

IL-33 signaling uncouples pulmonary regulatory T-cell specialization from suppressive function during type 2 inflammation

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All mice were housed and used according to the regulations of the Canadian Council of Animal Care Guidelines and Animal Care and Use Committees at McGill University.

Title: IL-33 signaling uncouples pulmonary regulatory T-cell specialization from suppressive function during type 2 inflammation

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Abstract: Regulatory T (T_{REG}) cells are critical mediators of mucosal homeostasis, yet how they fail to restrain exaggerated type 2 immune responses remains poorly understood. This question is exemplified by neonatal respiratory syncytial virus (RSV) infection, which is strongly associated with asthma susceptibility later in life despite the persistent presence of pulmonary T_{REG} cells. Here, using complementary murine neonatal RSV infection and allergic airway inflammation models, we identify an IL-33–IL-4R α signaling axis that reprograms pulmonary T_{REG}-cell function. Mechanistically, IL-33 increased IL-4R α expression, enhancing glycolysis-dependent STAT6 signaling and promoting GATA3-dependent functional specialization of pulmonary T_{REG} cells. Functionally, IL-33 promoted the expansion and persistence of long-lived pulmonary T_H2 cells while simultaneously licensing IL-4-dependent loss of T_{REG}-cell suppressive function. These findings identify IL-33 as a developmental checkpoint that coordinately programs pathogenic T_H2 memory and dysfunctional pulmonary T_{REG} cell responses following exaggerated pathogenic type 2 responses.

Summary: The mechanisms by which IL-33 promotes asthma remain unclear. Here, we show how IL-33 simultaneously drives pathogenic T_H2 memory and licenses IL-4-dependent loss of pulmonary T_{REG}-cell suppressive function, uncoupling tissue adaptation from immune regulation.

INTRODUCTION

How inflammatory signals shape the long-term balance between immune regulation and pathogenic type 2 immunity remains a fundamental question in respiratory immunology. One of the clearest clinical examples is the well-established association between severe respiratory viral infections during infancy and an increased risk of asthma(1, 2). Primary infant RSV infection promotes persistent type 2-skewed memory responses, and severe disease is associated with an increased risk of recurrent wheezing later in life(2), underscoring the need to better understand these mechanisms for targeted intervention. This susceptibility reflects the unique characteristics of the neonatal immune system, which is developmentally immature and physiologically biased toward type 2 immunity(3, 4). Compared with adults, neonatal DCs, macrophages and ILCs are biased toward type 2 cytokine production, exhibiting reduced IL-12 and IFN γ alongside increased IL-4, IL-5 and IL-13(5). This cytokine milieu promotes type 2 helper T (T_H2) cell responses(4). How this neonatal environment is translated into durable alterations in tissue-adaptive immune regulation remains poorly understood.

FoxP3-expressing CD4⁺ regulatory T (T_{REG}) cells are central to maintaining tissue homeostasis in the lung(6), playing an essential role in restraining pulmonary immunity during RSV infection(7, 8). In contrast, they fail to adequately control exaggerated pulmonary inflammation in mice infected during the neonatal period(9), suggesting that early-life infection imprints long-lasting changes that alter T_{REG} cell suppressive function. Indeed, T_{REG} cells seed non-lymphoid tissues such as the lung within the first days of life in mice, where they maintain a largely stable transcriptional program and TCR repertoire into adulthood(10, 11), raising the possibility that neonatal inflammatory events, including RSV infection, shape the establishment and functional specialization of tissue-resident T_{REG} cells. Accordingly, deciphering how

pulmonary T_{REG} cells are programmed during neonatal infection is critical to understanding dysregulated recall immunity in the lung.

Pulmonary T_{REG} cells acquire a tissue-adapted phenotype characterized by expression of tissue-adaptive receptors and transcriptional regulators that support their local maintenance and function, including ST2, the IL-33 receptor subunit, as well as transcription factors such as GATA3, BATF, and IRF4(12-15). In adult mice, ST2⁺ T_{REG} cells restrain IL-17A-producing $\gamma\delta$ T cells(16) and promote tissue repair through amphiregulin production during viral infection(13). Paradoxically, the same IL-33 signaling pathway that supports pulmonary T_{REG} cell maintenance and tissue repair is also associated with asthma severity (17, 18) and type 2 immunopathology (19) by shifting the balance away from T_{REG}-mediated regulation toward T_H2 responses in the lung(20). The mechanisms by which IL-33 promotes pathogenic type 2 immunity instead of supporting T_{REG}-mediated tissue adaptation and homeostasis remain poorly understood. We therefore hypothesized that neonatal RSV infection durably reprograms IL-33-dependent pulmonary T_{REG}-cell responses, promoting pathogenic type 2 immunity at the expense of tissue-adaptive immune regulation.

Although IL-33 is a critical regulator of both ST2⁺ T_{REG} cells and type 2 effector responses, whether these pathways are differentially programmed during neonatal viral infection is unknown. Using complementary neonatal RSV infection and allergen-driven airway inflammation models, we investigated how IL-33-dependent signals shape the development, specialization and function of pulmonary ST2⁺ T_{REG} cells. We found that neonatal RSV infection establishes an a pulmonary T_{REG} cell program that becomes highly responsive to antigen-independent IL-33 stimulation. Using inducible genetic models during allergen challenge, we further uncoupled the respective roles of GATA3 and IL-33, demonstrating that GATA3 preserves tissue-adapted T_{REG} cell identity,

whereas IL-33 is required for their suppressive function. Finally, we identify an IL-33–IL-4R α signaling axis that metabolically reprograms pulmonary T_{REG} cells and impairs their suppressive capacity within type 2 inflammatory environments. Together, these findings establish a context-dependent mechanism by which IL-33 promotes tissue adaptation while simultaneously compromising immune regulation, providing a framework for understanding how early-life inflammation durably shapes susceptibility to type 2 inflammatory disease.

