UNIVERSITY OF CALIFORNIA SAN DIEGO

IL-18 Controls ILC2 Cytokine Production by Acting as a Mediator of Plasticity

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by

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The Thesis of Michael Bruno Amadeo is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

I would like to thank Dr. Taylor Doherty for the opportunity to conduct my Master's with him and for letting me be a part of his lab. I am very grateful for the experience and knowledge that I gained from being a part of his team.

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A portion of this thesis titled, Identification of conventional and unconventional ILC2s, is coauthored with Cavagnero, Kellen and Badrani, Jana. The thesis author was the primary author of this portion.

ABSTRACT OF THE THESIS

IL-18 Controls ILC2 Cytokine Production by Acting as a Mediator of Plasticity

by

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Master of Science in Biology

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Allergic asthma is characterized by airway inflammation, hyperresponsiveness, and remodeling. Group 2 Innate Lymphoid cells (ILC2s) are a relatively newly discovered cell type that likely contributes to the pathogenesis of asthma. ILC2s produce type 2 cytokines IL-5 and IL-13, which leads to the development of the allergic asthma phenotype that includes tissue eosinophilia and mucus production. ILC2s belong to a class of immune cells collectively known as innate lymphocytes (ILCs), and recent literature has shown that plasticity occurs between the ILC subtypes under specific conditions. Our studies demonstrate that IL-18 may control the plasticity of ILC2s in vivo through modulation of cytokine production. IL-18R$^{-/-}$ mice, which lack the IL-18 receptor, demonstrated an enhanced asthma phenotype that was characterized by

an increase in lung and BAL eosinophils. The ILC2s in the IL-18R$^{-/-}$ mice displayed unconventional cytokine production and were found to more actively produce the ILC3 cytokine IL-17A. Further studies into the plasticity of ILC2s may lead to the discovery of new treatments for asthma by controlling the activity of ILC2s by taking advantage of their plasticity.

INTRODUCTION

Asthma is an airway disease with contributions from genetics and external factors such as viruses and allergens. Its major characteristics include bronchial hyperresponsiveness, airway inflammation and remodeling. Classically, these features of asthma have been thought to be orchestrated by CD4+ type 2 helper T (T_H2) cells [1]. In this pathway, upon inhalation of an allergen, T_H2 cells in the lung respond to activation by antigen presenting cells expressing allergen peptides and produce T_H2 cytokines IL-4, IL-5, and IL-13 [2]. IL-4 drives naïve helper T cells to differentiate into T_H2 cells and promotes their survival. This cytokine is also responsible for the antibody isotype switch and secretion of IgE by B cells, which binds with high-affinity to FcεRI on mast cells and basophils [3]. The cross-linking of IgE to an allergen derived antigen while bound to FcεRI leads to the activation of mast cells and the release of lipid mediators like prostaglandins and leukotrienes, cytokines, and histamine that promote early allergic responses in asthma [4-5]. The cytokine IL-5 has recently become a major target for asthma therapies due to its ability to promote the production, recruitment and survival of eosinophils in inflammation [6]. Eosinophils are mainly implicated in host defense against helminths, however, studies have shown that eosinophils contribute to asthma by promoting T_H2 cell responses through the secretion of cytokines, and by inducing cytotoxicity and airway hyperresponsiveness of host tissue through the secretion of cytotoxic granule proteins and reactive oxygen species [7]. IL-13 has been shown to induce airway hyperresponsiveness, remodeling features and increased mucus production [8].

Group 2 innate lymphoid cells (ILC2s) are a recently discovered cell type that likely plays a role in asthma based on emerging evidence. In 2010, three independent studies reported

the existence of a non-B and T innate immune cell in various tissues including adipose tissue, the mesenteric lymph node, the spleen, the liver, and the lung, that produced T_H2 cytokines IL-4, IL-5, and IL-13 [9-11]. These cells were known by various terms including nuocytes, innate type 2 helper cells, and natural helper cells, but are now collectively known as group 2 innate lymphoid cells (ILC2s) by consensus [9-12]. Through flow cytometry analysis, ILC2s are identified as lineage negative, as in, they lack surface markers used to identify other immune cells, such as CD3, CD4, CD8, TCRβ, TCRδ, CD5, CD19, B220, NK1.1, Ter119, Gr-1, Mac-1, CD11c, and FcεRIα [11]. Markers that ILC2s express include CD45, IL7Rα, IL17RB, T1/ST2, ICOS, c-kit, Sca-1, Thy1.2, CD44, CD25, CD38, CD69, CD27, and MHC class II [9-11]. ILC2s rely on the cytokine IL-7 for development into pre-ILC2s from common lymphoid progenitors (CLPs), and require the transcription factors GATA3 and RORα for development [12-15]. IL-7 is a member of the γ_c cytokine family as it binds to γ_c (along with IL-7Rα) to activate the JAK/STAT pathway through STAT5, and promote ILC2 development, maturation, and survival [18]. GATA3 is a zinc-finger transcription factor that is required for T cell development and differentiation into T helper type 2 (T_H2) cells, but has recently been shown to be required for ILC2 maturation as well as controlling ILC2 cytokine production [19]. Interestingly, GATA3 has also been shown to control the ability for ILC2s to respond to inflammatory cytokines through induction of IL-33R and IL-25R expression [20]. Finally, retinoic-acid-receptor-related orphan nuclear receptor alpha (RORα) has been shown in mouse models to be more specifically required for ILC2 development, and in the absence of this transcription factor, helminth expulsion was impaired, and in the lungs, the ability to induce eosinophilic inflammation in response to protease allergens was also inhibited [17, 21].

ILC2s produce T_H2 cytokines when activated by the epithelial cytokines IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) in response to damage to the epithelial barrier from foreign entities such as helminths or protease-based allergens [10, 16-17]. IL-33 is an IL-1 cytokine family member that has been detected in epithelial and endothelial cells, and it is the ligand for the orphan receptor ST2 located on many immune cells, such as T cells, ILC2s, mast cells, NK cells, eosinophils, and macrophages [22]. IL-33 is constitutively expressed and stored in the cell nucleus and is called an alarmin, and after infection or tissue damage, it is quickly released passively from necrotic cells, or secreted from stressed cells into the extracellular space to initiate immune responses [22]. IL-25, also known as IL-17E, is a member of the IL-17 cytokine family that has been found to be secreted by epithelial cells, and binds to a heterodimeric receptor complex of IL-17RB and IL-17RA to activate MAPK and NF-κB signaling [13, 23]. TSLP is a cytokine that binds to a heterodimer of TSLPR and IL-7Rα (CD127), and induces IL-5 and IL-13 cytokine production from ILC2s through the induction of GATA3 by phosphorylating STAT5 [24-25].

Recently, new studies have revealed that mediators of ILC2 activation are not limited to IL-33, IL-25, and TSLP, but include other diverse ligands. Very recently, three studies reported the novel discovery that ILC2s can respond to neuromedin U (NMU) through the neuropeptide receptor NMUR1. Stimulation with NMU was reported to activate ILC2s to proliferate more and to produce type 2 cytokines [26-28]. ILC2 cytokine production was reported to be regulated by NMU through ERK1/2 and a calcium-influx activation of calcineurin and nuclear factor of activated T cells (NFAT) [27]. In addition, one of the studies reported how combining the alarmin IL-25 with NMU stimulation lead to an amplification of allergic inflammation [28]. Another class of molecules that ILC2s respond to includes the eicosanoid family members,

prostaglandins (PGs) and leukotrienes (LTs), which are products of arachidonic acid metabolism [29]. PGD2 binds to CRTH2 (chemoattractant receptor-homologous molecule expressed on T_H2 cells) expressed by ILC2s and promotes ILC2 chemotaxis and type 2 cytokine production [30-31]. ILC2s also express cysteinyl leukotriene receptor 1 (CysLT1R) that could bind to leukotrienes LTC4, LTD4, and LTE4 to activate ILC2s, and to potentiate ILC2 activation induced by alarmins [29, 32-34]. Recently, and after our IL-18R studies commenced, another study showed that ILC2s in the skin expressed the IL-18 receptor, IL-18R1, and that skin ILC2s were preferentially activated by the cytokine IL-18 over the typical IL-33 and IL-25 alarmins compared with other tissue ILC2s. The study also showed that certain subsets of ILC2s in the lungs expressed IL-18R1 [35]. However, the effects of IL-18 on lung ILC2s remains unknown.

IL-18 is an IL-1 family cytokine that is produced by macrophages and epithelial cells and binds to a complex of IL-18Rα and IL-18Rβ to induce type 1 helper T (T_H1) cells to release IFN-γ [36]. It was traditionally thought that the activation of T_H1 cells by IL-18 would lead to a corresponding inhibition of a T_H2 response in accordance with the phenomenon that the products of T_H1 and T_H2 cells suppress the development of each other [36]. However, further studies have shown the complex actions of IL-18 to also illicit T_H2 responses. One study demonstrated that a combination of IL-18 and IL-12 created an anti-allergic T_H1 response by inhibiting B cell production of IgE by stimulating the B cells to produce IFN-γ [36-37]. However, another study demonstrated that a combination of IL-18 with IL-2 induced the opposite affect and led to increased serum IgE levels by inducing the production of IL-4 and IL-13 from T cells [38]. The ability of IL-18 to induce a T_H2 response raises the question of whether IL-18 plays a role in the pathogenesis of T_H2 related diseases, such as asthma. Studies have shown that a correlation exists between high serum levels of IL-18 and IL-18 receptor and asthmatics [39]. Another study

showed how in a mouse model of allergic asthma utilizing the allergen ragweed, the addition of IL-18 could augment the sensitization of mice to an allergen [40]. Additional studies have shown that polymorphisms in the IL-18 and IL-18R1 genes correlated with asthma severity [41-42]. However, the exact role IL-18 plays in asthma has yet to be determined, but a potentially important pathway may be IL-18 regulation of IL-18R1-expressing ILC2s in asthma.

The effect of IL-18 on ILC2s is largely unexplored, though one possibility is that IL-18 controls the plasticity of ILC2s. Recent literature has demonstrated the capacity of ILC2s to express characteristics of other subsets of innate lymphoid cells (ILCs) based on the conditions they experience. The other ILCs include group 1 ILCs (ILC1s) and group 3 ILCs (ILC3s), and like ILC2s, these cells are lineage negative and express IL-2Rα (CD25) and IL-7Rα (CD127), yet the ILC groups differ from each other based on the transcription factors that are highly expressed, the effector cytokines they respond to, and the effector cytokines they produce [43]. ILC1s require the transcription factor T-bet, and ILC3s require RORγt [43-45]. ILC1s respond to the cytokines IL-12 and IL-18 to produce interferon-γ (IFN-γ) and tumor necrosis factor (TNF) to promote innate immunity to viruses and intracellular pathogens [43, 46]. ILC3s respond to the cytokines IL-23 and IL-1β to produce IL-22 and IL-17A, which contributes to immunity against extracellular bacterial infections [43, 47]. Importantly, recent studies have shown that ILC1s, ILC2s, and ILC3s, have functional plasticity between each other. In one study, it was reported that ILC1s expressed IL-1R and IL-23R, and that they produced less IFN-γ and differentiated into IL-22 producing ILC3s after being cultured with IL-1β, IL-2, and IL-23 [48]. Another study showed how a subset of ILC2s called inflammatory ILC2s (iILC2s) had the ability to produce both the type 2 cytokine IL-13, and the ILC3 cytokine IL-17A after allergen challenge with house dust mite (HDM) [49]. Additional studies demonstrated ILC2 plasticity in response to

viral infection and cigarette smoke with lung ILC2s showing less GATA3 expression, increased T-bet expression, and production of the ILC1 cytokine IFN-γ in response to IL-12 and IL-18 [50].

Though reports have shown interactions between IL-18 and ILC2s, the specific role IL-18 plays in asthma is unclear, and may involve interactions between ILC2s and IL-18 that induce plasticity and contribute to the pathogenesis of asthma. To explore these questions, IL-18R$^{-/-}$ mice were given intranasal challenges of the fungal allergen *Alternaria alternata*, and the phenotypic lung inflammatory changes were examined to understand the impact IL-18 has on the pathogenesis of asthma features in mice. Also, ILC2s were examined for changes in transcription factor expression and cytokine production that are associated with other ILCs to observe whether potential plasticity occurred or evidence of mixed ILC subsets were involved. Thus, the plasticity of the innate lymphoid cells may be a significant factor to amplify the effects of asthma already brought about by natural ILC2s.

MATERIALS AND METHODS

Mice

Female 6-10 week old C57BL/6 mice were obtained from Jackson Labs. IL-18R$^{-/-}$ mice were obtained from Dr. Hal Hoffman at UC San Diego. IL-18R$^{-/-}$ mice were bred in house on a C57/BL/6 background. All experiments were approved by the UC San Diego Institutional Animal Care and Use Committee.

Alternaria alternata allergen challenge model

WT mice were anesthetized with a 1:1 ratio of isofluorane and O_2, and then intranasally challenged with 50 μg of *Alternaria alternata* extract (Greer, NC). One, three, and twelve hours after challenge, mice were euthanized in a CO_2 chamber. The bronchoalveolar lavage (BAL) was collected using 2% bovine serum albumin (BSA), and used to measure levels of IL-33 and IL-18 by ELISA. In most experiments, WT mice were anesthetized with a 1:1 ratio of isofluorane and O_2, and then intranasally challenged with 50 μg of *Alternaria alternata* extract (Greer) on days 0, 1, and 2. On day 3, mice were euthanized in a CO_2 chamber. The BAL was collected using 2% BSA, and lungs were then collected. Naïve WT mice that did not experience allergen challenge served as negative controls. In kinetics experiments. WT and IL-18R$^{-/-}$ mice were anesthetized with a 1:1 ratio of isofluorane and O_2, and then intranasally challenged with 50 μg of *Alternaria alternata* extract (Greer) at varying time points. After challenges, mice were euthanized in a CO_2 chamber. The BAL was collected using 2% BSA, and lungs were then collected. The time course of the allergen challenges and the BAL and lung harvest were as follows: mice were challenged on day 0 and tissues were collected on day 1, mice were challenged on days 0 and 1 and tissues were gathered on day 2, or, mice were challenged on days 0, 1, and 2, and then tissues were

harvested on either day 3 or day 7. Naïve WT and IL18R$^{-/-}$ that did not experience allergen challenges served as negative controls.

BAL and Lung Processing

After BAL and lungs were collected, the BAL was centrifuged for 5 minutes at 4°C and at a speed of 1500 RPM using an Allegra X-14R Centrifuge (Beckman Coulter, Carlsbad, CA). The supernatant was kept and stored at -20°C for ELISA of cytokines. The cells from the BAL were analyzed via flow cytometry. The lungs were digested using a gentleMACS dissociator (Miltenyi Biotec, San Diego, CA) following the manufacturer's protocol. The lungs were then filtered through a 40μm filter, centrifuged at 4°C for 5 minutes at 1500 RPM using an Allegra X-14R Centrifuge (Beckman Coulter), and then the lung cells were resuspended in 1 mL of Roswell Park Memorial Institute medium (RPMI).

Flow Cytometry

A portion of BAL and lung single cell suspensions were diluted 1:100 in a PBS solution of 10% FBS and 0.01% sodium azide (FACs Buffer) for determining cell counts using a Novocyte Flow Cytometer (Acea Biosciences, Inc., San Diego, CA). The samples were then aliquoted into equal numbers of cells, and then washed with FACs buffer to prepare for staining. Eosinophils were identified as CD45.2+Siglec-F+CD11c- cells, and neutrophils as CD45.2+GR-1+SiglecF- cells using anti-CD45.2 conjugated to PerCP (Biolegend, San Diego, CA), anti-Siglec-F conjugated to PE (BD Biosciences, La Jolla, CA), anti-CD11c conjugated to FITC (Biolegend), and anti-GR-1 conjugated to APC (Biolegend). Lung cells were stained with a cocktail of FITC-conjugated lineage antibodies that includes the following: A premade cocktail

including CD3, Gr1, CD11b, B220, and Ter-119 (Biolegend), along with the additions CD11c (Biolegend), TCRβ (Biolegend), TCRγδ (Biolegend), NK1.1 (Biolegend), FcεR1α (Biolegend), and CD5. (Biolegend) In addition to the lineage cocktail, lung cells were with CD45.2 conjugated to PerCP (Biolegend), Thy1.2 conjugated to eFluor 450 (eBiosciences, San Diego, CA), ST2 conjugated to APC (Biolegend), and CD127 conjugated to PE-Cy7 (Biolegend). Lung ILC2s were identified as CD45.2 positive, lineage negative, Thy1.2 positive, ST2 positive, CD127 positive lymphocytes. Stained ILC2s were also surface stained for IL-18R expression using anti-IL18R conjugated to PE. Intracellular transcription factor analysis was performed using PE-conjugated antibodies, including anti-Ki67 (eBiosciences), anti-GATA3 (eBiosciences), anti-RORγt (eBiosciences), or T-bet (Biolegend) after surface staining and permeabilization of whole lung cells using a FoxP3 intracellular staining kit according to the manufactuerers protocol (eBiosciences). Intracellular cytokine analysis was performed using anti-IL-5 conjugated to PE (Biolegend), anti-IFN-γ conjugated to APC-Cy7 (Biolegend), and anti-IL-17A conjugated to eFluor 506 (eBiosciences) after surface staining and permeabilization using a BD intracellular staining kit according to the manufacturers protocol (BD Biosciences) of whole lung cells that were cultured for 3 hours in T cell media (TCM) that contained RPMI, penicillin/streptomycin, glutamine, 2-Mercaptoethanol, and fetal bovince serum. PMA ionomycin and Golgi-Plug was added to stimulate the cultured cells during the 3 hours.

ELISA

An ELISA for IL-18 was performed on BAL using an Invitrogen Mouse IL-18 ELISA kit according to the manufacturer's protocol (Invitrogen, Carslbad, CA). An ELISA for IL-33 was performed on BAL using a Mouse IL-33 DuoSet ELISA DuoSet kit according to the

manufacturer's protocol (R&D Systems, Minneapolis, MN). ELISA plates were then read on a model 680 microplate reader (Bio-Rad, Hercules, CA) at 450nm with correction at 550 nm.

Data Analysis

Flow cytometry data plots were analyzed using FlowJo version 10.2 (Tree Star, Ashland, OR), Microsoft Excel 2016, and Graphpad Prism (GraphPad, La Jolla, CA). ELISA data was analyzed using Microsoft Excel 2016 and GraphPad Prism version 7 (GraphPad).

Statistics

For all experiments, statistical analysis was performed using the GraphPad Prism version 7 (GraphPad). The Mann-Whitney U test was performed for all experiments. Statistical p-values less than 0.05 were considered statistically significant.

