differentially expressed in SLAMF1⁺SLAMF6⁻) or green (differentially expressed in SLAMF1⁻SLAMF6⁺). Row-normalized heatmaps of differentially expressed genes are shown at right. Receptors, signal transducers, transcriptional regulators, cytokine/cytokine receptors, and GPCRs that exhibited a fold-change of greater or less than 2.5 are shown. Gene expression data are from a single experiment. B) Predicted upstream regulators of differentially expressed genes. Predicted regulators were ranked according to their activation z-score. Positive z-scores suggest activity in SLAMF1⁺SLAMF6⁻ and negative z-scores suggest activity in SLAMF1⁻SLAMF6⁺ subsets. C) SLAMF1 and SLAMF6 distinguish ROR γ t⁻ and EOMES-expressing $\gamma\delta$ T cell subsets. Dot plots depict SLAMF1 and SLAMF6 expression on lung and lymph node $\gamma\delta$ T cell subsets. ROR γ t⁻ and EOMES-expressing $\gamma\delta$ T cells are highlighted in red and green, respectively. All other cells are shown in grey. Data are

representative of two separate experiments, 4-5 male C57BL/6J mice, 13 weeks of age per group.

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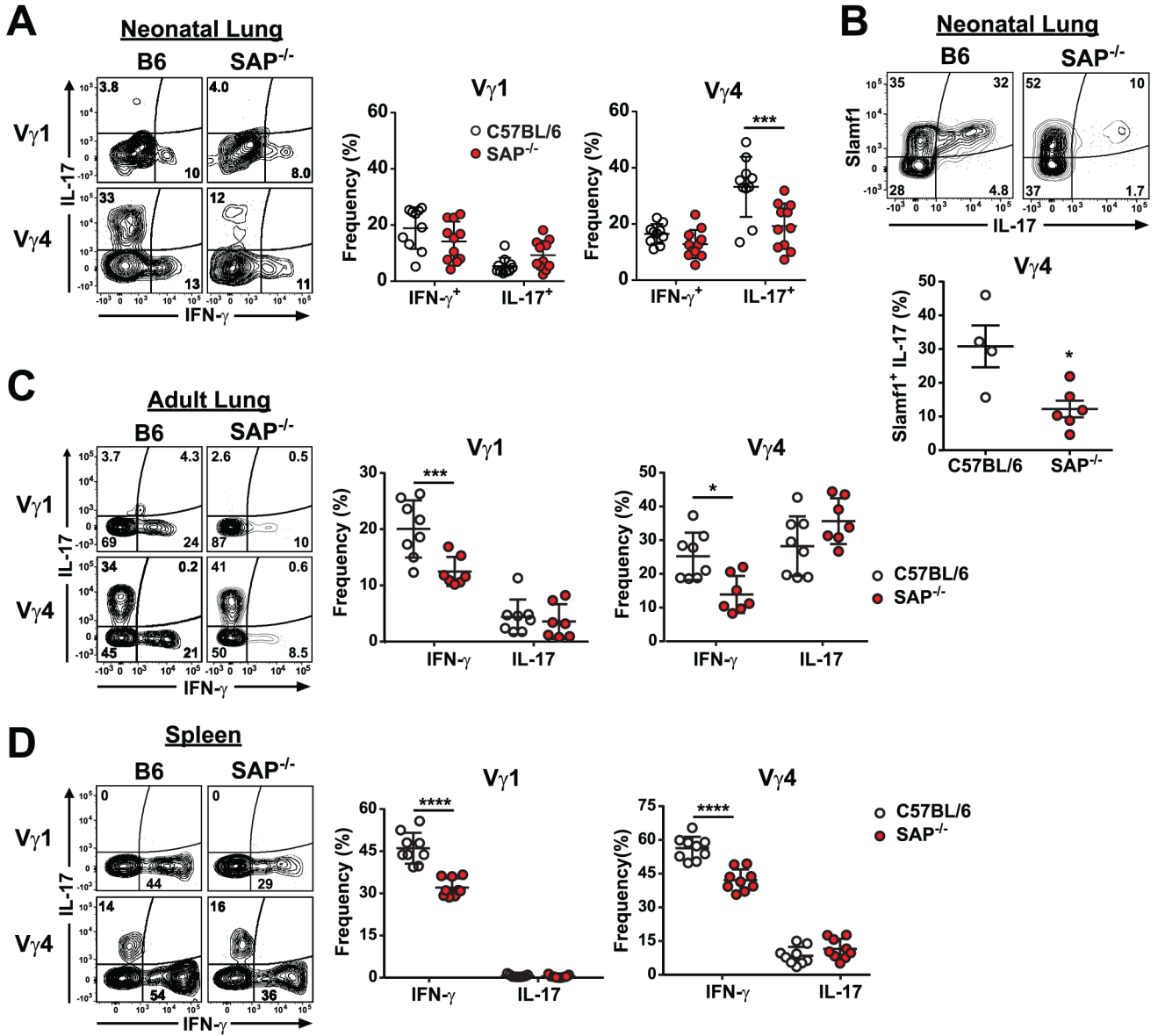


Fig. 7. Impaired $\gamma\delta$ T cell function in the peripheral tissues in SAP-deficient mice.
 A) Decreased lung $V\gamma 4$ IL-17 production in B6.SAP^{-/-} neonates. Representative contour plots of IL-17 and IFN- γ production from neonate lung $V\gamma 1$ and $V\gamma 4$ T cells in B6 and B6.SAP^{-/-} mice are shown at left. Numbers indicate the percentage of IL-17 and IFN- γ production in each subset. Cumulative frequencies of IL-17 and IFN- γ -producing cells from $\gamma\delta$ T cell subsets are shown at right. Data are the combined results of 2 independent experiments, day 6 to 7 after birth, sex was not determined. *** $p < 0.001$ as determined by two-way ANOVA followed by Sidak's multiple comparisons test. B) Decreased SLAMF1⁺IL-17⁺ lung $V\gamma 4$ in neonate day 7 B6.SAP^{-/-} mice. Representative contour plots of SLAMF1⁺IL-17⁺ lung $V\gamma 4$ T cells are shown above. The data are representative of 2 independent experiments, * $p < 0.05$ as determined by an unpaired, two-tailed t-test. The sex of the mice was not determined. C) Decreased $V\gamma 1$ and $V\gamma 4$ IFN- γ production in adult

B6.SAP^{-/-} lung (C) and spleen (D). Representative contour plots of IL-17 and IFN- γ production are shown at left. Frequencies of IL-17 and IFN- γ -producing cells are shown at right. Data representative the combined results of 2 to 3 independent experiments with male and female mice, 10 to 12 weeks of age, ***p < 0.001, ****p < 0.0001 as determined by two-way ANOVA followed by Sidak's multiple comparisons test.

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