RESULTS

1. Neonatal RSV infection promotes expansion of ST2⁺ GATA3⁺ T_{REG} cells despite pathogenic type 2 recall responses.

Neonatal RSV infection predisposes mice to develop exaggerated type 2 immune responses upon reinfection in adulthood, providing a useful model to investigate how early-life respiratory infections shape long-term pulmonary immunity. To determine whether neonatal RSV infection durably alters pulmonary T_{REG} cells, we analyzed their phenotype and function upon reinfection in adulthood. Neonate BALB/c pups (5-7 days old) were infected intranasally with RSV A2 and reinfected (secondary) alongside mice receiving their first infection (primary) (**Fig. 1A**). To characterize the memory T cell responses induced by neonatal infection, lungs were collected 4 days after (re)infection. As reported(21), secondary infection induced rapid weight loss (**Fig. 1B**), accompanied by increased pulmonary infiltration of CD45⁺ leukocytes and CD4⁺ T cells compared with primary infection (**Fig. 1C**). Unbiased dimensionality reduction (UMAP) and clustering (FlowSOM) revealed increased abundance of eosinophils (cluster 1) and neutrophils (cluster 4) (**Fig. 1D-F; Fig. S1**). A significant increase in ILC2 abundance was observed (**Fig. S2A**), further supporting the development of a type 2-biased inflammatory response.

CD8⁺ T cells were more abundant in the lungs of secondary infected mice, although the CD4⁺:CD8⁺ T cell ratio remained skewed toward CD4⁺ T cells (**Fig. S2B**). While the frequency of IFN γ ⁺ CD8⁺ T cells remained unchanged (**Fig. S2C**), secondary infection selectively amplified pulmonary cytokine-producing CD4⁺ T cells, with IL-4⁺ and IL-13⁺ populations predominating over IFN γ ⁺ cells(**Fig. 1G**), revealing a T_H2-skewed recall response following RSV reinfection(7). The number of FoxP3⁺ T_{REG} cells was higher following secondary infection, although their proportion amongst CD4⁺ T cells and expression of the proliferation marker Ki67 remained similar between groups (**Fig. 1H-I**). Secondary infection increased the number of pulmonary ST2⁺ T_{REG} cells (**Fig. 1K**), which positively correlated with T_H2 cells, eosinophils, and B cells, but not T_H1 cells, CD8⁺ T cells, or neutrophils (**Fig. 1L**). These ST2⁺ T_{REG} cells expressed higher levels of GATA3 (gMFI; **Fig. 1M**), and a small proportion produced IL-13 rather than IFN γ (**Fig. 1N**), identifying a type 2-associated regulatory population that expands alongside exaggerated type 2 inflammation.

2. Neonatal RSV infection promotes the establishment of pulmonary ST2⁺ T_{REG} cells.

To determine how neonatal RSV infection influences the establishment of tissue-localized ST2⁺ T_{REG} cells and IL-4-producing T_H2 cells, neonatal pups were infected with RSV A2 (**Fig. 2A**). CD45⁺ leukocytes, including ILC2s and alveolar macrophages (AMs), accumulated in the lungs 4 days post-infection (**Fig. 2B; Fig. S3A-C**). AMs from infected pups displayed higher MHC-II (gMFI; **Fig. S3D**) and increased expression level of Arginase 1 (**Fig. S3E**), indicating M2 differentiation. In these pups, the number of antigen-experienced (CD44^{high}CD62^{low}) CD4⁺, but not CD8⁺ T cells, increased (**Fig. 2C-D**), and the frequency of IL-4⁺, but not IFN γ ⁺, CD4⁺ T cells were also higher (**Fig. 2E**). T_{REG} cells accumulated in the lungs (**Fig. 2F**), although CD44^{hi} T_{EFF}

expanded to a greater extent (**Fig. 2G**). Specifically, although the frequency of IL-4⁺ CD4⁺ T cells increased, ST2⁺ T_{REG} cells did not (**Fig. 2H-I**), suggesting a delay in the accumulation of these cells compared to T_H2 cells. To assess whether this observation reflected a reduced capacity of neonatal T_{REG} cells to differentiate into ST2⁺ T_{REG} cells, splenic CD4⁺CD25^{hi} T_{REG} cells from neonatal pups were activated in the presence or absence of IL-33 and their phenotype compared with those from 8-week-old adults. No difference in the generation of ST2⁺ T_{REG} cells was observed between the two groups (**Fig S4A-C**), demonstrating that neonatal T_{REG} cells retain the intrinsic capacity to acquire an ST2⁺ phenotype in response to IL-33. Interestingly, neonatal T_{REG} cells exhibited significantly lower expression of GATA3 than their adult counterparts (**Fig. S4D-E**). Given neonatal T cells are known to produce less IL-2 than adult T cells (22), limited IL-2-STAT5 signaling may represent a developmental constraint on GATA3 expression in neonatal T_{REG} cells, as observed during T_H2 differentiation(23).