RESULTS

Lung Innate Lymphoid Cells Express IL-18R During Allergen Challenge

Previous literature has reported that ILC1s are the primary innate lymphoid cell that responds to IL-18, however, ILC3s and ILC2s express functional IL-18 receptor [35, 43, 51]. In regard to ILC2s, cells located in the skin were shown to have much greater IL-18R expression compared to ILC2s in other tissues, yet a portion of lung ILC2s were shown to express IL-18R [35]. To investigate whether IL-18 plays a role in ILC2s and asthma pathogenesis, an ELISA was performed on BAL for IL-18 expression within hours of challenge with 50μg of *Alternaria alternata* extract in comparison to a PBS challenged mouse. The concentration of IL-18 in the BAL was found to be significantly increased in one hour after the allergen was administered in comparison to the PBS challenged control (Figure 1A). An ELISA for IL-33 was also performed as a positive control of successful allergen challenge (Figure 1B). We then investigated whether conventional ST2+ ILC2s in the lungs express the IL-18 receptor as reported in the literature, and what effect allergen challenge causes on IL-18 receptor expression. WT mice were challenged with 50μg of *Alternaria alternata* extract or PBS on days 0, 1, and 2, and then lungs were harvested on day 3. ILC2s were identified as Lineage-Thy1.2+ST2+ lymphocytes, and staining for IL-18Rα revealed that lung ILC2s have increased IL-18Rα expression after allergen challenge in comparison to the control PBS challenged group (Figure 2A-B).

A portion titled, Lung Innate Lymphoid Cells Express IL-18R During Allergen Challenge, is coauthored with Cavagnero, Kellen and Badrani, Jana. The thesis author was the primary author of this portion.

Exacerbated lung eosinophilia in IL-18R$^{-/-}$ Mice

To evaluate the effects of IL-18 on asthma, IL-18R$^{-/-}$ mice were utilized in a model of allergic asthma. WT and IL-18R$^{-/-}$ mice were challenged on days 0, 1, and 2 with 50μg of *Alternaria alternata* extract, and the lungs and BAL were harvested on day 3. Given the potential for ILC plasticity at early time points, we performed kinetic analysis for ILC2 responses and lung eosinophilia. Thus, lungs and BAL were also harvested between challenges on days 1 and 2 (Figure 3). From granulocyte staining by FACS, eosinophils (SiglecF+ CD11c-) were significantly increased in both the BAL and lungs of IL-18R$^{-/-}$ mice at days 1, 2, and 3 (Figure 4). Interestingly, a decrease in the frequency of neutrophils was observed in the BAL at day 3, yet this phenotype was not reflected in the lungs (Figure S1).

Identification of conventional and unconventional ILC2s

To evaluate the effects of IL-18 on ILCs in the aforementioned kinetics protocol, we collected single-cell lung suspensions and stained for surface markers of ILC2s from WT and IL-18R$^{-/-}$ mice. Total innate lymphoid cells (ILCs) in general were defined as Lineage-Thy1.2+ lymphocytes, and conventional ILC2s were defined as ST2+CD127+ cells from the ILC population. A representative parent gating scheme for ILC2s is shown in Figure 5. From the literature, various surface markers have been identified that ILC2s express, and two common surface markers include ST2 and CD127 [54]. Thus, ILCs that are positive for ST2 and CD127 are conventionally determined to be ILC2s, while other populations such as ST2+CD127-, ST2-CD127+, and ST2-CD127- ILCs are thought of as not being ILC2s. However, another defining criteria of ILC2s is whether they express the transcription factor GATA3, and whether they produce type 2 cytokines such as IL-5 [55]. Intracellular staining for GATA3 and IL-5

expression in the populations of ILCs that are not ST2+CD127+ after allergen challenge reveals that these populations of cells also significantly express GATA3 and produce IL-5 (Figure 6A-D). Thus, we termed these populations of cells as unconventional ILC2s based on our work showing that these populations also produce some level of T_H2 cytokines despite being negative for one or both classical CD127 and ST2.

Examining conventional and unconventional ILC2 population shifts

From our kinetics model of allergic asthma, we considered changes in the population sizes of the ST2 CD127 ILC subpopulations to be suggestive, but not proof of, plasticity. There was no significant change in the cell frequency or number of total ILCs (Figure 7A-B). When considering the ST2 and CD127 subpopulations of ILCs, we observed no significant changes in the total number of conventional ILC2s, however, we did see an across the board trending decrease in the frequency of conventional ILC2s in IL-18R$^{-/-}$ mice. (Figure 8A-B). In the ST2+CD127- unconventional ILC2 population, there was a modest increase in the frequency of this population by day 2 in IL-18R$^{-/-}$ mice (Figure 8C). We also observed modest increases in the total number of ST2+CD127- ILCs by day 2 and day 3 in IL-18R$^{-/-}$ mice (Figure 8D). We observed a significant increase in the frequency of ST2-CD127+ unconventional ILC2s by day 3 and a modest increase in the total cells by day 2 and day 3 in IL-18R$^{-/-}$ mice (Figure 8E-F). Lastly, we detected a modest increase in the frequencies of the ST2-CD127- unconventional ILC2 population by day 1, and modest increases in the total cells by day 3 in IL-18R$^{-/-}$ mice (Figure 8G-H).

A portion of this thesis titled, Identification of conventional and unconventional ILC2s, is coauthored with Cavagnero, Kellen and Badrani, Jana. The thesis author was the primary author of this portion.

Increased Proliferation of ILC2s in IL-18R$^{-/-}$ Mice

To assess whether ILCs had changes in proliferation in IL-18R$^{-/-}$ mice, we performed nuclear staining of ILCs for the proliferation marker Ki67 on single-cell lung suspensions. By day 1 of challenge, there was a significant increase in the frequency of Ki67 expression and a significant increase in the total amount of Ki67 expressing cells in total ILCs in IL-18R$^{-/-}$ mice (Figure 9A-B). We also saw similar trends in the conventional and unconventional ILC2 subpopulations. We observed a significant increase in the frequency of Ki67 expression by conventional ST2+CD127+ ILC2s day 1, but no changes in the totals in IL-18R$^{-/-}$ mice (Figure 9C-D). In the ILC2 subpopulations we observed a significant increase in the total number of Ki67 expressing cells by day 1 in our ST2+CD127- and ST2-CD127- populations in IL-18R$^{-/-}$ mice (Figure 9F & 9J). We also observed modest increases in the total number of Ki67 expressing cells by days 2 and 3 for all the unconventional ILC2 subpopulations in IL-18R$^{-/-}$ mice (Figure 9F, 9H, and 9J). No significant trends were observed in the frequency of Ki67 expression in any of the unconventional ILC2 subpopulations (Figure 9E, 9G, and 9I).

Increased Unconventional Cytokine Activity from IL-18R$^{-/-}$ Mice

Despite the rigorous phenotyping of the various surface markers of innate lymphoid cells, the classifications for ILCs are primarily defined by the cytokines that the cells produce, with IFN-γ production being a feature of ILC1s, the type 2 cytokines IL-5 and IL-13 being

characteristic of ILC2s, and IL-17A and IL-22 production being associated with ILC3s [45]. Our primary measure of plasticity is through intracellular cytokine staining representative of ILC1s, ILC2s, and ILC3s performed on single-cell lung suspensions from WT and IL-18R$^{-/-}$ mice from the kinetics model (Figure 3). Staining for transcription factors specific to ILCs was also performed, but the early results failed to show any statistical significance between the two groups in GATA3 (Figure S3). However, the percent of RORγt expression showed modest increases in the ILC populations in IL-18R$^{-/-}$ mice at day 1, and increases in percent and total RORγt expression in most ILC populations from IL-18R$^{-/-}$ mice at day 2, suggesting an effect on IL-17A production in the IL-18R$^{-/-}$ mice (Figure S4). Lung ILCs were stained for IL-5, IFN-γ, and IL-17A after culture of lung cells for 3 hours with PMA ionomycin and a protein transport inhibitor.

Staining for the type 2 cytokine IL-5 showed no overall significant change in expression between WT and IL-18R$^{-/-}$ mice in any ILC population (Figure 10A-K). However, by day 2 of the challenges, we can see a modest trending increase in the total number of IL-5 producing cells amongst total ILCs, and in both the conventional ILC2 and unconventional ILC2 subsets in IL-18R$^{-/-}$ mice (Figure 10A-K). Overall, we found that IL-18R$^{-/-}$ mice display a modest enhancement in IL-5 production, and suggests that IL-18 plays a role in inhibiting characteristic ILC2 cytokine production.

IFN-γ expression was shown to be very low in the ILCs, however, some interesting trends were observed. We saw a modest increase by day 1 in the frequency of IFN-γ expression in total Thy1.2 ILC population in IL-18R$^{-/-}$ mice, but this trend was not reflected in the total number of IFN-γ expressing cells (Figure 11A-B). We see a similar trend in the conventional ILC2 subpopulation, with a modest increase by day 1 in the frequency of IFN-γ expression in IL-

18R$^{-/-}$ mice, but this trend was also not reflected in the total number of ST2+CD127+ IFN-γ expressing cells (Figure 11C-D). We see similar trends in the unconventional ILC2 subpopulations, but with some key differences. In the ST2+CD127- subpopulation, we saw significant increases by day 1 in the frequency of expression and in the total number of IFN-γ expressing cells in IL-18R$^{-/-}$ mice (Figure 11F-G). In the ST2-CD127+ unconventional ILC2 subpopulation, we saw only a modest increase in the frequency of IFN-γ expression by day 1, but we do see a significant increase in the total number of IFN-γ producing cells by day 1 in IL-18R$^{-/-}$ mice (Figure 11H-I). The ST2-CD127- subpopulation showed only a modest increase in percent and total IFN-γ expression by day 1 (Figure 11-J-K). Overall, these results demonstrate that IL-18 affects IFN-γ production from ILC2s.

Lastly, staining for IL-17A in ILCs revealed some significant changes when comparing IL-18R$^{-/-}$ mice to WT mice. (Figure 11A-K). From staining of total ILCs, we observed significant increases in the expression of IL-17A by days 1, 2, and 3 in IL-18R$^{-/-}$ mice (Figure 12A). We only observed modest increases in the total number of IL-17A producing ILCs by day 2 and day 3 (Figure 12B). Flow cytometry data for the conventional ILC2 subpopulation revealed significant increases in the frequency of IL-17A expression at baseline and by day 1 of the model, with modest increases by day 2 and day 3 in IL-18R$^{-/-}$ mice (Figure 12C). We also saw a significant increase in the total number of IL-17A producing conventional ILC2s at baseline and by days 1 and 2, with a modest increase seen in day 3 in IL-18R$^{-/-}$ mice (Figure 12D). Next, we assessed the IL-17A expression in unconventional ILC2 subpopulations. We detected no significant changes in the ST2+CD127- subpopulation and only found modest increases in IL-17A expression by days 1, 2 and 3 in both frequency and total cells in IL-18R$^{-/-}$ mice (Figure 12F-G). In the ST2-CD127+ unconventional ILC2 subpopulation, we observed a

significant increase in the frequency of IL-17A expression by days 1, 2, and 3 in IL-18R$^{-/-}$ mice (Figure 12H). We also saw a modest increase in the total number of IL-17A expressing cells by day 2, and a significant increase by day 3 in IL-18R$^{-/-}$ mice (Figure 12I). Lastly, in our ST2-CD127- subpopulation, we observed modest increases in IL-17A expression by days 1 and 3, and significant increases in IL-17A expression at baseline and by day 2 of the model in IL-18R$^{-/-}$ mice (Figure 12J-K). Since IL-17A staining showed the most dramatic changes in IL-18R$^{-/-}$ mice, we also performed a preliminary examination of IL-5 and IL-17A dual staining in conventional and unconventional ILC2s to illustrate plasticity in ILC2s. In a first look of FACs plot data at day 2 of our challenges, we demonstrate that both conventional and unconventional ILC2s in IL-18R$^{-/-}$ mice have an expansion of IL-5 IL-17A double positive cells (Figure 12L). Overall, these findings suggest that IL-18 controls the production of the characteristic ILC3 cytokine IL-17A from ILC2s, and portrays a potential role for IL-18 to affect ILC2 cytokine plasticity.

DISSCUSION

Our study demonstrates that IL-18 plays a role in controlling airway eosinophilic inflammation and acts as a potential factor for controlling ILC2 plasticity. Preliminary data exploring factors induced by the fungal allergen *Alternaria alternata* revealed an increase in airway IL-18 production, and an increase in the expression of IL-18Rα in both ST2+ and ST2- ILCs, as shown from Figure 1 and Figure 2. Without the IL-18 receptor, airway eosinophilia in mice was exacerbated in an innate model of allergic asthma, as shown in Figure 4. This initially suggests that IL-18 inhibits eosinophilic inflammation, and a possible mechanism for this phenomenon is through ILC2 plasticity.

Since the focus of this study is the plasticity of ILC2s, it became important to define the characteristics of ILC2s. From the literature, ILC2s are defined as lineage negative lymphocytes that express surface markers CD127 and ST2, that highly express the transcription factor GATA3, and produce type 2 cytokines including IL-5 [54-55]. Thus, a conventional method for identifying ILC2s is to stain innate lymphoid cells for ST2 and CD127, and to pick the population that is double positive for both markers, with the ST2+CD127-, ST2-CD127+, and ST2-CD127- subpopulations of ILCs being considered not ILC2s or not interrogated. However, from phenotyping data that our lab has collected (Figure 6), relying on surface marker expression for identifying ILC2s was shown to be insufficient. Unconventional ILC2 populations that are not traditionally thought of as ILC2s show a significant increase in the total numbers of GATA3 expressing cells, and a significant increase in IL-5 production from these populations after allergen challenge. Thus, these cells that lacked the surface marker expression of conventional

ILC2s still displayed characteristics of ILC2s, thus, we defined ST2+CD127-, ST2-CD127+, and ST2-CD127- innate lymphocytes as unconventional ILC2s.

To explore whether IL-18 affects plasticity in our conventional and unconventional ILC2 populations, we considered the surface marker expression for ILCs in IL-18R$^{-/-}$ mice to see if ILCs were favoring the expansion of one subset over another. From the findings in Figure 9, we observed modest shifts in the expression patterns of the ILCs that may be indicative of plasticity. The frequency of conventional ILC2s amongst all ILCs showed a modest decrease, while unconventional ILC2 populations showed modest increases in both frequency and totals in IL-18R$^{-/-}$ mice. Interestingly, despite observing decreases in the frequency of conventional ILC2s, there were no observed changes in the total number of conventional ILC2s. One explanation could be that the frequency of conventional ILC2s is dependent on the frequencies of the unconventional ILC2s, thus, if the unconventional ILC2s are increasing in number at a faster pace than the conventional ILC2s, this would translate to observing a decreased frequency in conventional ILC2s. This is supported by modest increases in Ki67 expression in unconventional ILC2s from IL-18R$^{-/-}$ mice in comparison to conventional ILC2s. From Figures 9F, 9H, and 9J, we demonstrated modest increases in the total number of Ki67 expressing ILCs by days 2 and 3, and significant increases in the total number of Ki67 expressing ILCs by day 1 in the ST2+CD127- and ST2-CD127- ILC subpopulations. Thus, our studies suggest that IL-18 may affect ILC subtype through a shift away from conventional ILC2s in favor of our unconventional ILC2 subpopulations. Finally, we did not assess levels of apoptosis and survival in these studies that could contribute to such findings.

After examining the surface marker expression and proliferation amongst the ILCs, we next considered the pattern of cytokine production the ILCs had as another method to examine

plasticity. We stained for cytokines that are typically associated with a certain classification of ILC, with IL-5 for ILC2s, IFN-γ for ILC1s, and IL-17A for ILC3s [45]. It is important to note that the data that follows was obtained from lung cells that were first cultured in PMA ionomycin, thus, the cytokine data represents the "potential" for the cells to produce cytokine and may not be representative of what happens in vivo.

For IL-5 levels, we observed no significant changes in the frequency of IL-5 expression amongst the ILC subpopulations, but we did observe a modest increase in the total number of IL-5 expressing cells amongst all the subpopulations by day 2 in IL-18R$^{-/-}$ mice (Figure 10). IL-5 plays a role in eosinophil activation [6], and interestingly, we detected increases in eosinophils by day 1 in IL-18R$^{-/-}$ mice (Figure 4), even though we did not detect changes in IL-5 until day 2. A possible explanation for this discrepancy is that intracellular FACs staining lacks sensitivity that other assays may have, and requires large amounts of cytokine to be present for detection. Thus, it is possible that our technique was not sensitive enough to capture subtle changes in IL-5 levels between WT and IL-18R$^{-/-}$ mice by day 1. Another explanation could be that there exist other recruiters or activators of eosinophils that our stains do not account. Some examples include IL-13, which is another type 2 cytokine that ILC2s produce that recruits eosinophils [52]. Another example being the chemokines, eotaxin 1 (CCL11) and eotaxin 2 (CCL24), which are known to be potent chemoattractants and activators for eosinophils by binding to CCR3 [62-63]. Interestingly, one study demonstrated that eosinophils express IL-18Rα, and that stimulation with IL-18 leads to increased production of IL-8 from eosinophils [64]. However, it remains unknown whether IL-18 directly affects the proliferation and expansion of eosinophils.

The next cytokine we examined was IFN-γ production in the ILCs. Interestingly, from the data shown in Figure 11, we observed modest to significant increases in IFN-γ production at day

1 of the model in all the ILC subpopulations in IL-18R$^{-/-}$ mice. This result is surprising since one of the main functions of IL-18 is to promote the release of IFN-γ [36], thus, we would normally expect from an IL-18R$^{-/-}$ mouse a decrease in IFN-γ production. A possible explanation for this discrepancy is due to that our findings regarding ILC numbers and proliferation demonstrated increases in ILC numbers and proliferation. Thus, this could suggest that since there are more ILCs present, then the amount of cytokine produced increases, which could lead to observed increases in IFN-γ production. Another explanation could be due to how we stimulated the cells first with PMA ionomycin, thus, the resulting IFN-γ production that we observe from the ILCs is not representative of what happens in vivo.

The last cytokine we examined as a measurement of plasticity was IL-17A. From the data collected in Figure 12, we saw the most dramatic increases in the percent and total expression of IL-17A in all ILC subpopulations at every time point in IL-18R$^{-/-}$ mice. We also observed an expansion of conventional and unconventional ILC2s in IL-18R$^{-/-}$ mice that had dual cytokine expression of IL-5 and IL-17A, which further supports that plasticity is occurring in ILC2s from the IL-18R$^{-/-}$ mice. This suggests that IL-18 plays a role in affecting ILC2 plasticity by controlling IL-17A production from ILC2s. The exact role that IL-17A plays in our model of allergic asthma is unknown. From the literature, IL-17A is a potent activator of neutrophils, and has been associated with autoimmunity [56]. However, we did not observe any changes in the total number of neutrophils in the lungs or BAL from IL-18R$^{-/-}$ mice, as shown in Figure S1. We did observe a significant decrease in the frequency of neutrophils by day 3 in the BAL in IL-18R$^{-/-}$ mice (Figure S1A). However, we also observed increases in the total numbers of eosinophils in the BAL by day 3 (Figure 4B). Thus, this decrease in neutrophil frequency is most likely a consequence of the large increase in eosinophils, and not due to an actual decrease in

neutrophilia. This conclusion is supported from how the total neutrophils in the BAL by day 3 looks similar between groups (Figure S1B). Another potential role for IL-17A can be explained by a study in 2013 that demonstrated that IL-17A was increased in patients with high levels of peripheral blood eosinophils, and how IL-17A enhanced the activity of eosinophils [53]. Thus, the increases in IL-17A that we observe in IL-18R$^{-/-}$ mice may be exacerbating existing eosinophilia and worsening eosinophilic inflammation.