ST2⁺ T_{REG} cell accumulation was therefore assessed at a later stage after infection (Day 14; weaning(24)) (**Fig. 2J**). At this time-point, the proportion of ST2⁺ cells among pulmonary FoxP3⁺ T_{REG} cells remained unchanged (**Fig. 2K**), but infection resulted in a marked increase in the number of ST2⁺ T_{REG} cells (**Fig. 2L**). This delayed accumulation of ST2⁺ T_{REG} cells was accompanied with T_H2 biased, RSV-specific recall responses as *ex vivo* RSV stimulation selectively induced IL-4⁺ but not IFN γ ⁺ T cells (**Fig. 2M**). We next examined the expression of IRF4, a transcription factor associated with effector T_{REG} cell differentiation and persistence(25-27). In naïve adult mice, IRF4 expression was highest in pulmonary ST2⁺ Helios⁺ T_{REG} cells compared with Helios⁺ T_{REG} and Helios⁻ T_{REG} (**Fig. 2N-O**). ST2⁺ T_{REG} cells displayed increased IRF4 expression compared with ST2⁺ T_{REG} cells from uninfected lungs (**Fig. 2P**), indicating acquisition of an activated effector state(25-28). Thus, neonatal RSV infection establishes a

temporally delayed but persistent ST2⁺ effector-like T_{REG} cell program that develops alongside RSV-induced T_H2 immunity.

3. IL-33 preferentially imprints pathogenic T_H2 memory over durable regulatory programs.

To determine whether IL-33 availability during neonatal RSV infection shapes the long-term balance between regulatory and pathogenic type 2 immunity, we examined the consequences of experimentally altering IL-33 signaling during primary infection. Since pulmonary IL-33 peaks within 12 hours post-RSV infection(29) and is minimal in neonatal lungs at steady state(30), we first tested whether IL-33 administration impacts the generation pulmonary ST2⁺ T_{REG} cells in neonates. Recombinant murine IL-33 was provided intra-nasally at the time of neonatal RSV A2 infection and the lungs were analyzed four days later (**Fig. 3A**). Recombinant IL-33 resulted in an increase in pulmonary T_{REG}, including ST2⁺ T_{REG} cells (**Fig. 3B**). Concomitantly, IL-33 markedly increased the number of IL-4⁺ and IL-13⁺ T_H2 cells, as well as IL-17A⁺ CD4⁺ T cells, in the lungs of infected neonates (**Fig. 3C**), indicating that IL-33 amplifies both inflammatory T cell and T_{REG} responses during neonatal infection.

To examine whether increased pulmonary IL-33 exposure during neonatal infection alters long-term immune memory, neonatal mice received recombinant IL-33 I.N. or I.P. during primary RSV A2 and were subsequently reinfected as adults (**Fig. 3D**). Elevated neonatal IL-33 exposure did not increase the abundance of pulmonary ST2⁺ T_{REG} cells during adult reinfection (**Fig. 3E**). Instead, IL-33-exposed mice developed significantly greater numbers of IL-13⁺ CD4⁺ T cells following reinfection (**Fig. 3F**), resulting in a pronounced shift toward T_H2- over T_H1-dominant immunity (**Fig. 3G**). Consistent with this, intranasal, but not intra-peritoneal, IL-33 administration increased eosinophil accumulation (**Fig. 3H**) without affecting neutrophil recruitment (**Fig. 3I**).

Importantly, the ratio of ST2⁺ T_{REG} cells to IL-13⁺ T_H2 cells was significantly reduced in these mice (**Fig. 3J**), further demonstrating that pulmonary neonatal IL-33 exposure preferentially promotes the establishment of long-lived pathogenic T_H2 responses over durable regulatory responses.

To further define the contribution of endogenous IL-33 during the initiation of neonatal immune responses, we next assessed the consequences of IL-33 neutralization. To specifically target the initiation phase of the response, neonatal mice received a single dose of neutralizing anti-IL-33 monoclonal antibody or isotype control at the time of RSV infection (**Fig. 3K**). Acute IL-33 neutralization did not alter the total number of pulmonary T_{REG} cells but further reduced the already limited population of ST2⁺ T_{REG} cells detected four days after infection (**Fig. 3L**). While sustained blockade of IL-33 throughout neonatal RSV infection was shown to prevent the establishment of pathogenic adult T_H2 responses(29), the numbers of IFN γ -, IL-4-, and IL-13-producing pulmonary T cells were unaffected by this treatment (**Fig. 3M**), indicating that early IL-33 availability contributes to ST2⁺ T_{REG} cell establishment but is insufficient to determine the magnitude of neonatal type 2 priming.

Finally, we examined whether IL-33 was sufficient to promote this phenotype in the context of neonatal RSV imprinting. Adult RSV^{NEO} and RSV⁻ mice received intranasal recombinant IL-33 (0.5 μ g/mouse) and pulmonary T cell responses were assessed (**Fig. 3N**). IL-33 selectively amplified IL-4⁺ and IL-13⁺ CD4⁺ T cells in RSV^{Neo} mice, while increasing the frequency, number and proliferation of ST2⁺ T_{REG} cells (**Fig. 3O-P**). Thus, IL-33 promotes antigen-independent expansion of pulmonary T_H2 cells and type 2 adapted ST2⁺ GATA3⁺ T_{REG} cells during neonatal RSV infection. Together, these findings demonstrate that sustained IL-33 signaling during

neonatal RSV infection reinforces both durable pathogenic type 2 responses and type-2 adapted ST2⁺GATA3⁺ T_{REG} cells in an antigen-independent manner.