The results of our study support that IL-18 can control the plasticity of ILC2s, for in the absence of IL-18 receptor signaling, there was an increase in cell numbers and proliferation of unconventional ILC2 populations. Our study also suggests a novel role for IL-18 in its ability to control IL-17A production from ILC2s. Possible mechanisms for how IL-18 controls ILC2 IL-17A production would have to be explored in future experiments. One of the known roles of IL-18 is to activate ILC1s, which rely on T-bet expression for development, to produce IFN-γ [43, 46]. In two studies involving a T-bet knockout mouse, there was an observed increase in the production of IL-17A from lung, gut, and lymph node ILCs [57-58]. Another study demonstrated that IFN-γ signaling inhibited the production of IL-17A from Th17 cells through the induction of indoleamine 2,3 dioxgenase (IDO), and that in an IFN-γ knockout mouse, there was increased production of IL-17A from Th17 cells [59]. Thus, based on these studies, it is possible that IL-18 inhibits IL-17A production from ILCs by promoting the production of IFN-γ. However, this would not explain why IL-18R$^{-/-}$ mice have increased IFN-γ as we saw in Figure 11. Another possible mechanism can be seen from signaling pathways of IL-18 and of IL-17A production. Studies have shown that IL-18 signaling activates the JAK/STAT pathway through STAT3, and interestingly, another study demonstrated that IL-23-induced IL-17 production from CD4+ T cells also requires activation of STAT3 [60-61]. Thus, there is the possibility that signaling

through IL-18 prevents signals for IL-17A production from signaling through STAT3 effects. To test whether IL-18 controls IL-17A production, and to test whether IL-17A production is driving the increase in eosinophilia observed in IL-18R$^{-/-}$ mice, future experiments are needed. One experiment to perform would involve stimulating purified ILC2s with IL-18 and analyzing IL-17A cytokine production via qPCR and ELISA to test whether IL-18 controls IL-17A production from ILC2s. Another experiment to perform in the future would be to block IL-17A in IL-18R$^{-/-}$ mice during allergen challenge and observe the effects on eosinophilia. Finally, to determine ILC intrinsic effects of IL-18R, we could utilize mixed bone marrow chimera mice that had bone marrow from both a WT mouse and an IL-18R$^{-/-}$ mouse, and then examine the phenotype after allergen challenge.

FIGURES

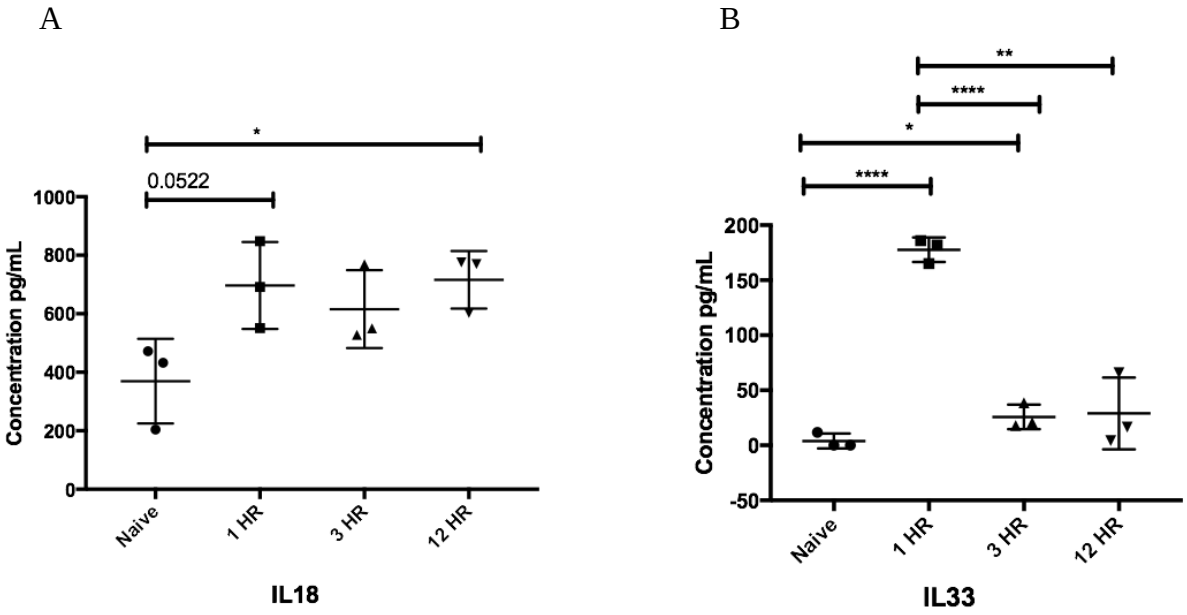


Figure 1: IL-18 present in models of allergic asthma. WT mice were challenged with *Alternaria alternata*, and then 1, 3, and 12 hours after intranasal challenge, mice were euthanized and BAL was collected for analysis via ELISA. Concentration in pg/mL of IL-18 (A) and IL-33 (B).

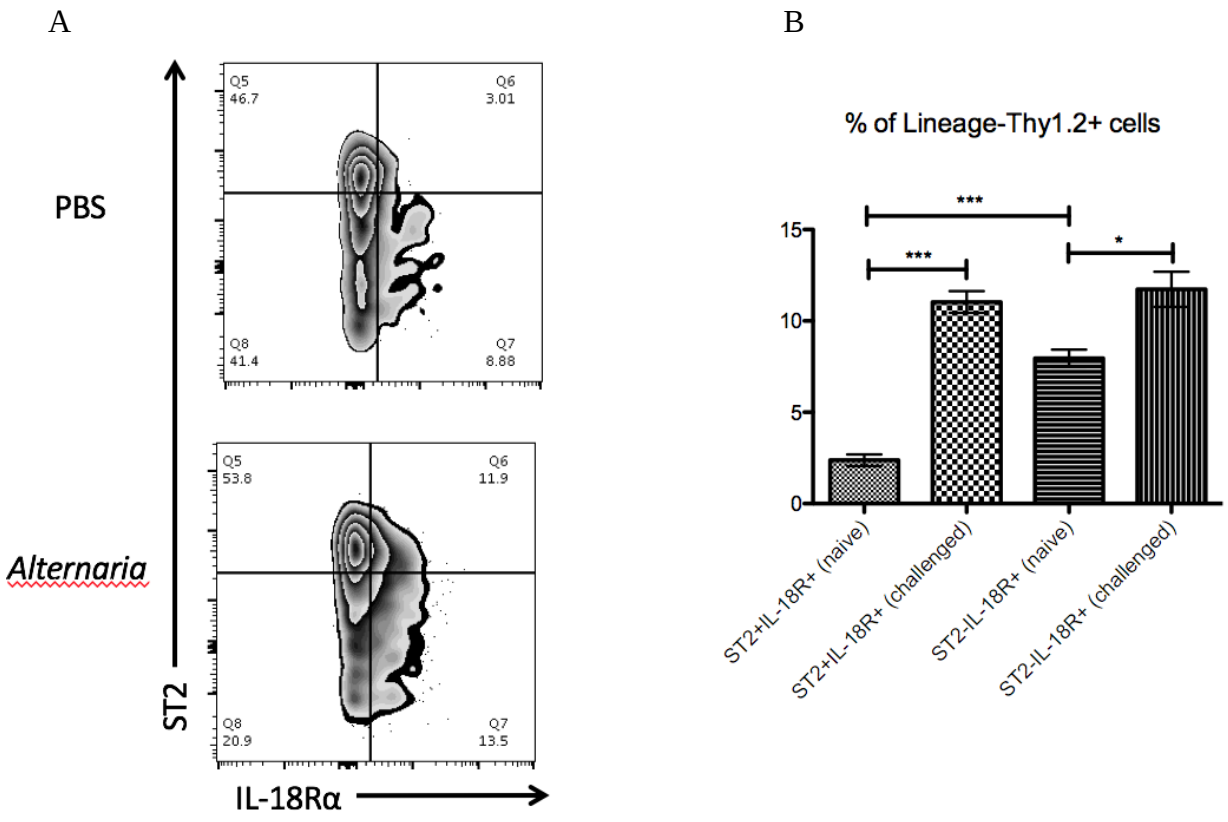


Figure 2: ILC2 expression of IL-18Rα increases with allergen challenge. WT mice were challenged with 50μg of *Alternaria alternata* or PBS once a day for 3 days. On day 4, the mice were euthanized, and lungs were collected. FACs plots of IL-18Rα expression of ILCs in PBS challenged and *Alternaria alternata* challenged mice (A). Percentages of lung ILCs expressing IL-18Rα (B).

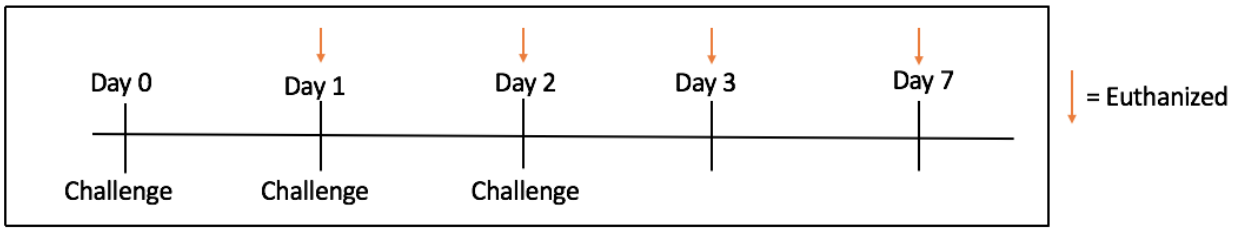
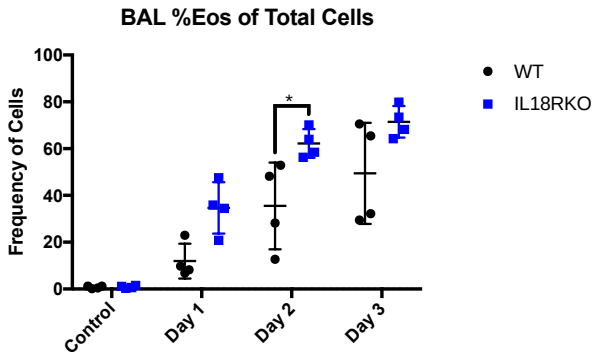


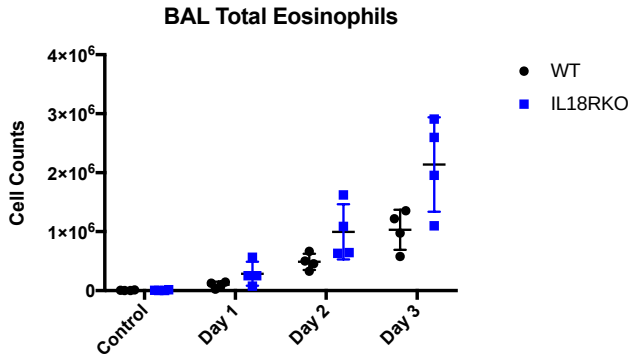
Figure 3: Kinetics asthma model. WT and IL-18R$^{-/-}$ mice were intranasally challenged on days 0, 1, and 2 with 50μg *Alternaria alternata*. Mice were euthanized either in the middle of their challenges on days 1 and 2, or euthanized on day 3, and their lungs and BAL were collected. A group of mice were also given 4 days of rest after challenges, and on day 7, mice were euthanized and lungs and BAL were collected.

Figure 4: Asthma phenotype exacerbated in IL-18R$^{-/-}$ mice. Mice were challenged as shown in Figure 4. The frequency of eosinophils (A) and the total eosinophils (B) in BAL from mice. The frequency of eosinophils (D) and the total eosinophils (E) in lungs from mice. Flow cytometry plots of BAL (C) and Lung (F) eosinophils. Data shown are representative of two independent experiments with two mice per group. *$p < 0.05$, unpaired *t* test.

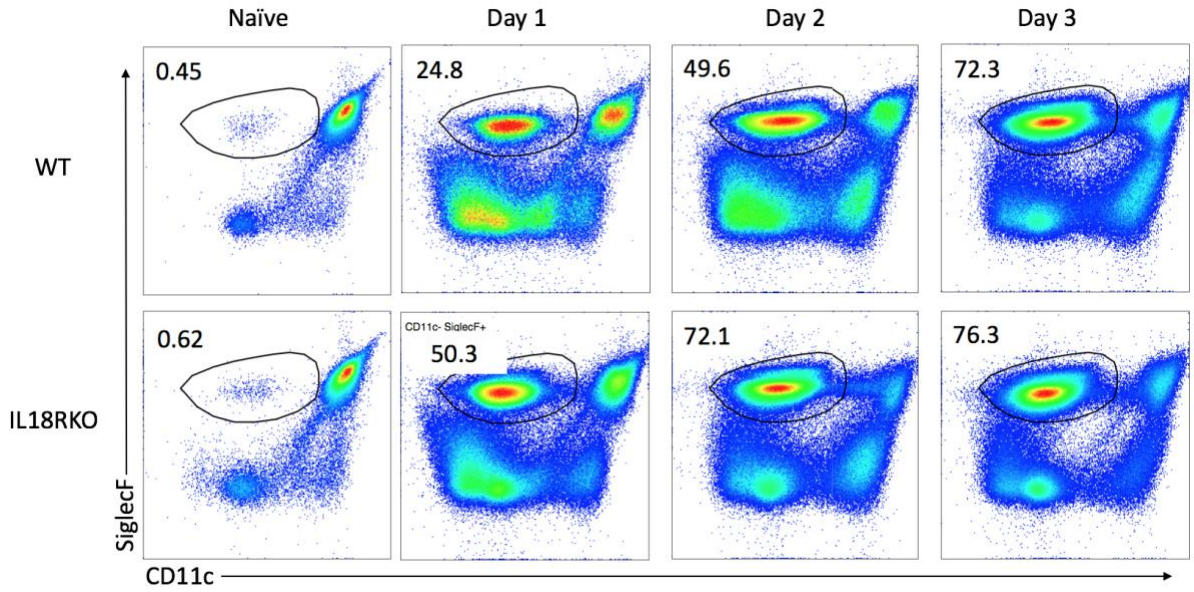
A


BAL %Eos of Total Cells
Frequency of Cells
100
80
60
40
20
0
Control
Day 1
Day 2
Day 3
*
WT
IL18RKO

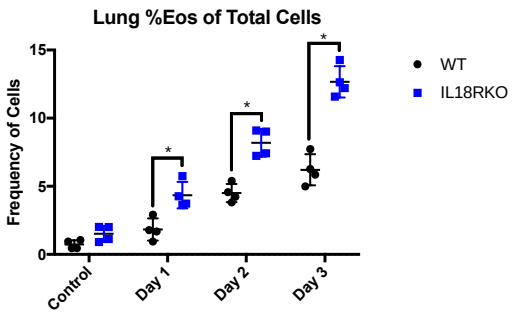
B


BAL Total Eosinophils
Cell Counts
4×10^6
3×10^6
2×10^6
1×10^6
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

C


Naïve
Day 1
Day 2
Day 3
WT
IL18RKO
0.45
24.8
49.6
72.3
0.62
CD11c- SiglecF+
50.3
72.1
76.3
SiglecF
CD11c

D


Lung %Eos of Total Cells
Frequency of Cells
15
10
5
0
Control
Day 1
Day 2
Day 3
*
WT
IL18RKO

E

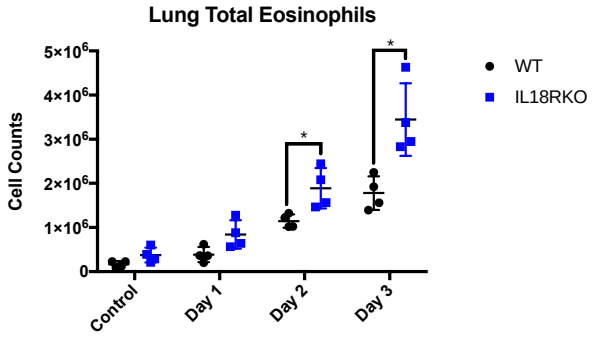
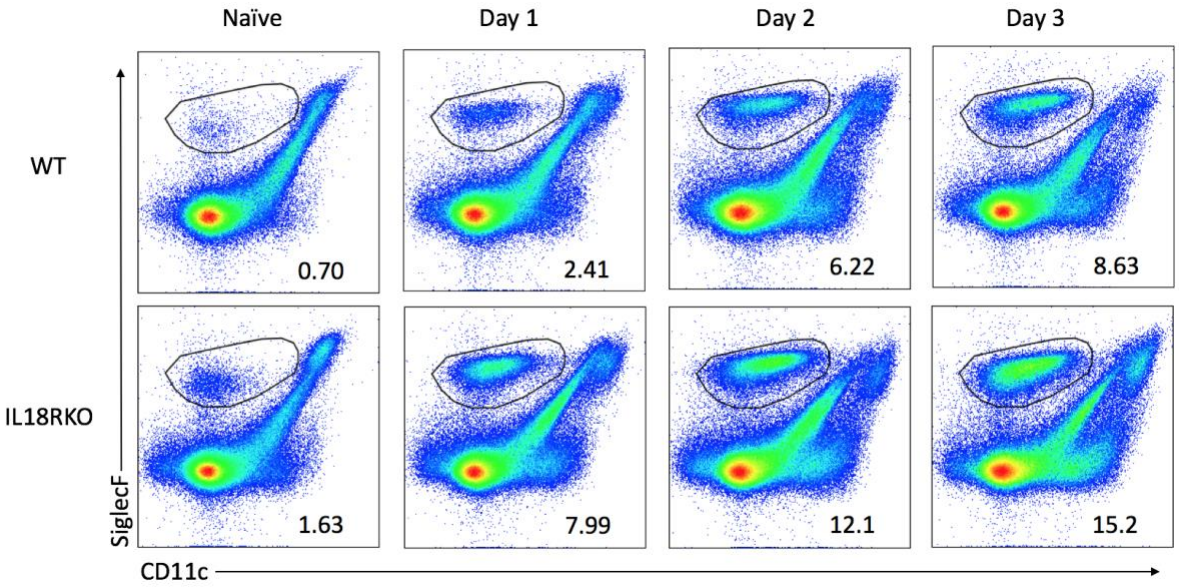

Lung Total Eosinophils
Cell Counts
5×10^6
4×10^6
3×10^6
2×10^6
1×10^6
0
Control
Day 1
Day 2
Day 3
*
WT
IL18RKO

Figure 4 Continued

F



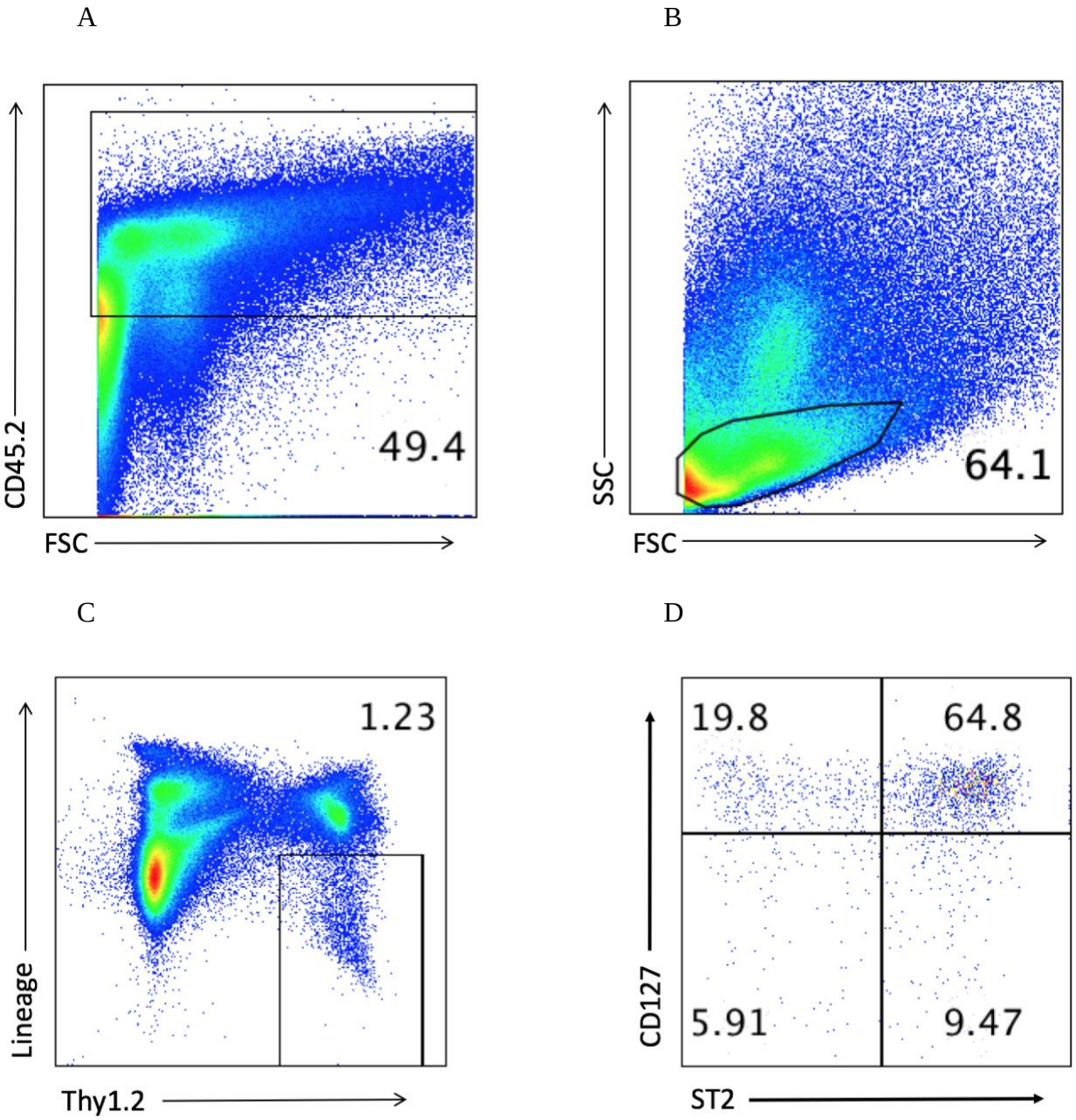


Figure 5: Parent gating scheme for ILC ST2 CD127 subsets. Representative FACs plots of CD45.2+ cells (A), Lymphocytes (B), Total ILCs (C), and ST2 CD127 gating (D).

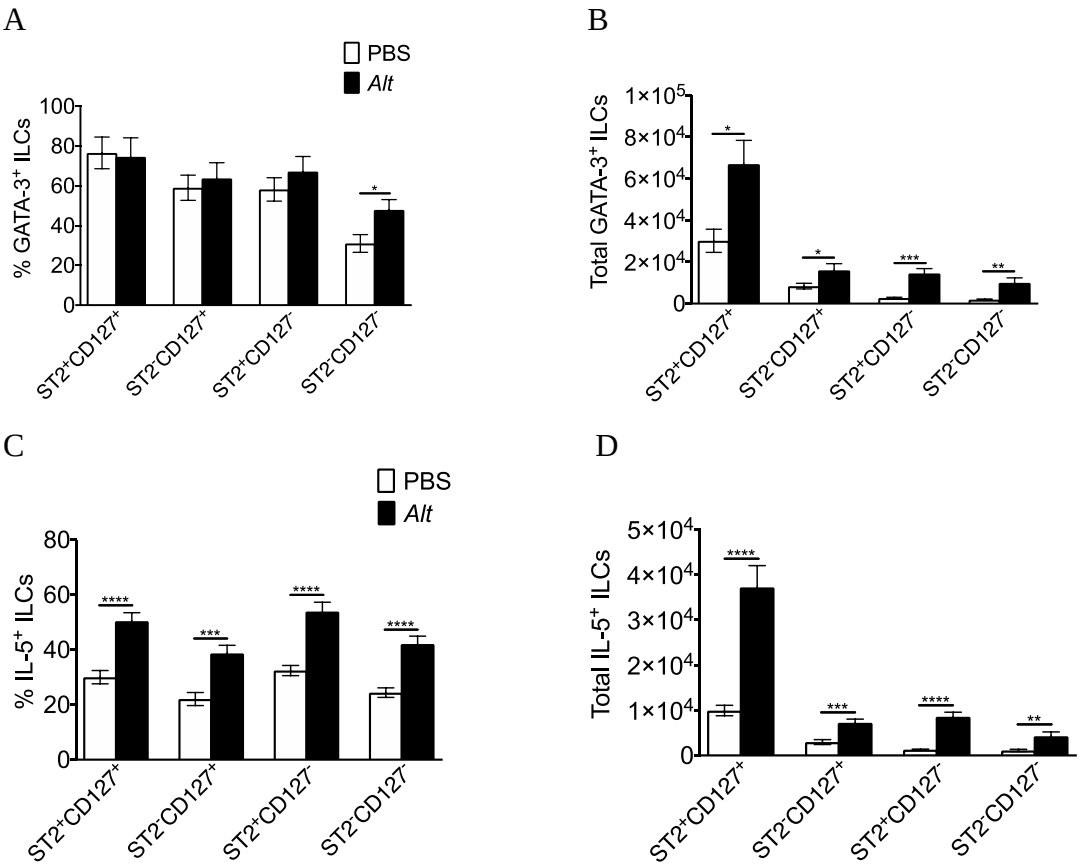


Figure 6: Designations of conventional and unconventional ILC2s. WT mice were challenged with 50μg of *Alternaria alternata* extract on days 0, 1, and 2, and lungs were collected for flow cytometry analysis on day 3. Innate lymphoid cells (ILCs) were gated as CD45.2 positive, lineage negative, Thy1.2 positive lymphocytes. Intracellular transcription factor expression and cytokine production from various subsets of ILCs using the markers ST2 and CD127 was stained for. GATA3 frequency (A) and total (B) expression, and IL-5 frequency (C) and total (D) expression.

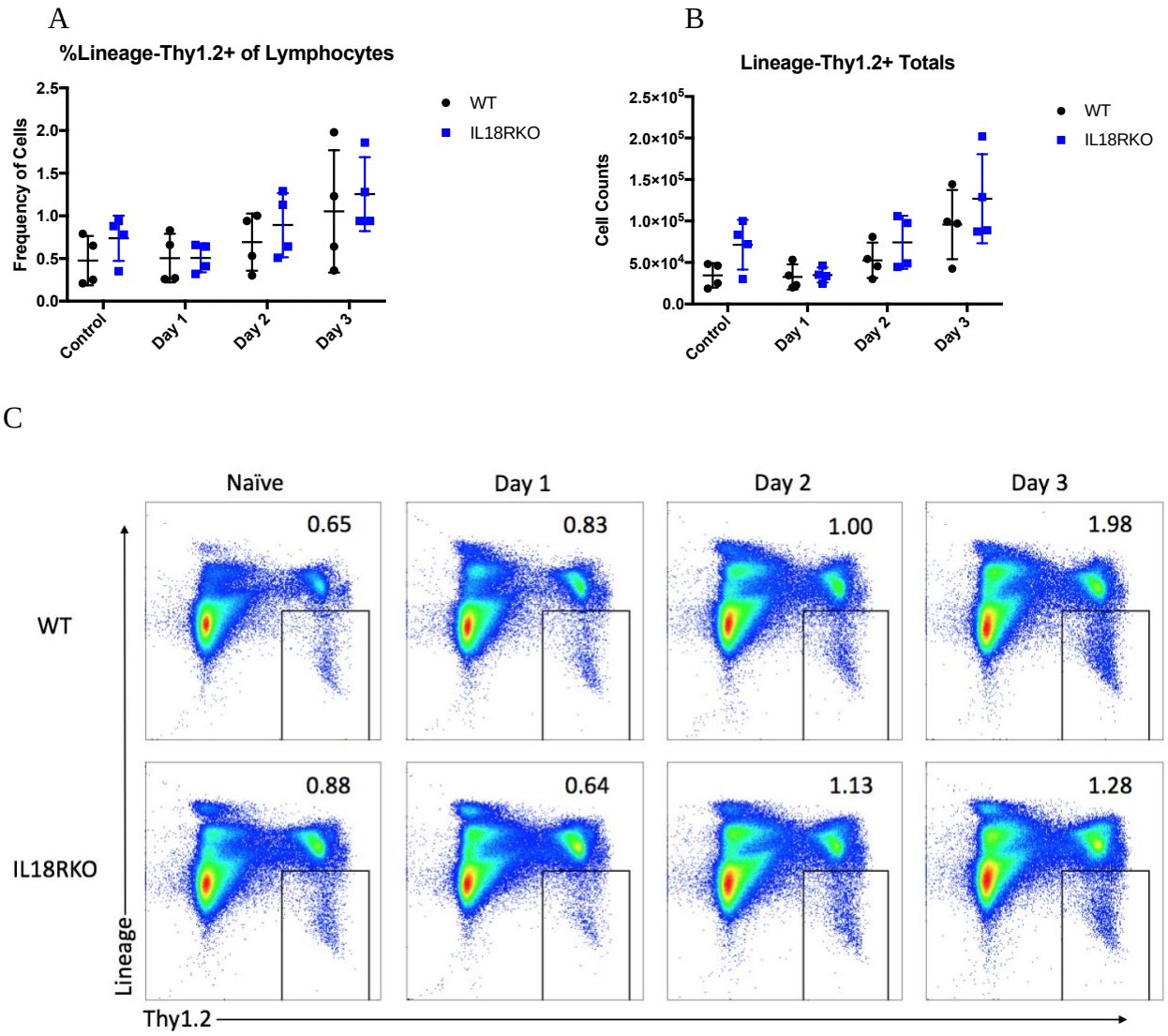


Figure 7: ILC frequencies and numbers. Mice were challenged as shown in Figure 4. ILCs were gated as Lineage-Thy1.2+ lymphocytes. The frequency (A) and total number (B) of ILCs. Flow cytometry plots of the lineage and Thy1.2 gating (C).

Figure 8: ILC subpopulation frequencies and numbers. Mice were challenged as shown in Figure 4. The ST2+CD127+ ILC frequency (A) and total number (B), the ST2+CD127- ILC frequency (C) and total number (D), the ST2-CD127+ ILC frequency (E) and total number (F), and the ST2-CD127- ILC frequency (G) and total number (H). Flow cytometry plots of the ST2 and CD127 quadrants (I). Data shown are representative of two independent experiments with two mice per group. $*p < 0.05$, unpaired *t* test.

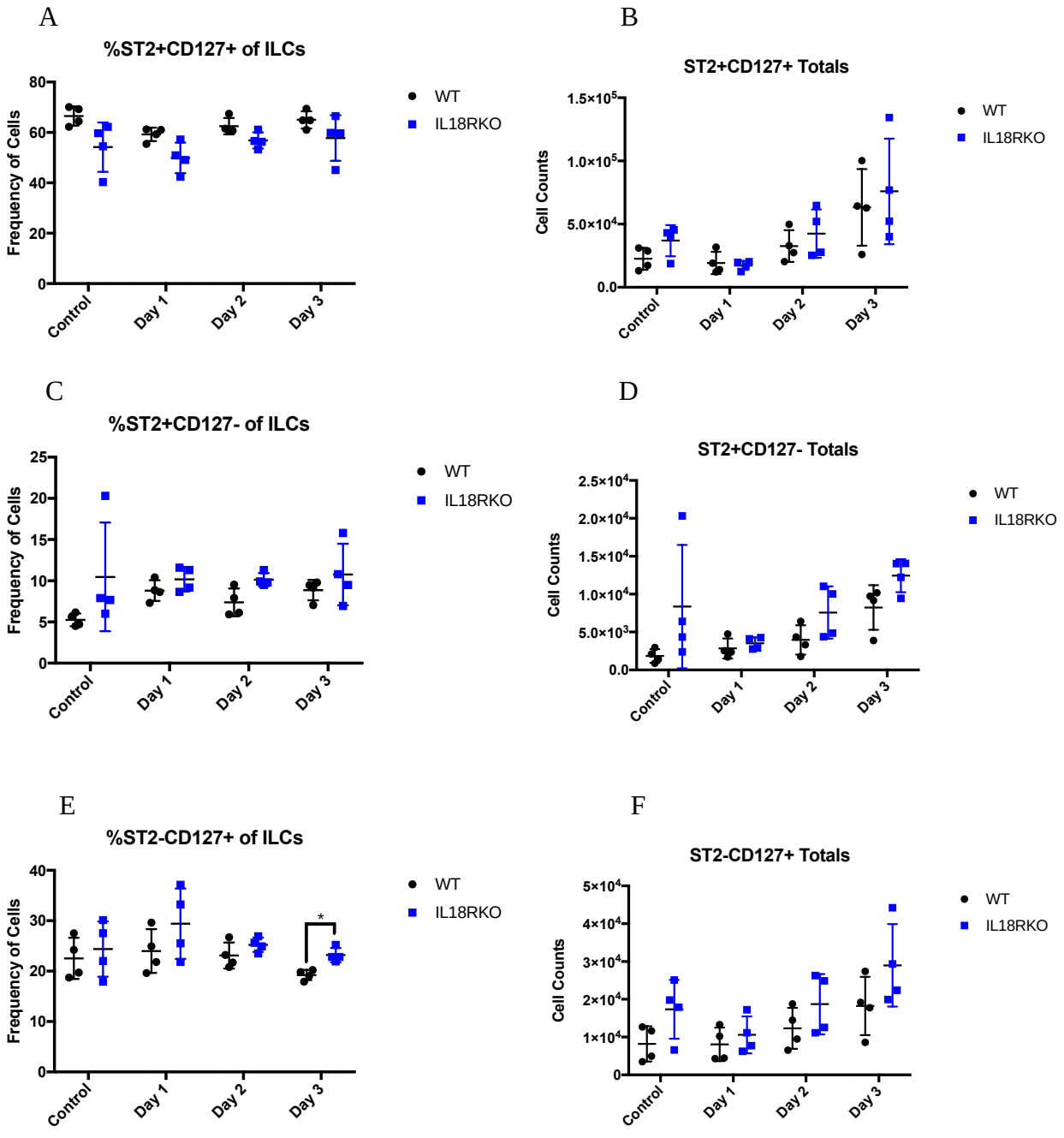
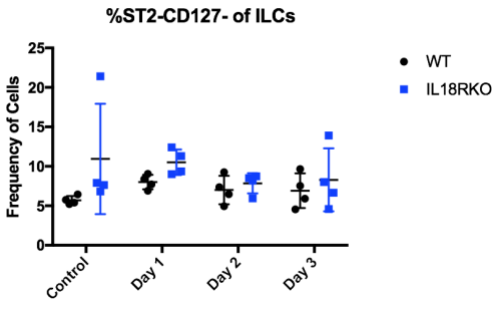
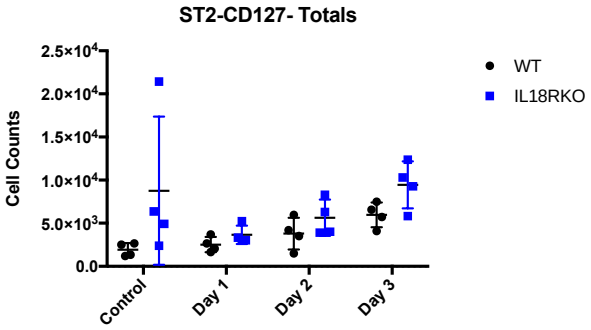

A
%ST2+CD127+ of ILCs
Frequency of Cells
80
60
40
20
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO
B
ST2+CD127+ Totals
Cell Counts
1.5×10^5
1.0×10^5
5.0×10^4
0.0
Control
Day 1
Day 2
Day 3
WT
IL18RKO
C
%ST2+CD127- of ILCs
Frequency of Cells
25
20
15
10
5
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO
D
ST2+CD127- Totals
Cell Counts
2.5×10^4
2.0×10^4
1.5×10^4
1.0×10^4
5.0×10^3
0.0
Control
Day 1
Day 2
Day 3
WT
IL18RKO
E
%ST2-CD127+ of ILCs
Frequency of Cells
40
30
20
10
0
*
Control
Day 1
Day 2
Day 3
WT
IL18RKO
F
ST2-CD127+ Totals
Cell Counts
5×10^4
4×10^4
3×10^4
2×10^4
1×10^4
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

Figure 8 Continued

G



H



I

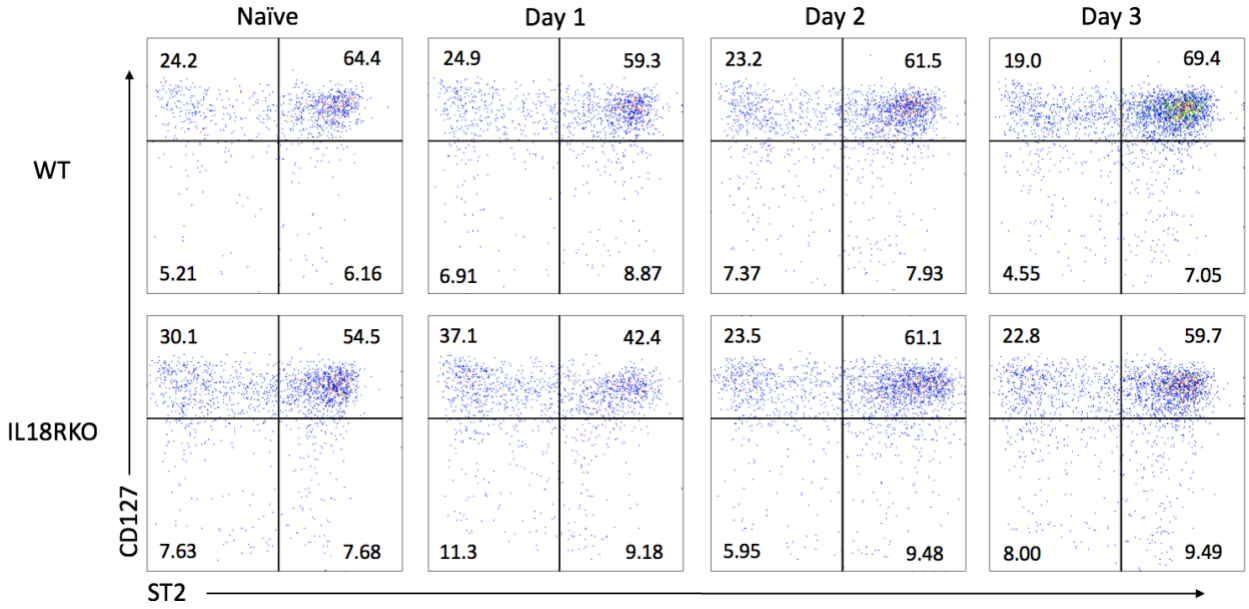
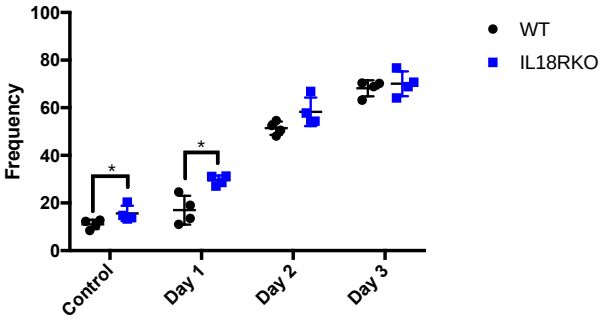
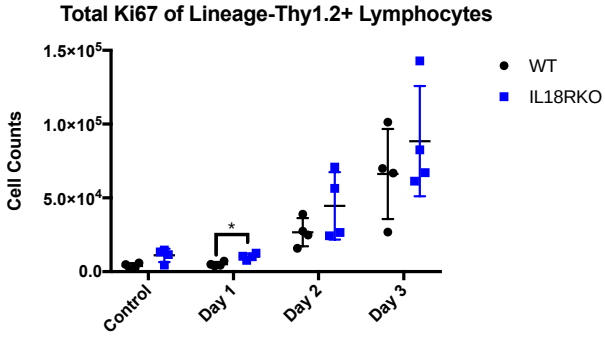


Figure 9: Increased ILC2 proliferation in IL-18R$^{-/-}$ mice. Mice were challenged as shown in Figure 4. The frequency of Ki67 expression (A) and the total number of Ki67 expressing cells (B) in the innate lymphoid cell population. Ki67 expression was also extended to the ST2 and CD127 ILC subpopulations (C-J). Data shown are representative of two independent experiments with two mice per group. *$p < 0.05$, unpaired *t* test.