4. GATA3 preserves the tissue-adapted identity of pulmonary T_{REG} cells during type 2 inflammation.

To investigate the effect of GATA3 on pulmonary T_{REG} cells during this rechallenge period, we next exploited the well described HDM (*D. pteronyssinus* extract) murine model of allergic airway inflammation. Here, the impact of GATA3 on the innate and adaptive immune responses upon antigenic rechallenge remains ill-defined. Thus, we first dissected the effect of GATA3 on the generation of exaggerated type 2 immunity upon antigen recall using tamoxifen-inducible global deletion of GATA3 (Rosa26^{ERT2-CRE} Gata3^{loxP}; ΔGATA3) (**Fig. S5A**). We observed reduced pulmonary eosinophilia and IL-13⁺ T cells while IL-17A⁺ T cells increased (**Fig. S5B-H**), consistent with its established role in maintaining type 2 immunity. Importantly, GATA3 deficiency reduced pulmonary FoxP3⁺ T_{REG} cell abundance (**Fig. S5I**), suggesting a role in sustaining tissue-adapted T_{REG} cell fitness.

To better define the role of GATA3 in T_{REG} cells, we generated tamoxifen-inducible FoxP3-specific GATA3-deficient mice (Foxp3-^{ERT2-GFP-CRE} GATA3^{loxP}; T_{REG}^{ΔGATA3})(**Fig. 4A**). Loss of GATA3 expression in T_{REG} cells significantly reduced both the frequency of ST2⁺ T_{REG} cells and the level of ST2 expression (gMFI; **Fig. 4B**), indicating that GATA3 promotes ST2 expression in pulmonary T_{REG} cells, similar to its role in conventional T_H2 cells(31). Contrary to the pan-GATA3 deletion, T_{REG}-specific GATA3 deletion did alter pulmonary T_{REG} cells abundance(**Fig. 4C**), indicating that the reduced T_{REG}-cell numbers observed following global GATA3 deletion are driven, at least in part, by GATA3-dependent extrinsic signals from other

immune or tissue-resident cells. Rather, $T_{REG}^{\Delta GATA3}$ cells displayed reduced FoxP3 expression (gMFI; **Fig. 4D**) and IL-10 production (**Fig. 4E-F**) accompanied by increased ROR γ T expression (**Fig. 4G**) and IL-17A production (**Fig. 4H-I-J**). However, in this experimental window, little to no changes on the innate response was observed (**Fig. 4K-M**), suggesting little functional impact. Thus, GATA3 is dispensable for the short-term maintenance and function of T_{REG} cells during allergen rechallenge but is required for preserving their type 2-associated tissue identity during inflammation.

5. IL-33 and GATA3 differentially regulate functional specialization and lineage stability of pulmonary T_{REG} cells.

To define the distinct contributions of IL-33 signaling in pulmonary T_{REG} cell adaptation, we generated mice with a FoxP3-specific deletion of ST2 (B6.FoxP3^{ERT2-CRE} Il1rl1^{loxP}; $T_{REG}^{\Delta ST2}$) and challenged them with HDM (**Fig. 5A**). Loss of ST2 expression resulted in increased frequencies and numbers of eosinophils (**Fig. 5B**) and abundant mucus deposition (**Fig. 5C**), together with enhanced recruitment of neutrophils (**Fig. 5D**) and IL-4⁺, IL-13⁺, IFN γ ⁺ CD4⁺ T cells (**Fig. 5E**). ST2 deficiency also impaired the ability to control IL-17A⁺ $\gamma\delta$ T cell responses (**Fig. 5F**), confirming a broad requirement for IL-33 responsiveness in limiting pulmonary inflammation(16). Despite these functional defects, the number and frequency of pulmonary T_{REG} cells among CD4⁺ T cells remained unchanged (**Fig. 5H**). Instead, $T_{REG}^{\Delta ST2}$ mice displayed reduced IRF4 expression while maintaining FoxP3 expression (**Fig. 5I**), accompanied by impaired IL-10 production (**Fig. 5J-K**), indicating that IL-33 signaling promotes acquisition of an effector phenotype.

Finally, the role of IL-33 and GATA3 in maintaining T_{REG} identity was assessed using a fate-tracking system (RFP⁺, **Fig. 5L**). Whereas GATA3-deficient T_{REG} cells readily lost FoxP3 expression, ST2-deficient T_{REG} cells maintained lineage stability (**Fig. 5L-M**). This effect was restricted to the lung, as FoxP3 expression and exT_{REG} cell generation remained unchanged in the draining mediastinal lymph nodes. Together, these findings indicate that IL-33-dependent functional specialization occurs independently of T_{REG} cell lineage stability, raising the question of how this pathway alters T_{REG} cell behavior within a type 2 inflammatory environment.

6. IL-33 licenses pulmonary T_{REG} cells for IL-4-dependent metabolic and functional reprogramming.

To identify mechanisms underlying the emergence of type 2-associated T_{REG} cells, we analyzed publicly available single-cell RNA-sequencing data from bronchoalveolar lavage fluid (BALF) of asthmatic and non-asthmatic donors before and after allergen challenge(32). We identified nine clusters (**Fig. S6A**), largely recapitulating the original study(32), including T_{REG} and T_{H2} populations that expanded following allergen exposure in asthmatic individuals (**Fig. S6B**). T_{REG} cells from asthmatic donors expressed canonical T_{REG} cell markers, including FOXP3 and IKZF2, while also displaying increased expression of both GATA3 and IL4RA compared with non-asthmatic controls (**Fig. S6C-D**), suggesting enhanced sensitivity to IL-4 signaling during type 2 inflammation.