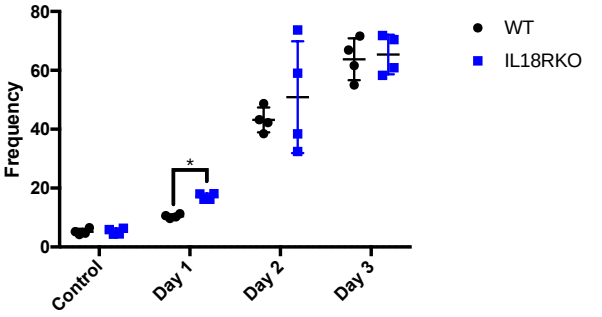
A


%Ki67 of Lineage-Thy1.2+ Lymphocytes
Frequency
100
80
60
40
20
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

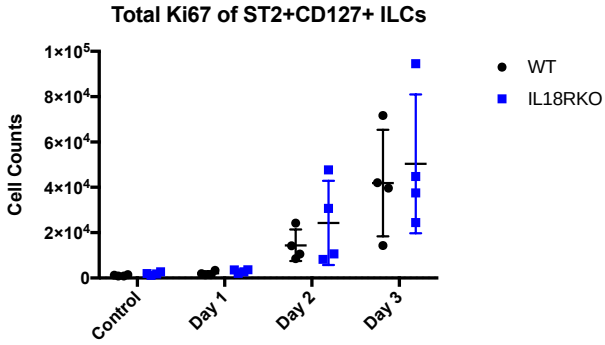
B


Total Ki67 of Lineage-Thy1.2+ Lymphocytes
Cell Counts
1.5×10^5
1.0×10^5
5.0×10^4
0.0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

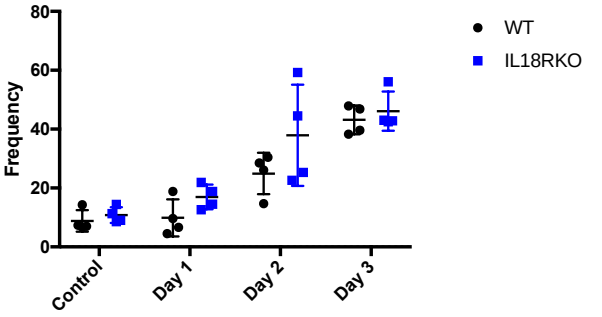
C


%Ki67 of ST2+CD127+ ILCs
Frequency
80
60
40
20
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

D


Total Ki67 of ST2+CD127+ ILCs
Cell Counts
1×10^5
8×10^4
6×10^4
4×10^4
2×10^4
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

E


%Ki67 of ST2+CD127- ILCs
Frequency
80
60
40
20
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

F

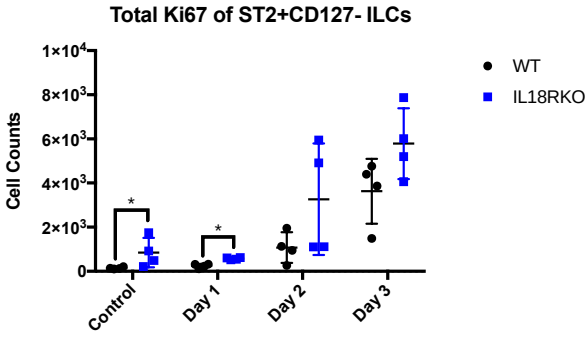

Total Ki67 of ST2+CD127- ILCs
Cell Counts
1×10^4
8×10^3
6×10^3
4×10^3
2×10^3
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

Figure 9 Continued

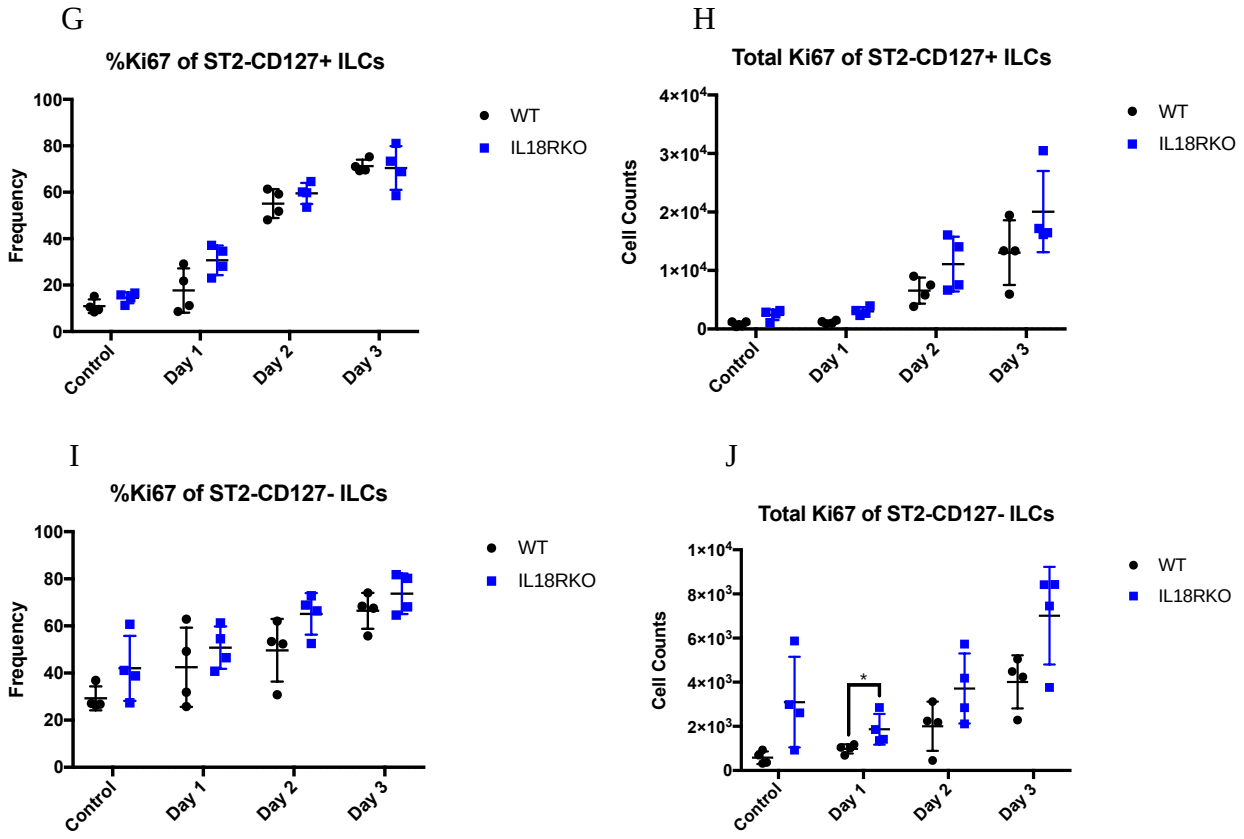


Figure 10: ILC IL-5 expression. Mice were challenged as shown in Figure 4. The frequency of IL-5 expression (A) and the total number of IL-5 expressing cells (B) in the innate lymphoid cell population. Flow cytometry plots of IL-5 expression in the ST2+CD127+ ILCs (C) IL-5 expression was also extended to the ST2 and CD127 ILC subpopulations (D-K).

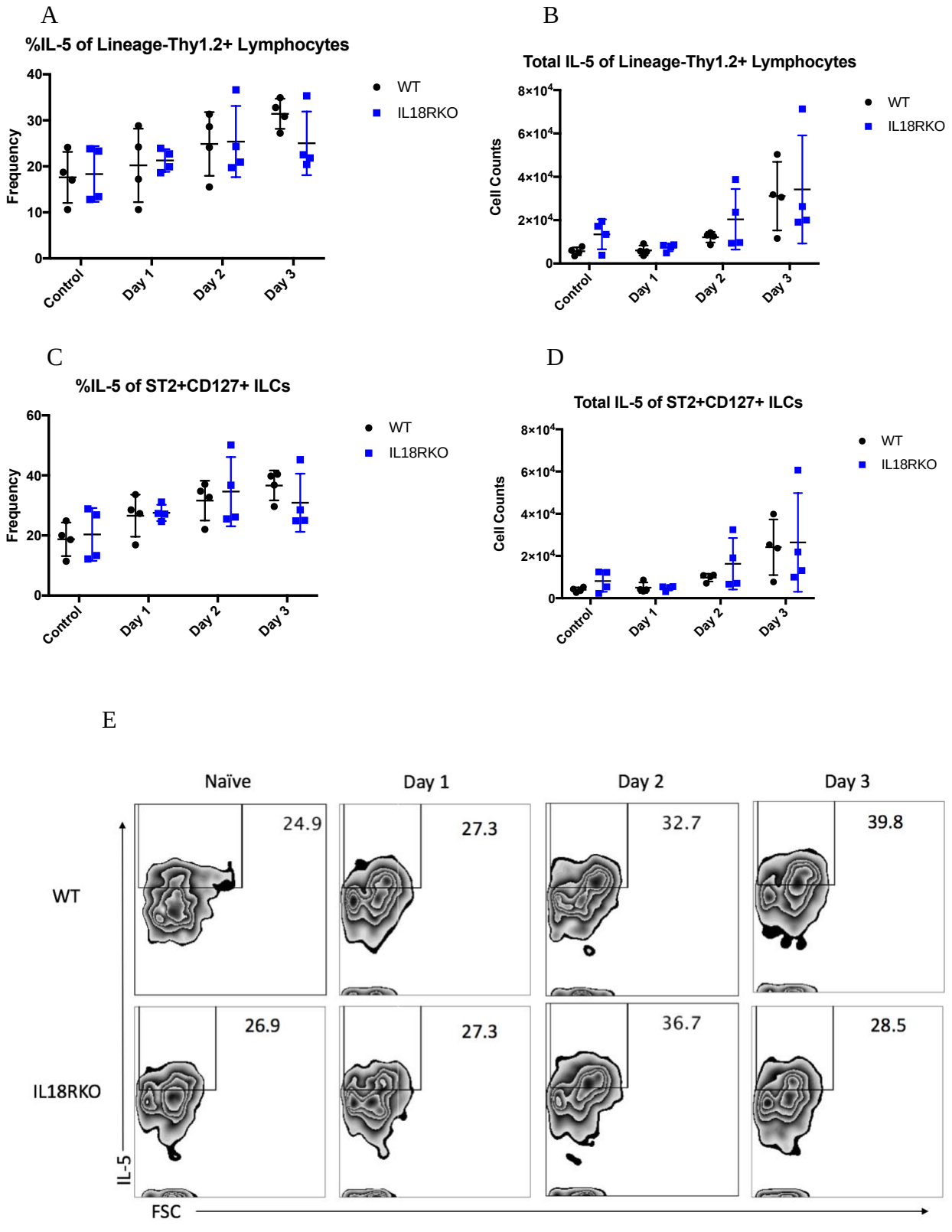
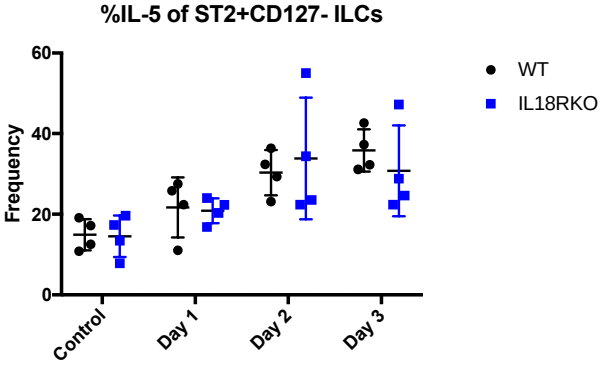
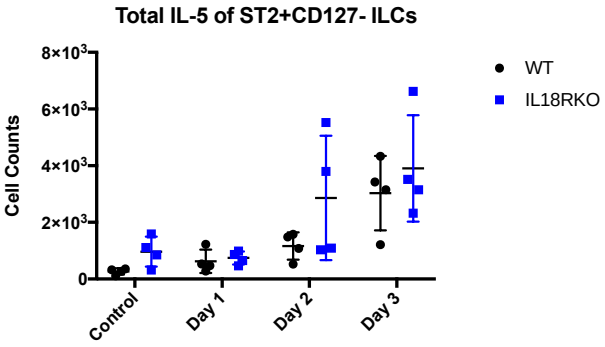

A
%IL-5 of Lineage-Thy1.2+ Lymphocytes
Frequency
40
30
20
10
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO
B
Total IL-5 of Lineage-Thy1.2+ Lymphocytes
Cell Counts
8×10^4
6×10^4
4×10^4
2×10^4
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO
C
%IL-5 of ST2+CD127+ ILCs
Frequency
60
40
20
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO
D
Total IL-5 of ST2+CD127+ ILCs
Cell Counts
8×10^4
6×10^4
4×10^4
2×10^4
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO
E
Naïve
Day 1
Day 2
Day 3
WT
IL18RKO
24.9
27.3
32.7
39.8
26.9
27.3
36.7
28.5
IL-5
FSC

Figure 10 Continued

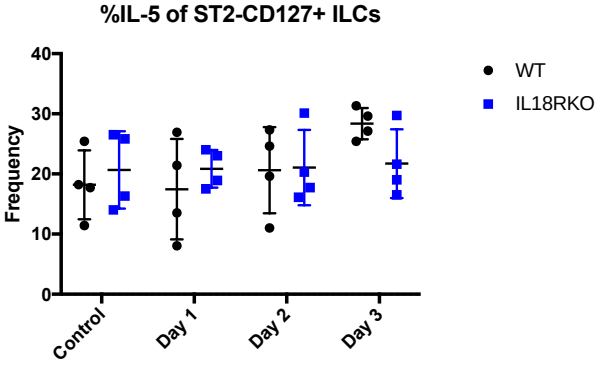
F



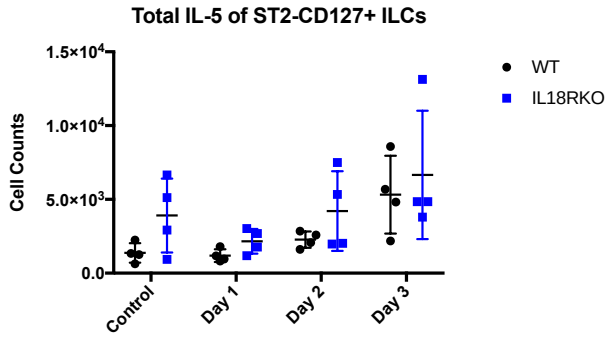
G



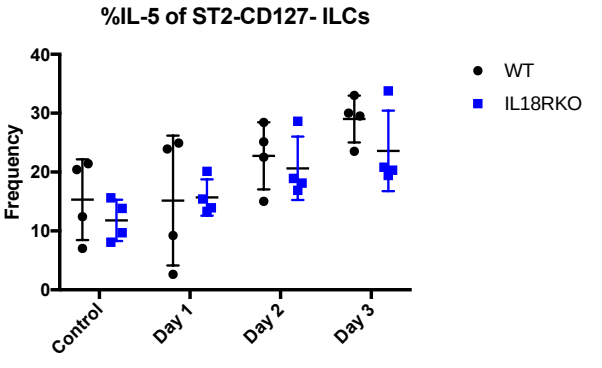
H



I



J



K

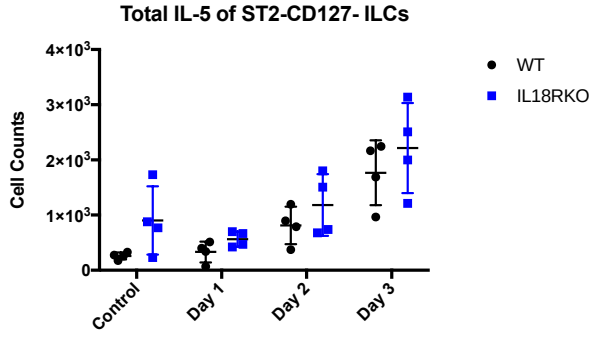
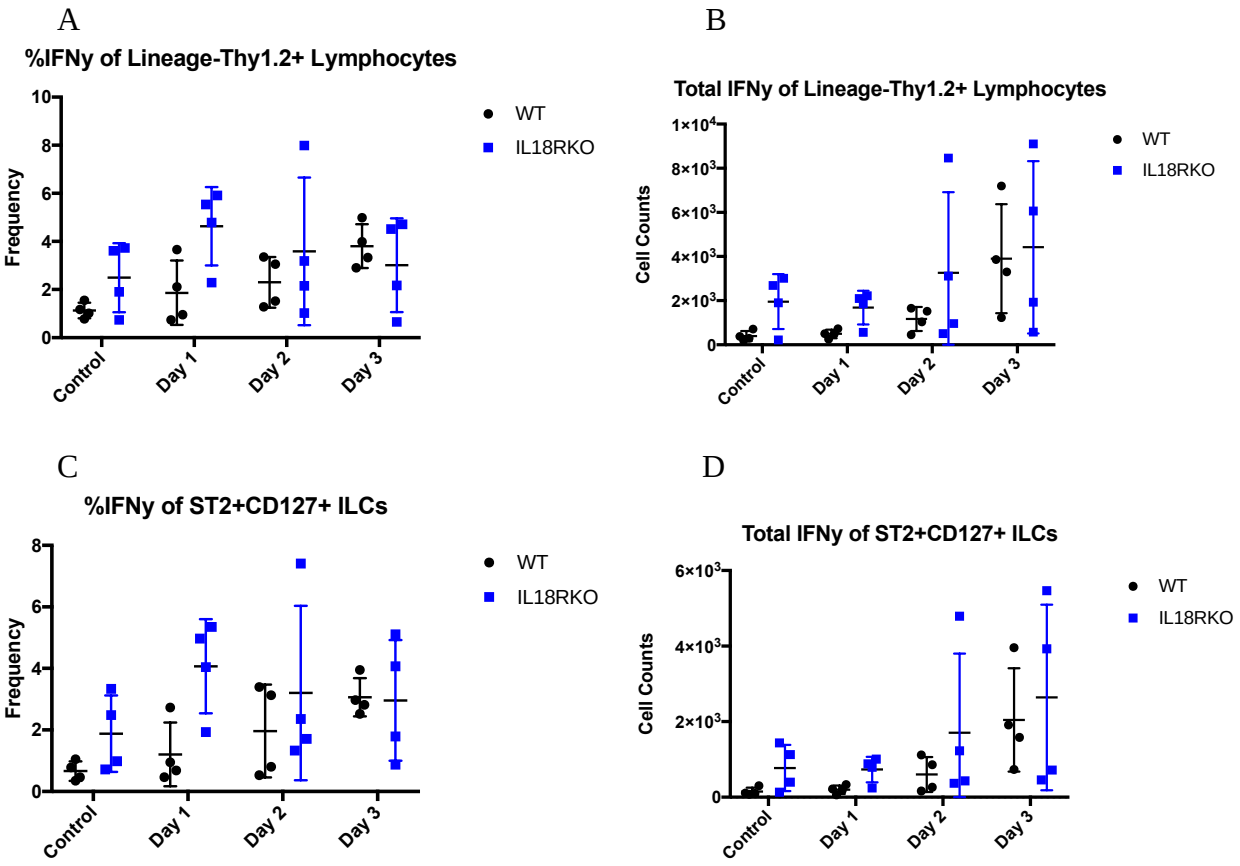


Figure 11: ILC IFN-γ expression. Mice were challenged as shown in Figure 4. The frequency of IFN-γ expression (A) and the total number of IFN-γ expressing cells (B) in the innate lymphoid cell population. Representative flow cytometry plots of IFN-γ in ST2+CD127+ ILCs is shown (C). IFN-γ expression was also extended to the ST2 and CD127 ILC subpopulations (D-K). Data shown are representative of two independent experiments with two mice per group. $*p < 0.05$, unpaired *t* test.


A
%IFNy of Lineage-Thy1.2+ Lymphocytes
Frequency
10
8
6
4
2
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO
B
Total IFNy of Lineage-Thy1.2+ Lymphocytes
Cell Counts
1×10^4
8×10^3
6×10^3
4×10^3
2×10^3
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO
C
%IFNy of ST2+CD127+ ILCs
Frequency
8
6
4
2
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO
D
Total IFNy of ST2+CD127+ ILCs
Cell Counts
6×10^3
4×10^3
2×10^3
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

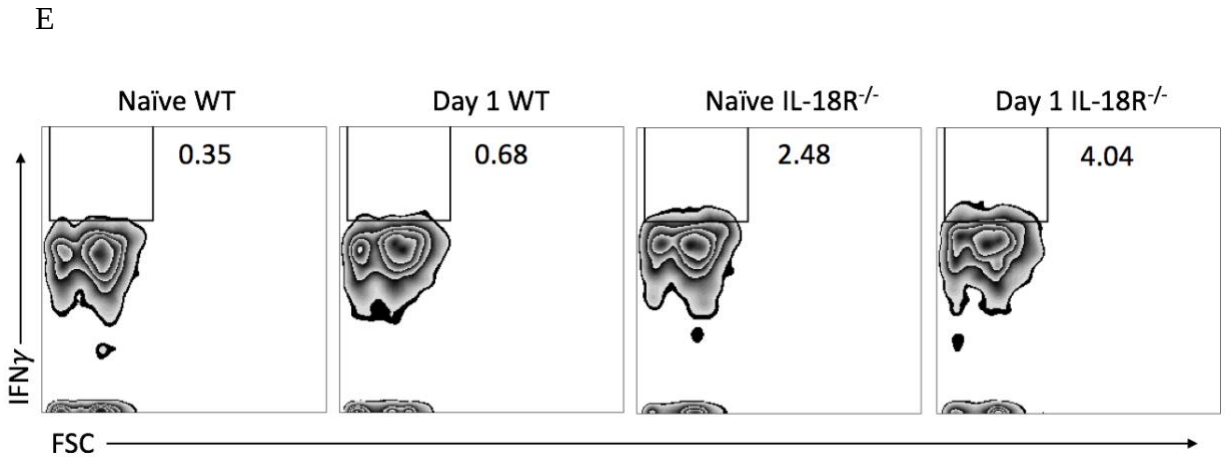

E
Naïve WT
Day 1 WT
Naïve IL-18R-/-
Day 1 IL-18R-/-
0.35
0.68
2.48
4.04
IFNγ
FSC

Figure 11 Continued

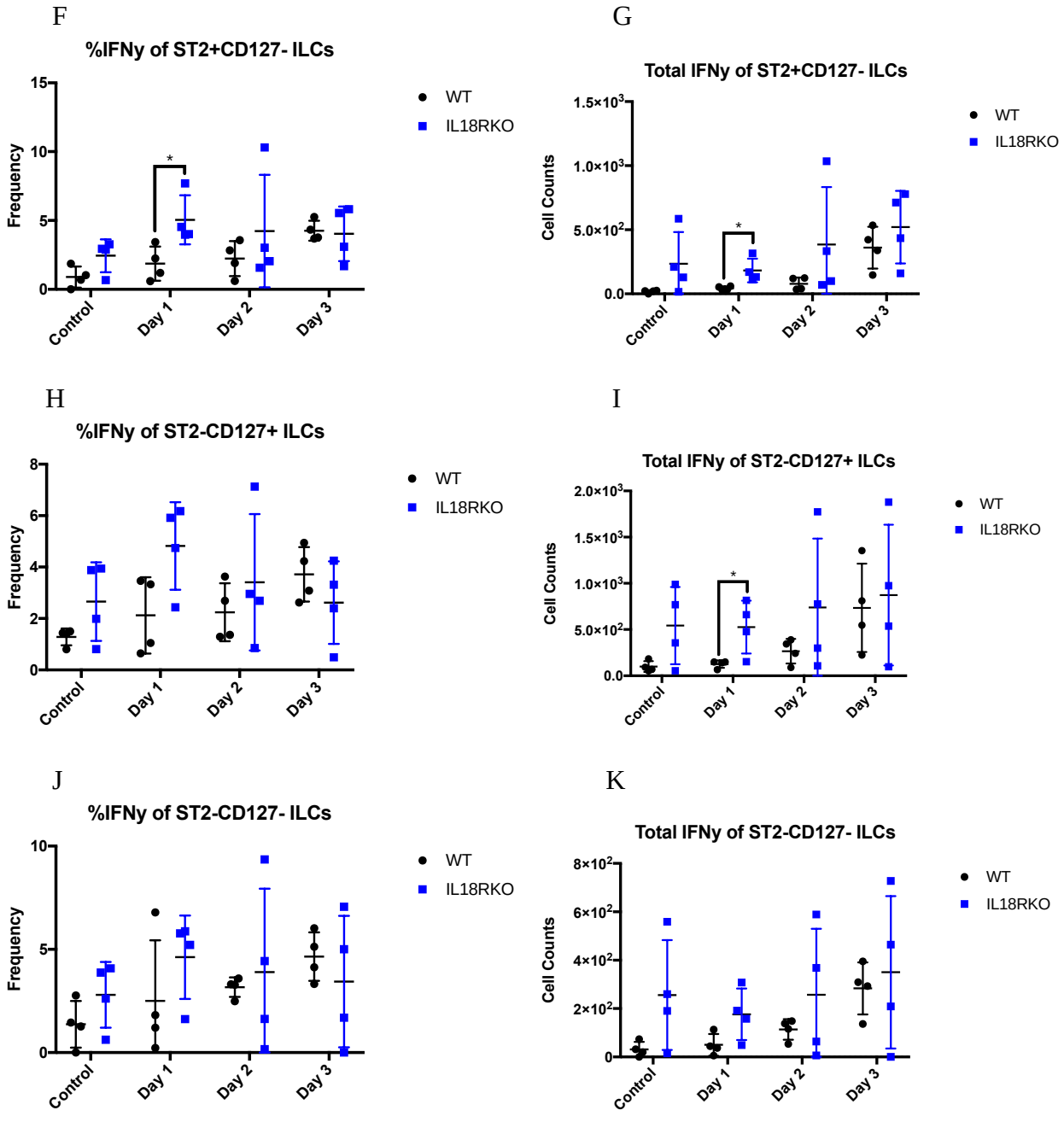
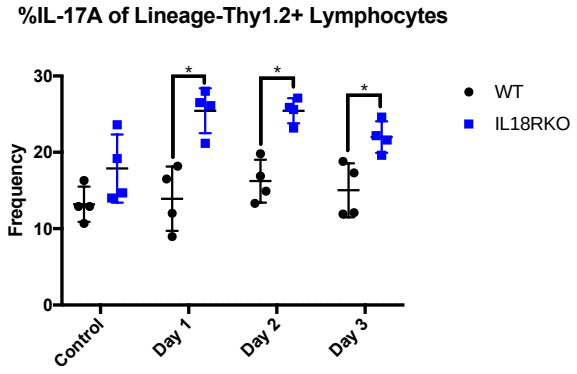
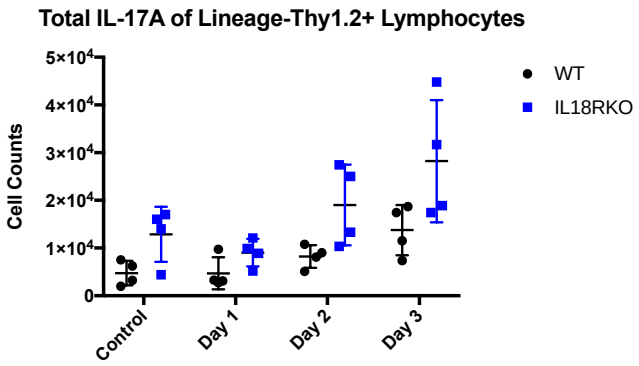


Figure 12: ILC IL-17A expression. Mice were challenged as shown in Figure 4. The frequency of IL-17A expression (A) and the total number of IL-17A expressing cells (B) in the innate lymphoid cell population. Representative flow cytometry plots of IL-17A expression in ST2+CD127+ ILCs is shown (C). IL-17A expression was also extended to the ST2 and CD127 ILC subpopulations (D-K). Representative FACs plot of double positive expression of IL-17A and IL-5 in ILCs at day 2. Data shown are representative of two independent experiments with two mice per group. *$p < 0.05$, unpaired *t* test.

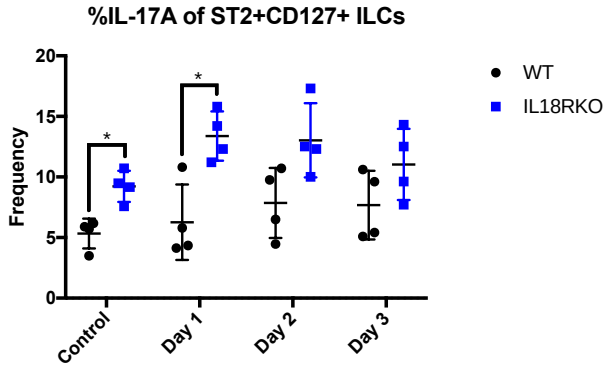
A


%IL-17A of Lineage-Thy1.2+ Lymphocytes
Frequency
30
20
10
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

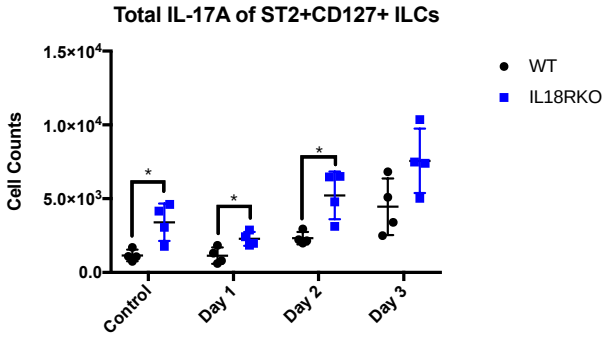
B


Total IL-17A of Lineage-Thy1.2+ Lymphocytes
Cell Counts
5×10⁴
4×10⁴
3×10⁴
2×10⁴
1×10⁴
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

C


%IL-17A of ST2+CD127+ ILCs
Frequency
20
15
10
5
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

D


Total IL-17A of ST2+CD127+ ILCs
Cell Counts
1.5×10⁴
1.0×10⁴
5.0×10³
0.0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

E

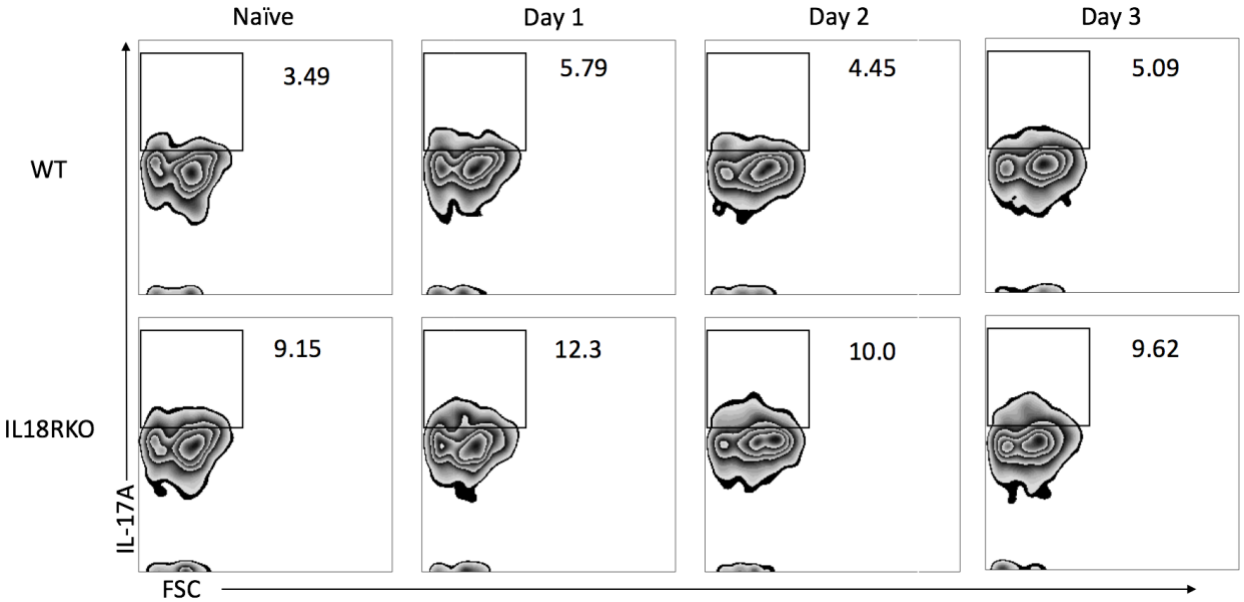
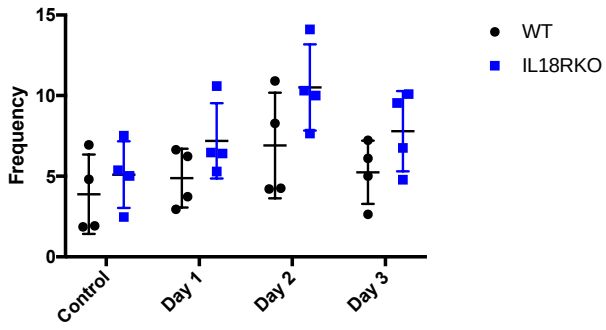
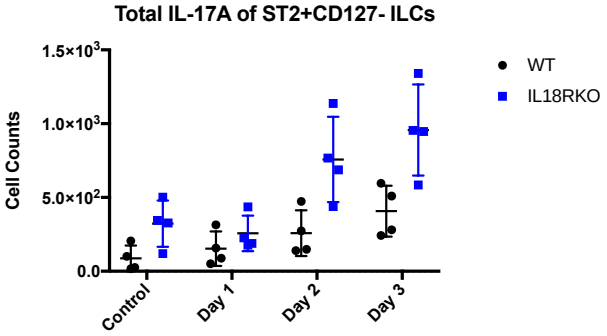

Naïve
Day 1
Day 2
Day 3
WT
IL18RKO
3.49
5.79
4.45
5.09
9.15
12.3
10.0
9.62
IL-17A
FSC