We next examined whether IL-4 signaling contributes to the development of ST2⁺ T_{REG} cells. Activation of WT or STAT6-deficient T_{REG} cells under ST2-inducing conditions(12, 15) revealed that STAT6 was dispensable for ST2 induction but required for optimal GATA3 expression (**Fig. 6A-B**). In WT T_{REG} cells, IL-33 promoted ST2 expression, whereas the

combination of IL-4 and IL-33 further enhanced the frequency of ST2⁺ T_{REG} cells and the level of ST2 expression (gMFI; **Fig. 6C**) and selectively enhanced GATA3 expression (**Fig. 6C**). Both cytokines increased the expression of BATF and IRF4 (**Fig. 6D**), indicating cooperation between IL-33 and IL-4 in establishing a tissue-adapted type 2 phenotype(25, 33-35).

We next investigated whether IL-33 influences IL-4 responsiveness. IL-33 increased the expression of IL-4R α on T_{REG} cells (**Fig. 6E**), enhancing their IL-4 responsiveness and promoting their expansion following IL-4-IL-33 stimulation (**Fig. 6F**). This cooperative signaling increased glucose uptake (**Fig. 6G**), suggesting metabolic adaptation accompanies type 2 T_{REG} cell programming (fluorescent glucose analog 2-NBDG uptake; **Fig. 6G**). Accordingly, pharmacological inhibition of glycolysis (2DG), but not fatty-acid oxidation (etomixir), impaired the generation of GATA3⁺ ST2⁺ T_{REG} cells (**Fig. 6H-I**). Collectively, these findings identify an IL-33-IL-4R α feed-forward circuit that metabolically licenses T_{REG} cells for acquisition of a type 2-associated phenotype. However, whether this IL-33-driven specialization enhances regulatory function or contributes to the inability of T_{REG} cells to restrain pathogenic T_H2 responses remained unclear.

7. IL-33 and IL-4 cooperatively destabilize T_{REG} cell suppressive function.

To investigate whether IL-4 and IL-33 influences T_{REG} cell suppressive function, Foxp3⁺ T_{REG} cells were activated in the presence of IL-2, IL-33, and/or IL-4. IL-33 stimulation induced IL-13 production, which was further enhanced by the addition of IL-4 (**Fig. 7A**). Multiplex analysis revealed that combined IL-33 and IL-4 stimulation also increased the production of IL-5, IL-10, GM-CSF, and IL-6, whereas IFN γ , IL-17A, IL-1 α , IL-1 β , IL-7, and MCP-2 remained

unchanged (**Fig. 7B; Fig. S7A, B**). In contrast, IL-4 alone induced only low levels of IL-12(p70), CXCL5, and MCP-1 (**Fig. S7B**). To confirm whether these effects required IL-4 signaling, we compared WT and STAT6-deficient T_{REG} cells. Loss of STAT6 markedly reduced GATA3 expression, and impaired IL-10, IL-5, and IL-13 production, demonstrating that IL-4/STAT6 signaling is required for acquisition of this type 2-associated T_{REG} cell program (**Fig. S7C–G**).

Next, we assessed if this functional reprogramming affected the suppressive capacity of T_{REG}. While IL-33 preserved T_{REG} cell mediated suppression, combined IL-33 and IL-4 significantly reduced the ability of T_{REG} cells to suppress responder CD4⁺Foxp3(GFP)^{neg} cells (% supp(36); **Fig. 7C**). To determine whether this effect was intrinsic to T_{REG} cells, WT or STAT6-deficient T_{REG} mice were co-cultured with STAT6-deficient conventional T cells (Δ STAT6 T_{conv}). IL-33 and IL-4 impaired T_{REG}-mediated suppression (division index; D.I.) only in the presence of WT, but not STAT6-deficient T_{REG} cells (**Fig. 7D**), demonstrating that IL-4/STAT6 signaling directly reprograms T_{REG} cell suppressive capacity.

Finally, we examined whether IL-33 was sufficient to promote this phenotype in the context of neonatal RSV imprinting. Adult RSV^{NEO} and RSV⁻ mice received intranasal recombinant IL-33 (0.5 μ g/mouse) and pulmonary T cell responses were assessed (**Fig. 7E**). IL-33 selectively amplified IL-4⁺ and IL-13⁺ CD4⁺ T cells in RSV^{Neo} mice, while increasing the frequency, number and proliferation of ST2⁺ T_{REG} cells (**Fig. 7F–H**). Thus, IL-33 promotes antigen-independent expansion of pulmonary Th2 cells and type 2 adapted ST2⁺ GATA3⁺ T_{REG} cells during neonatal RSV infection.

DISCUSSION

By dissecting a murine model of neonatal infection(21, 29), our study identifies a developmental mechanism by which IL-33 shapes the long-term balance between regulatory and pathogenic type 2 immunity following neonatal RSV infection. We demonstrate that IL-33 acts on both arms of the immune response, promoting the specialization of pulmonary T_{REG} cells through an IL-33–IL-4R α signaling axis while independently establishing durable pathogenic T_H2 memory. Rather than simply failing to suppress inflammation, neonatal RSV infection induces a qualitative reprogramming of pulmonary T_{REG} cells, resulting in a specialized GATA3⁺ ST2⁺ IL-4R α ^{hi} phenotype with reduced suppressive capacity. These findings extend the emerging concept that neonatal immunity is intrinsically biased toward type 2 responses(37) and provide a mechanistic explanation for how early-life viral infection establishes persistent pulmonary immune dysregulation.