Figure 12 Continued

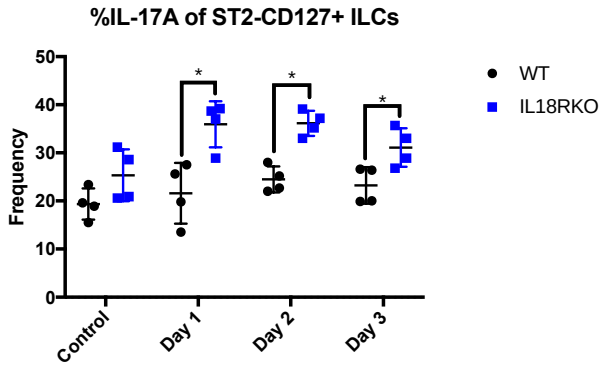
F



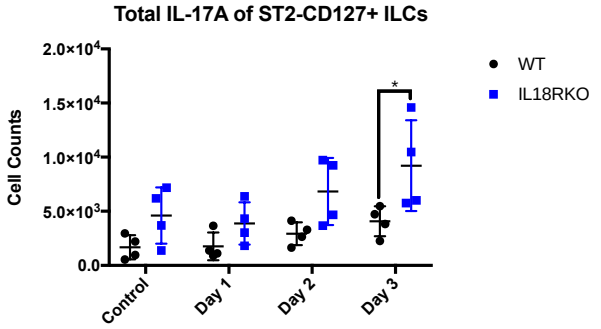
G



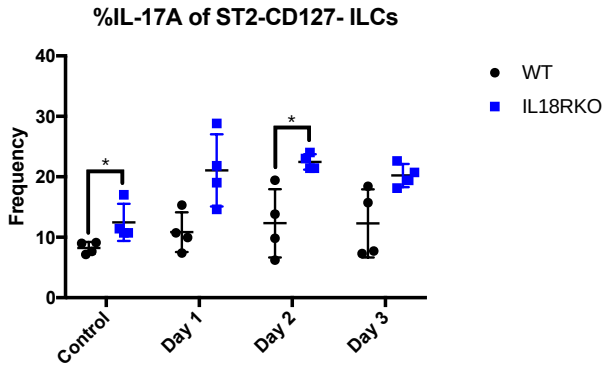
H



I



J



K

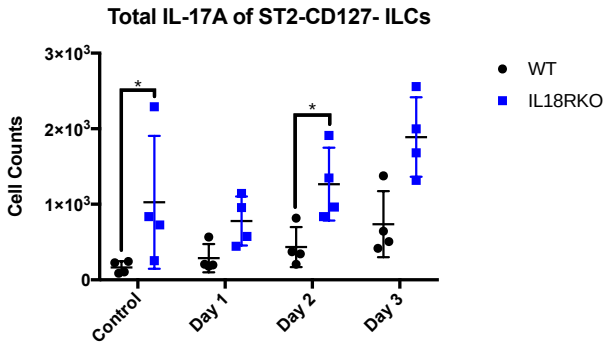


Figure 12 Continued

L

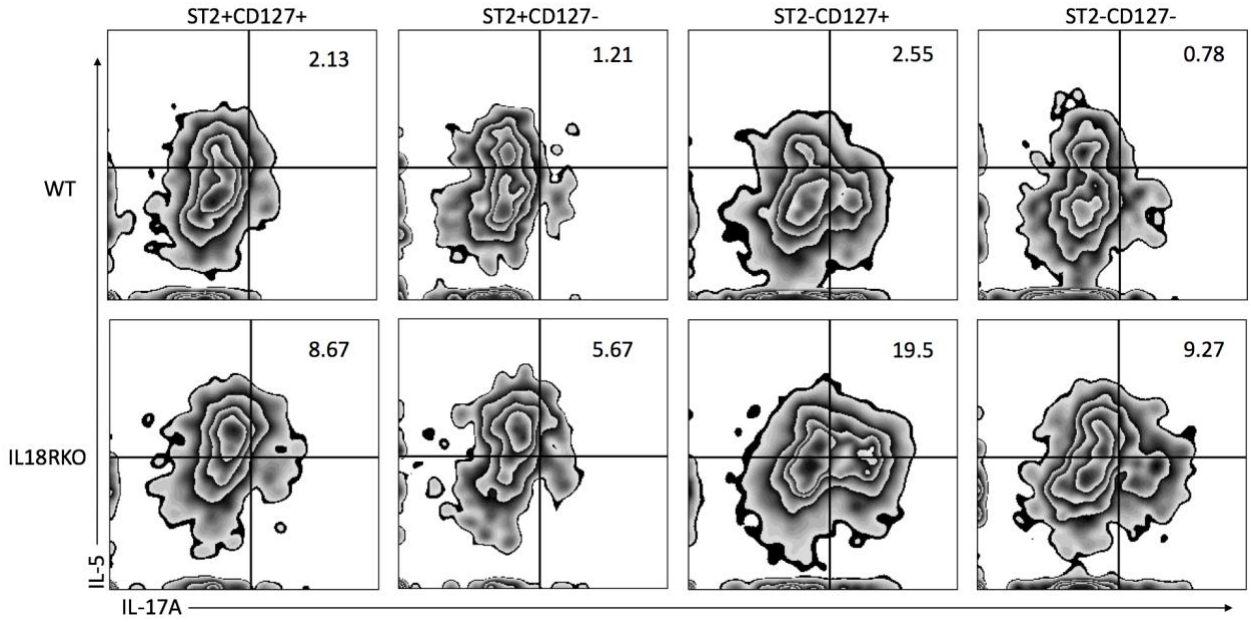
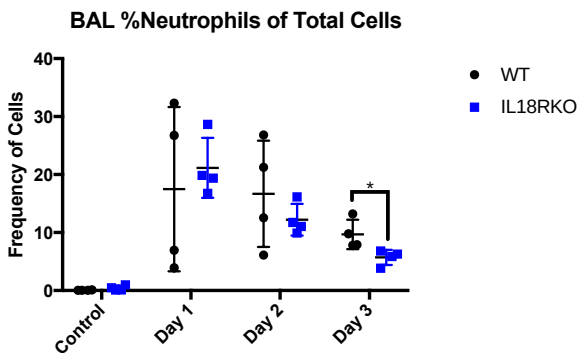
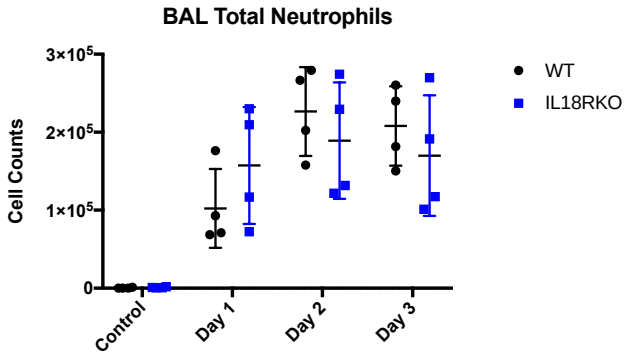


Figure S1: Neutrophilia in IL-18R$^{-/-}$ mice. Mice were challenged as shown in Figure 4. The frequency of neutrophils (A) and the total neutrophils (B) in BAL from mice. The frequency of neutrophils (D) and the total neutrophils (E) in lungs from mice. Representative flow cytometry plots of the neutrophil gating for BAL (C) and lungs (F) are shown. Data shown are representative of two independent experiments with two mice per group. *$p < 0.05$, unpaired *t* test.

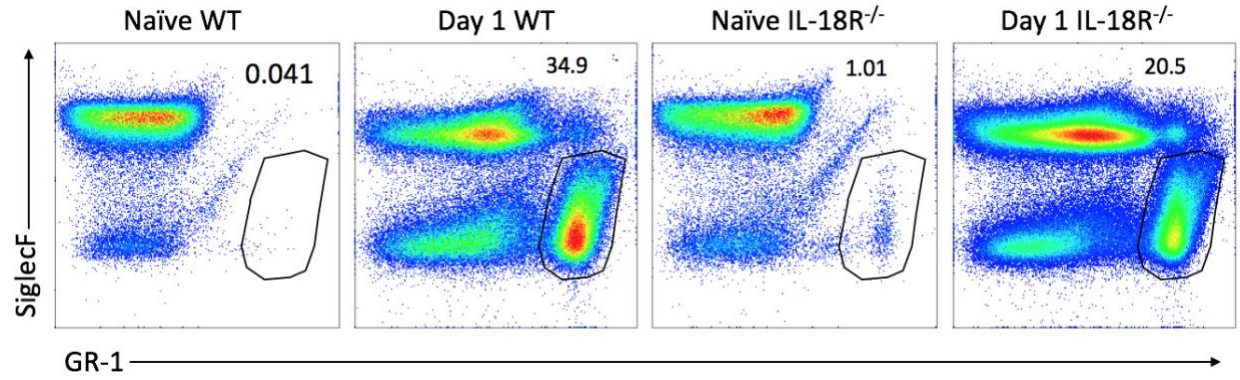
A


BAL %Neutrophils of Total Cells
Frequency of Cells
40
30
20
10
0
Control
Day 1
Day 2
Day 3
*
WT
IL18RKO

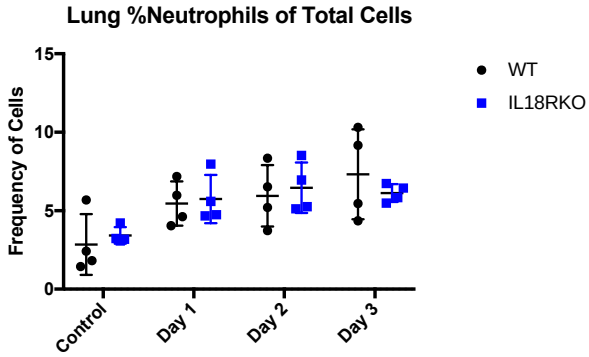
B


BAL Total Neutrophils
Cell Counts
3×10^5
2×10^5
1×10^5
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

C


Naïve WT
Day 1 WT
Naïve IL-18R-/-
Day 1 IL-18R-/-
0.041
34.9
1.01
20.5
SiglecF
GR-1

D


Lung %Neutrophils of Total Cells
Frequency of Cells
15
10
5
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

E

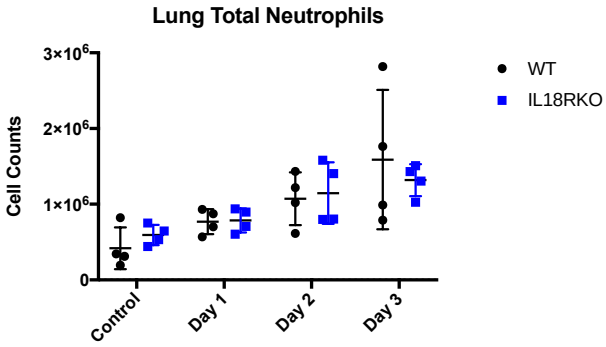

Lung Total Neutrophils
Cell Counts
3×10^6
2×10^6
1×10^6
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

Figure S1 Continued

F

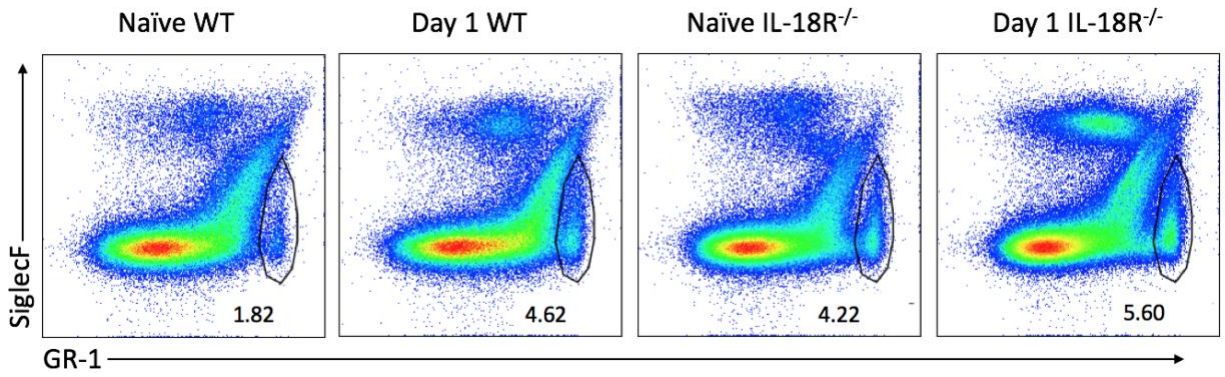
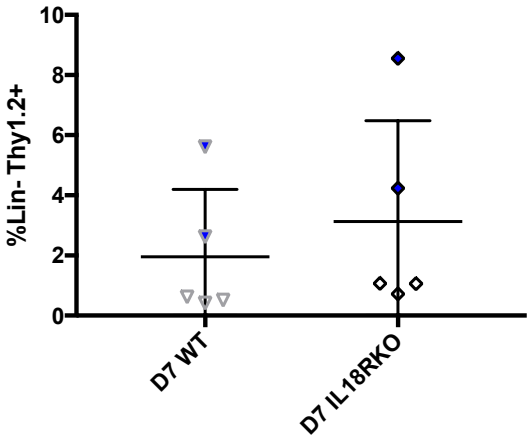
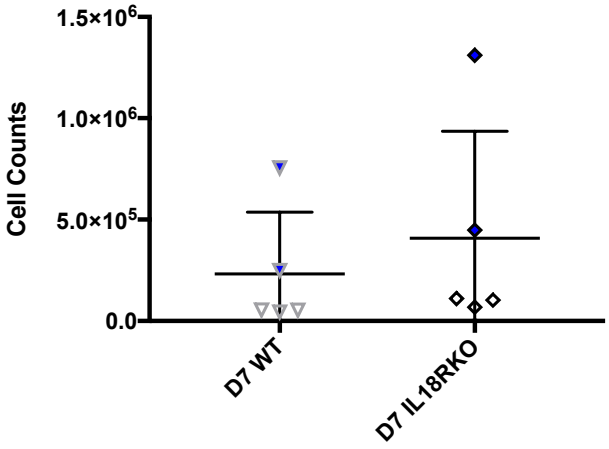


Figure S2: ILC frequencies and numbers after 4 days of rest. Mice were challenged as shown in Figure 4. The frequency (A) and total number (B) of all innate lymphoid cells in the lungs. Further staining of ILCs with ST2 and CD127 yielded 4 distinct populations of ILCs. The ST2+CD127+ ILC frequency (C) and total number (D), the ST2+CD127- ILC frequency (E) and total number (F), the ST2-CD127+ ILC frequency (G) and total number (H), and the ST2-CD127- ILC frequency (I) and total number (J). Data shown are representative of two independent experiments with two mice per group. $*p < 0.05$, unpaired *t* test.

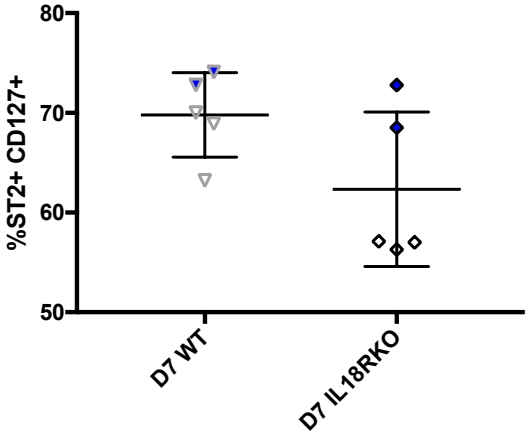
A


%Lin- Thy1.2+ of Lymphocytes
%Lin- Thy1.2+
10
8
6
4
2
0
D7 WT
D7 IL18RKO

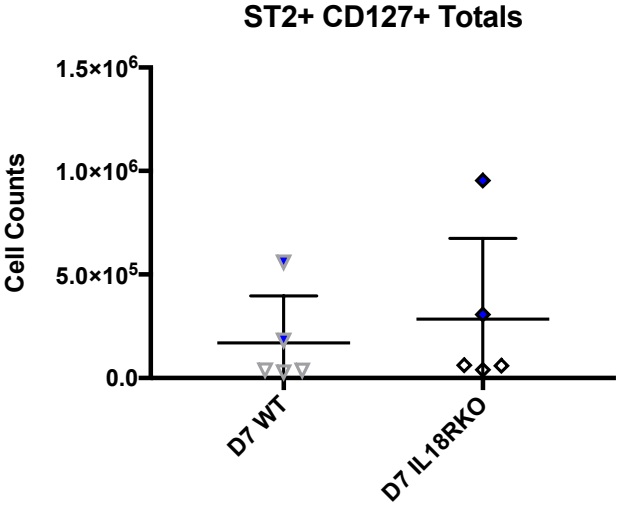
B


Lin- Thy1.2+ Totals
Cell Counts
1.5×10^6
1.0×10^6
5.0×10^5
0.0
D7 WT
D7 IL18RKO

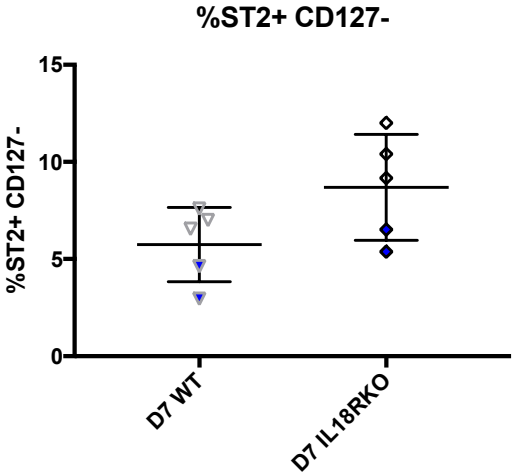
C


%ST2+ CD127+
%ST2+ CD127+
80
70
60
50
D7 WT
D7 IL18RKO

D


ST2+ CD127+ Totals
Cell Counts
1.5×10^6
1.0×10^6
5.0×10^5
0.0
D7 WT
D7 IL18RKO

E


%ST2+ CD127-
%ST2+ CD127-
15
10
5
0
D7 WT
D7 IL18RKO

F

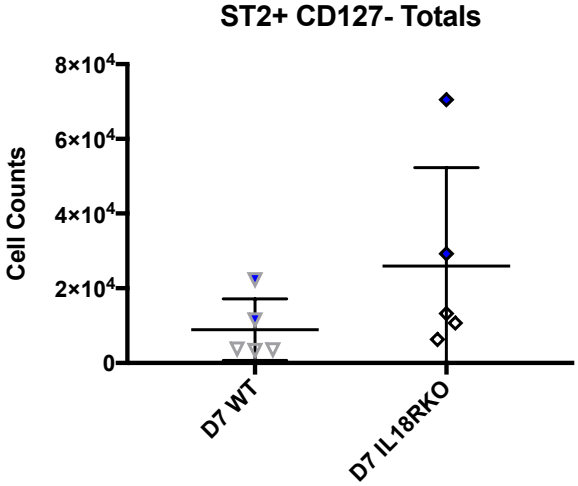
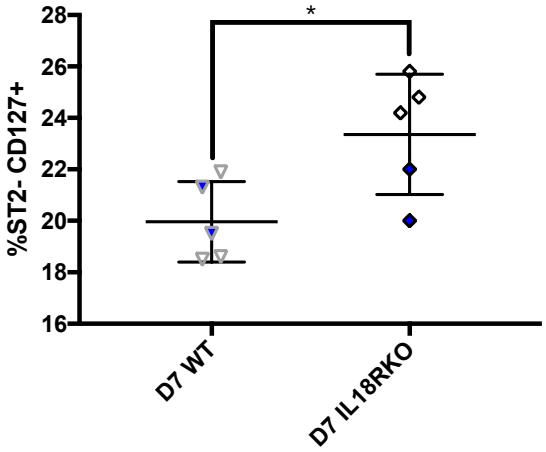
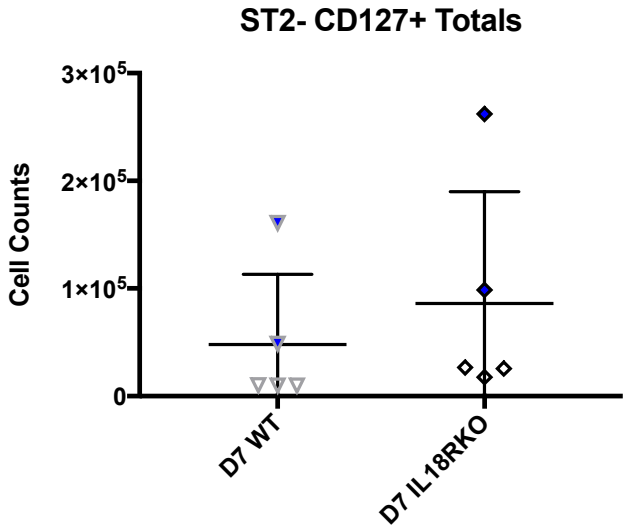