Our findings further redefine the role of IL-33 in tissue T_{REG} cell biology. IL-33 has generally been viewed as a key regulator of tissue adaptation, promoting FoxP3 stability, tissue residency and suppressive function in multiple organs(12, 14, 15). Consistent with this paradigm, we found that IL-33 promoted the differentiation and expansion of pulmonary ST2⁺ GATA3⁺ T_{REG} cells through a glycolysis-dependent program coupled to increased IL-4R α expression. GATA3, which is closely associated with T_{REG} cell stability(38), was for pulmonary T_{REG} cells to maintain this tissue-adapted phenotype, preserve FoxP3 expression and limit acquisition of ROR γ T-associated programs. Thus, IL-33 does not simply regulate T_{REG} cell abundance but actively shapes their functional identity within the inflammatory environment.

Acquisition of this specialized phenotype did not translate into improved immune suppression. Instead, IL-33 and IL-4 cooperated to amplify STAT6 signaling, promote GATA3-

dependent functional reprogramming and impair T_{REG} cell suppressive activity. These findings help reconcile apparently conflicting reports regarding IL-33 biology(20). Whereas IL-33 enhances T_{REG} cell function during T_H1- and T_H17-dominated inflammation(12, 14, 15), including adult viral infection, its effects appear fundamentally altered within the neonatal type 2 environment established following RSV infection. One explanation may be that IL-33 amplifies the dominant immune program already present within tissues. In type 1 settings IL-33 may reinforce regulatory adaptation and tissue repair, whereas in type 2-biased tissues it promotes a maladaptive regulatory state that becomes increasingly permissive to pathogenic inflammation. Although our experiments cannot distinguish the relative contribution of IL-4 versus IL-13, they identify IL-4R α -STAT6 signaling as a central pathway linking IL-33 sensing to altered T_{REG} cell function.

A key implication of our study is that IL-33 simultaneously controls T_{REG} cell specialization and the long-term establishment of pathogenic type 2 immunity. Although IL-33 promoted differentiation of specialized ST2⁺ T_{REG} cells, it ultimately favored the expansion and persistence of long-lived pulmonary T_H2 cells following neonatal RSV infection. This observation highlights the importance of developmental timing in shaping tissue immunity. T_{REG} cells seed non-lymphoid organs shortly after birth and retain much of their transcriptional identity and TCR repertoire throughout adult life (10, 11), suggesting perturbations during this developmental period may alter their functional trajectory. Our findings extend this concept by demonstrating that neonatal viral infection reprograms the developmental trajectory of pulmonary T_{REG} cells while independently imprinting pathogenic T_H2 memory, providing a mechanistic explanation for the association between severe infant RSV infection and asthma susceptibility.

Together, our findings support a model in which IL-33 functions as a developmental checkpoint coordinating tissue adaptation of pulmonary T_{REG} cells with establishment of pathogenic T_H2 memory. This model integrates alarmin signaling, metabolic programming and tissue-specific T_{REG} cell specialization into a novel framework explaining how early-life events influence long-term tissue homeostasis. The association of genetic variations in IL-33, IL-4 and IL-13 with asthma risk(39) further supports the relevance of the IL-33–IL-4R α –STAT6 axis as a potential therapeutic target for preventing chronic type 2 airway disease(40).

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COMPETING FINANCIAL INTERESTS

All authors declare that they have no competing financial interests.

DATA AND MATERIALS AVAILABILITY

All data are available in the main text or the supplementary materials

MATERIALS AND METHODS

Mice. Wild-type BALB/c and BALB/c STAT6^{-/-} mice were obtained commercially (Jackson Laboratory, Bar Harbor, ME) and were inbred in the RI-MUHC B6.FoxP3^{GFP} reporter knock-in (FoxP3^{GFPki}) mice were bred into the congenic background (Ly5.1⁺) for more than 10 generations. Sex-mixed BALB/c and STAT6^{-/-} on BALB/c background (Δ STAT6) mice breeding cages were setup up and monitored daily. Neonatal BALB/c mice were infected I.N. between 5-7 days old. Adult mice were re-infected at 6 weeks of age. B6.FoxP3tm9(EGFP/cre/ERT2)Ayr/J mice (B6.FoxP3^{GFP/cre/ERT2}) were provided by Dr Suh (McGill University, Montreal, QC) and crossed with Rosa26RFP^{stop-loxP} reporter mice (provided by Dr Jeffrey Bluestone, UCSF, San Francisco, CA) to obtain B6.Foxp3^{GFP/cre/ERT2} x Rosa26-RFP^{fl-stop-fl} (Foxp3-Fate⁺). Foxp3^{WT} dual reporter mice were further crossed to B6.ST2^{loxP} mice (provided by Dr Stephen J. McSorley, UC Davis, Davis, CA) (T_{REG} ^{Δ ST2}) or B6.GATA3^{loxP} (T_{REG} ^{Δ GATA3}). To obtain high numbers of age and sex matched groups, homozygote parents were crossed for 1-2 generations before the experiments. While the RSV experiment was performed on equal numbers of males and females, the HDM experiments were performed in female mice, as males are known to display lesser cellular infiltration in the lungs(41). All mice were crossed, housed, and bred in our facility under specific pathogen-free conditions and used according to institutional guidelines at McGill University and the Canadian Council on Animal Care (CCAC).

RSV purification. The human-purified RSV A2 virus (ATCC #VR-1540; ATCC, Manassas, VA) was propagated in human epithelial type 2 cells (Hep-2; ATCC #CCL-23) in DMEM (supplemented with 3% FBS, 10 mM HEPES, 2 mM glutamine, and 50 mg/ml gentamicin (Wisent,

St-Bruno, QC, Canada)) at 37°C. When extensive cytopathic effect was observed 4-5 d post-infection, the cells were scraped and the supernatant was collected, as previously described (40). In a 24-well plate, medium with virus was then added to 70-80% confluent Hep-2 cells. Virus was adsorbed to the monolayer for 90 minutes on a rocker at 4°C. RPMI+2.5% HI-FBS was used to wash the monolayer which was then cultured for 3 days at 37°C in fresh RPMI+2.5% HI-FBS. Once the cell monolayer exhibited cytopathic effect (CPE) over 40% of the surface under light microscopy, scraping was used to disrupt the monolayer. Two washes were performed, once with H₂O to disrupt intact cells, followed by supernatant from first wash, both spinning at 2000rpm for 5 minutes at 4°C, to remove debris. The supernatant was transferred to a T175 flask containing 80-90% confluent Hep2-cells and placed in a rocker for 90 minutes at for the virus to be adsorbed. The cells were washed with RPMI+ 2.5% HI-FBS, and the monolayer was cultured for 3 days at 37°C in RPMI+2.5% HI-FBS. Once CPE reached 70-80%, the cell monolayer was disrupted by scraping. To clarify the supernatant, centrifugation at 4°C for 10 minutes at 2095g was used (42). To further purify the virus, a sucrose gradient with centrifugation at 116 000 x g for 2 hours at 4°C was used (43). The virus-containing supernatants were aliquoted and stored at -80°C. RSV A2 titration was quantified by serial dilution on Vero cell monolayers incubated for a week and fixed in 1% formaldehyde (Millipore-Sigma, St. Louis, MO, USA). The positive plaques were assessed by immunofluorescence, using an anti-RSV FITC-conjugated antibody (*ab20391*, Abcam, Cambridge, MA, USA). Viral titers (TCID₅₀) were calculated by the Reed-Muench method (44).

Lymphocyte isolation. Isolation of T cells was performed using a previously described method(45). Briefly, immediately after CO₂ euthanasia, the lungs are perfused with cold PBS through the right ventricle. Whole lungs were collected and sterile scissors were used to cut the

lungs into small pieces. Lung pieces were then digested enzymatically for 30 minutes at 37°C, 5% CO₂ with a cocktail of DNase I (200µg/ml; Millipore Sigma, St-Louis, MO), LiberaseTM (100µg/ml; Roche, Indianapolis, IN), hyaluronidase 1a (1mg/ml; Life Technologies, Carlsbad, CA), and collagenase XI (250µg/ml; Life Technologies, Carlsbad, CA) in RPMI-1640 as described previously. Cells were washed with RPMI-1640 media containing 1% Penicillin/Streptomycin and 5% FBS. To lyse red blood cells sterile, filtered ammonium-chloride-potassium (ACK) buffer was used. A 0.7 µM strainer was used to filter the cells. The remaining viable cells were recovered. For the HDM experiments, the collected lungs were digested in RPMI 1640 with 5% FBS (Wisent Bioproducts, Saint-Bruno, QC) containing collagenase A (0.5mg/ml) and collagenase D (0.5mg/ml) in the presence of DNase I (200µg/ml; Millipore Sigma, St-Louis, MO) for 45 minutes at 37°C and then mechanically processed in the same manner as the mediastinal LN, the spleen and the inguinal lymph nodes. The cells were then filtered through a cell strainer and kept in complete RPMI medium. For *in vitro* culture, CD4⁺ splenocytes were enriched using an autoMACS (Miltenyi Biotec) followed by a FACS-isolation (FACSARIATM, BD Biosciences) of CD4⁺GFP⁺ (FoxP3⁺ T_{REG}) and GFP⁻ (T_{EFF}) when FoxP3^{GFPki} reporter mice were used or CD4⁺CD25^{HI} (T_{REG}) and CD25⁻ (T_{EFF}) when WT BALB/c and ΔSTAT6 mice were used. To assess T cell cytokine production, isolated cells were then activated with phorbol myristate acetate (PMA), ionomycin and BD GolgiStopTM (BD Biosciences) for 3 hours.

***In vitro* assays.** FACS-sorted CD4⁺GFP⁺ T_{REG} cells (FoxP3⁺, 5x10⁴) were activated in 96-well flat-bottomed (0.2 ml) plates previously coated with α-CD3 (5 µg/ml) and α-CD28 (2 µg/ml), and in the presence of IL-2 (200 U/ml). For these *in vitro* stimulation assays, IL-33 (10 ng/ml), IL-4 (10 ng/ml) and mouse recombinant TGFβ1 (1 ng/ml) (R&D biosystems) were added at the start of

the culture unless otherwise stated. For suppression assays, FACS-sorted, CellTrace™ Violet (CTV⁺, Thermo Fisher Scientific, Waltham, MA) labelled CD4⁺GFP⁻ (5.0×10^4) were co-cultured with titrated numbers of FACS-sorted CD4⁺GFP⁺ (FoxP3) T_{REG} cells [2.5×10^4 (1:2), 1.25×10^4 (1:4), 6.25×10^3 (1:8) and 3.75×10^3 (1:16)] in the presence of soluble α CD3 (1 μ g/ml) and 3.0×10^5 irradiated (30 Gys) feeder cells (CD4⁻ fraction from the MACs®) in RPMI (Wisent) supplemented with 10% FBS at 37°C for 72 hours.