ST2+ CD127- Totals
Cell Counts
8×10^4
6×10^4
4×10^4
2×10^4
0
D7 WT
D7 IL18RKO

Figure S2 Continued

G



H



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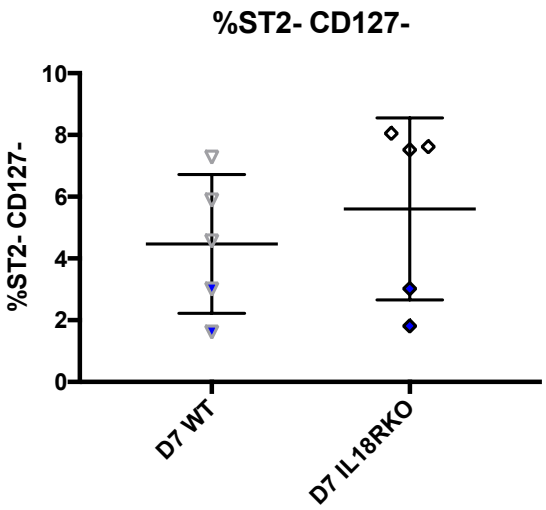


J

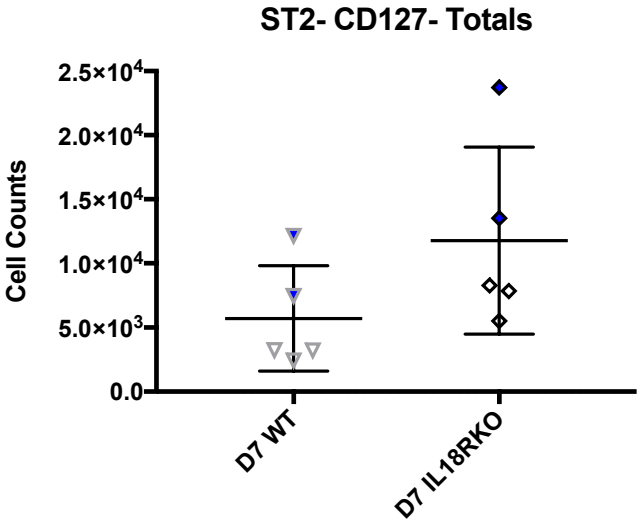
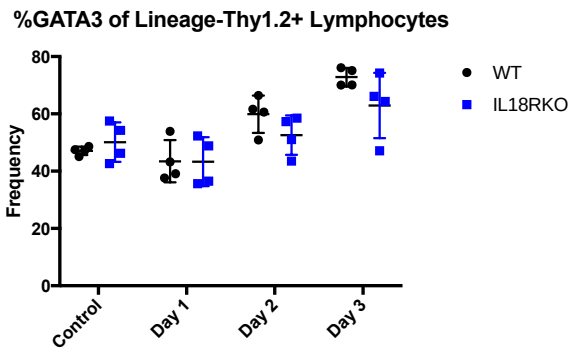
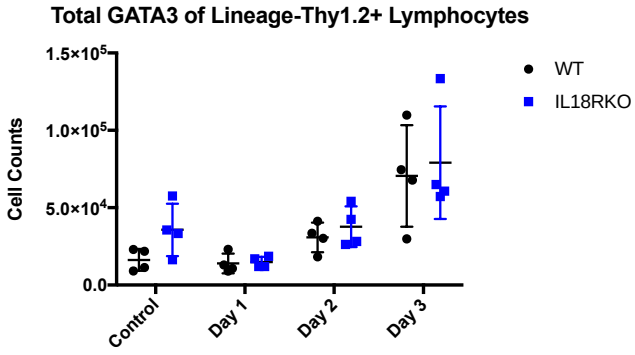


Figure S3: ILC GATA3 Expression. Mice were challenged as shown in Figure 4. The frequency of GATA3 expression (A) and the total number of GATA3 expressing cells (B) in the innate lymphoid cell population. GATA3 expression was also extended to the ST2 and CD127 ILC subpopulations (C-J).

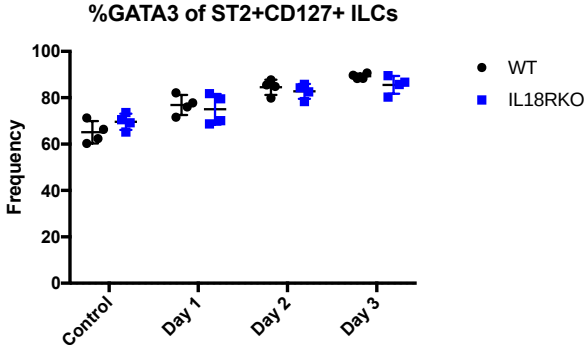
A


%GATA3 of Lineage-Thy1.2+ Lymphocytes
Frequency
80
60
40
20
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

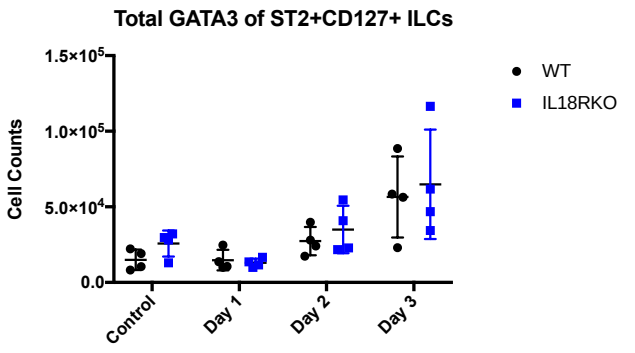
B


Total GATA3 of Lineage-Thy1.2+ Lymphocytes
Cell Counts
1.5×10^5
1.0×10^5
5.0×10^4
0.0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

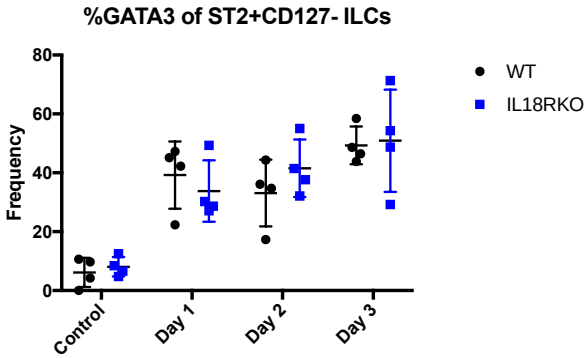
C


%GATA3 of ST2+CD127+ ILCs
Frequency
100
80
60
40
20
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

D


Total GATA3 of ST2+CD127+ ILCs
Cell Counts
1.5×10^5
1.0×10^5
5.0×10^4
0.0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

E


%GATA3 of ST2+CD127- ILCs
Frequency
80
60
40
20
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

F

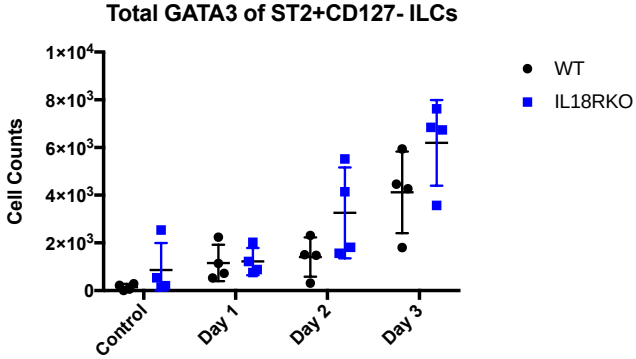

Total GATA3 of ST2+CD127- ILCs
Cell Counts
1×10^4
8×10^3
6×10^3
4×10^3
2×10^3
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

Figure S3 Continued

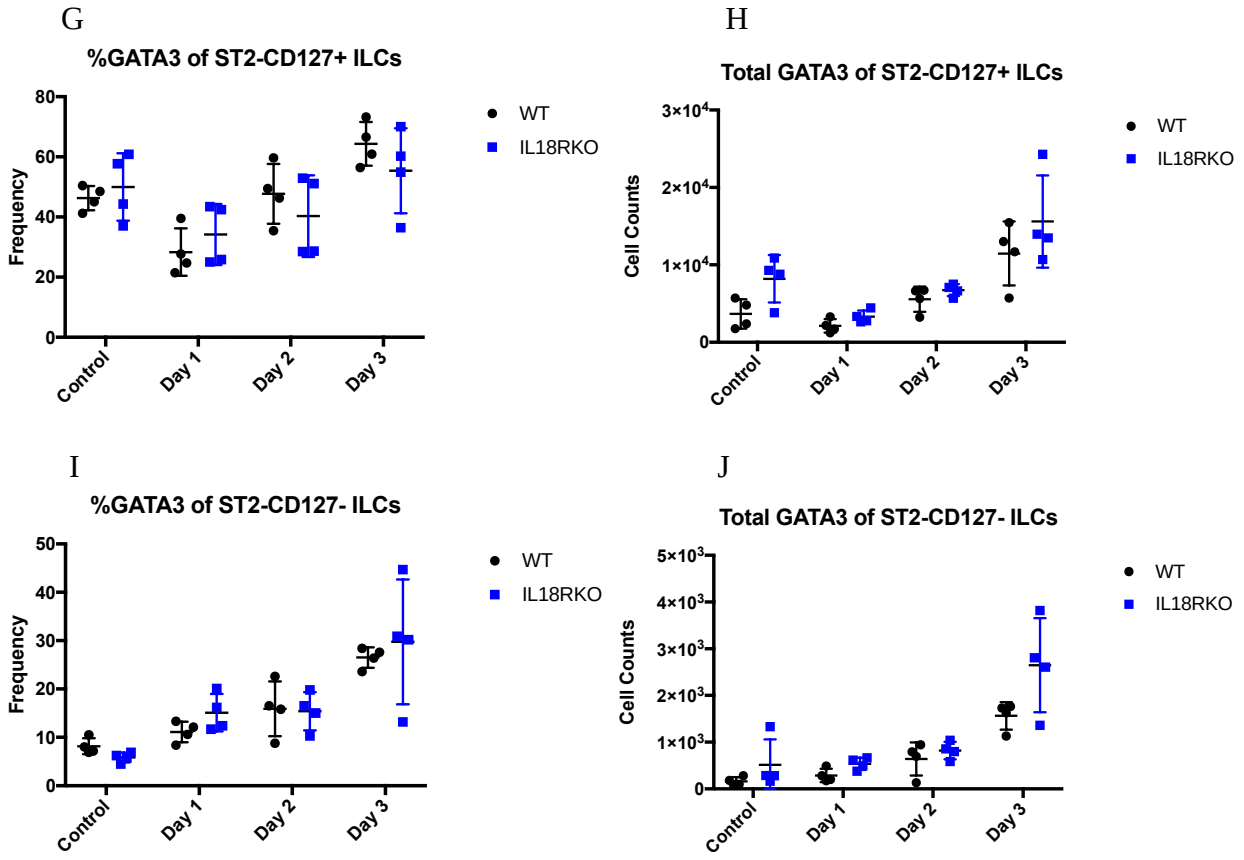
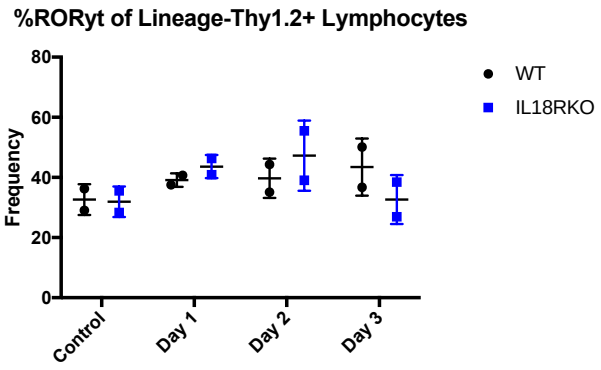
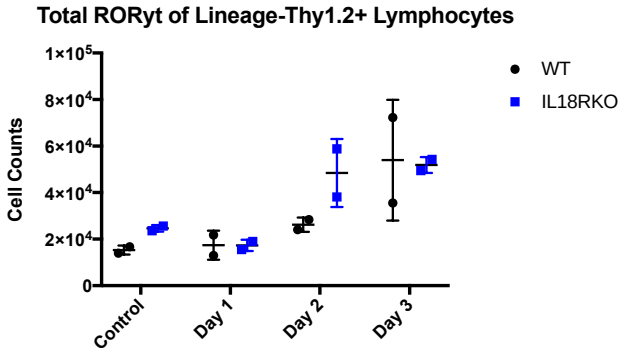


Figure S4: ILC RORγ Expression. Mice were challenged as shown in Figure 4. The frequency of RORγ expression (A) and the total number of RORγ expressing cells (B) in the innate lymphoid cell population. RORγ expression was also extended to the ST2 and CD127 ILC subpopulations (C-J).

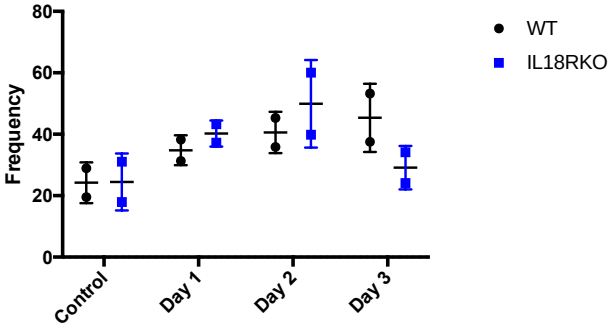
A


%RORyt of Lineage-Thy1.2+ Lymphocytes
Frequency
80
60
40
20
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

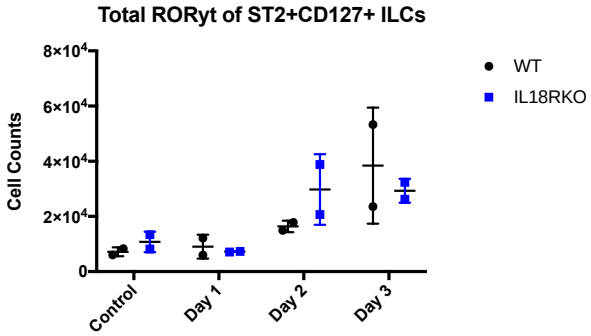
B


Total RORyt of Lineage-Thy1.2+ Lymphocytes
Cell Counts
1×10^5
8×10^4
6×10^4
4×10^4
2×10^4
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

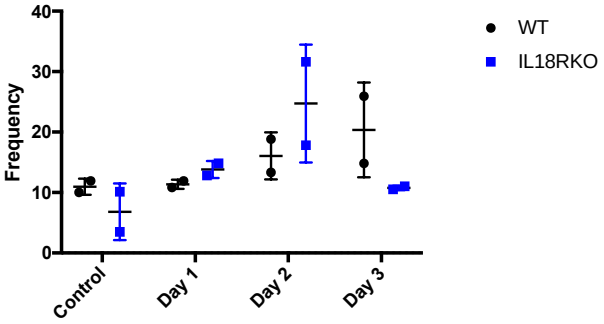
C


%RORyt of ST2+CD127+ ILCs
Frequency
80
60
40
20
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

D


Total RORyt of ST2+CD127+ ILCs
Cell Counts
8×10^4
6×10^4
4×10^4
2×10^4
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

E


%RORyt of ST2+CD127- ILCs
Frequency
40
30
20
10
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

F

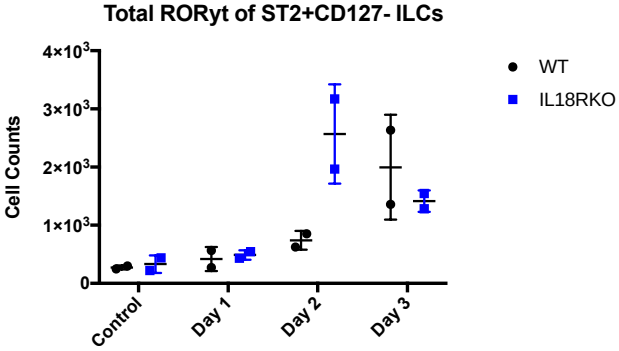
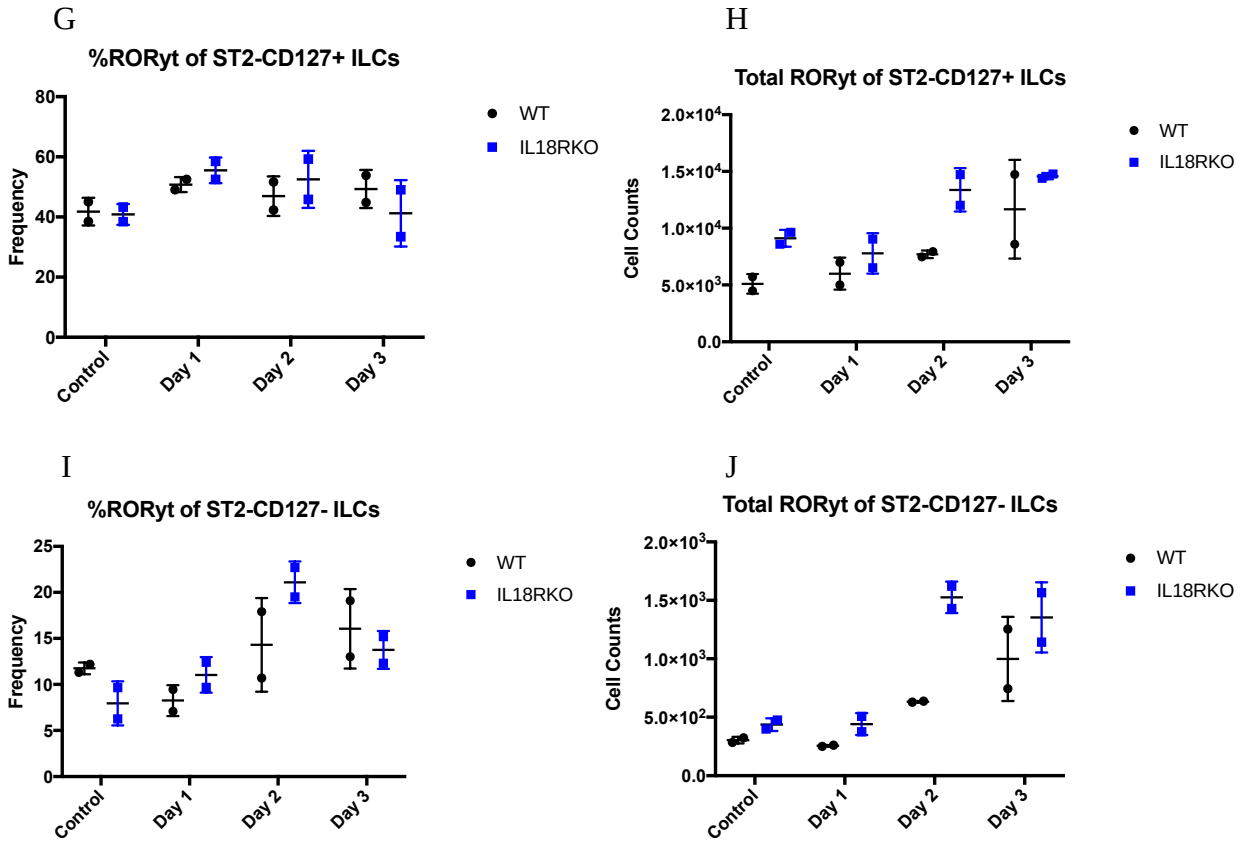

Total RORyt of ST2+CD127- ILCs
Cell Counts
4×10^3
3×10^3
2×10^3
1×10^3
0
Control
Day 1
Day 2
Day 3
WT
IL18RKO

Figure S4 Continued



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