Multiplex ELISA. FACS-sorted CD4⁺GFP⁺ (FoxP3; >99 % purity) T_{REG} cells from FoxP3^{GFPki} mice were cultured as described above. After 72 hours in culture, 300 μ l (100 μ l triplicates pooled as one experiment) of media supernatant from the 96 well plate culture was collected and immediately frozen at -80°C. In parallel, one experiment was also performed using CD4⁺CD25^{HI} FACS-sorted T_{REG} cells from BALB/c and STAT6^{-/-} mice. The samples from 3 distinct experiments were sent for multiplex ELISA to Eve Technologies using the Mouse High-Sensitivity Multiplex Discovery Assay-18 testing for GM-CSF, IFN γ , IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, LIX, IL-17A, KC, MCP-1, MCP-2, TFN α (Eve Technologies, Calgary, AB, Canada).

Intranasal infection with RSV. The human-purified RSV A2 virus (ATCC #VR-1540; ATCC, Manassas, VA) was propagated with human epithelial type 2 cells (ATCC #CCL-23) in DMEM (supplemented with 3% FBS, 10 mM HEPES, 2 mM glutamine, and 50 mg/ml gentamicin (all from Wisent, St-Bruno, QC, Canada)) at 37°C. When extensive cytopathic effect was assessed 4-5 d post-infection, cells were scraped and the supernatant was collected, as previously described (40). Cell pellets were subjected to 3 freeze/thaw cycles, and this additional supernatant was

collected. The compiled supernatants were concentrated with polyethylene glycol (PEG) 6000 (EMD Millipore, Billerica, MA, USA), as previously described (40), and stored at -80°C. RSV A2 titration was quantified by serial dilution on Vero cell monolayers incubated for a week and fixed in 1% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA). The positive plaques were assessed by immunofluorescence, using an anti-RSV FITC-conjugated antibody (*ab20391*, Abcam, Cambridge, MA, USA). Viral titers (TCID₅₀) were calculated by the Reed-Muench method (44).

For the neonatal infections, the breeder pairs were time mated, and pups within 5–7 days of birth were used for experiments. The pups were infected with RSV A2 intranasally (i.n.) (10⁶ TCID₅₀/g body weight, 2–3 g body weight), as described previously (40) and euthanized 4 days later. For the adult re-infection model, mice were infected as neonates, and re-infected 6 weeks later with RSV A2 (10⁶ TCID₅₀/g body weight; 19–22 g body weight), as described in Figure 3A. For the IL-33 instillation experiment, 5-7-day-old BALB/c pups were infected with 10⁶ TCID₅₀/g of RSV A2 and left to recuperate while control BALB/c mice received PBS. At 6 weeks, the mice received (0.25 µg/mouse) rIL-33 i.n. and were euthanized two days later. Here, female and male mice were distributed evenly between groups. To study cytokine production from antigen-specific T cells, isolated cells from the lungs were stimulated with 2 µg protein/ml of heat inactivated RSV A2 (30 minutes at 60°C) for 72 hours and in the presence of BD GolgiStop™ (BD Biosciences) for the last 6 hours. For the IL-33 neutralization, mixed sex 5-7 days old BALB/C pups received 50µl of anti-mouse IL-33 (R&D Systems; # MAB3626; 5 µg/200 µl) or its control IgG2A (R&D Systems; # MAB006; 5 µg/200 µl) intra-peritoneally (IP) on the day of RSV A2 infection. For the recombinant IL-33, 5-7 day old BALB/c pups received 50µl of recombinant IL-33 i.p. (R&D Systems; #3626-ML; 20µg/kg), the recombinant IL-33 in the intra-nasal (46) RSV A2 preparation

(20µg/kg diluted in 35µl) or the carrier BSA (vehicle; 0.03% diluted in 1/100 of the RSV preparation to obtain a final concentration of 0.0003%) on the day of infection.

Intranasal challenge with house dust mite. To sensitize and challenge the mice to HDM, 8-10 weeks old mice were anesthetized by isoflurane and 10µg in PBS of purified *Dermatophagoides pteronyssinus* extract (XPB82D3A2.5, Stallergene Greer Inc, Lenoir, NC) was instilled in each nostril (25 µl/nostril). The mice received 2 doses, 6 days apart, followed by 3 consecutive doses at day 14, 15 and 16 to induce the onset of asthma-like responses in the lungs. Tamoxifen (Sigma-Aldrich, St-Louis, MO) diluted in corn oil (Sigma-Aldrich) was administered intra-peritoneally on days 8 and 10 at 20 µg/mouse. Mice were euthanized 4 days after the last HDM dose and the cells from the lungs and mediastinal lymph nodes were isolated.

Flow cytometry. After lymphocyte isolation, single-cell suspensions were stained with the following fluorescence-conjugated mAbs, purchased from Thermo fisher Scientific (eBioscience®) unless otherwise stated: α-CD4–Alexa700 (GK1.5), α-CD8α-V500 (53-6.7) (BD Biosciences), α-CD8β-V510 (H35-17.2) (BD Biosciences), α-CD44-Alexa efluor780 (IM7), α-CD62L-PE (MEL-14), α-ST2-PerCP710 (RMST2-2), α-CD25-PECy7 (PC61, BD Biosciences), α-FoxP3-FITC or PE (FJK-16s), α-IL-13-PECy7 (eBio13a), α-IL-4-PE (11b11), α-IFNγ-PECy7 or BUV737 (XMG1.2), α-GATA3-Alexa647 (BD Biosciences), α-Helios-Pacific Blue or PE (22F6, Biolegend) and Thy1.2 (CD90.2)-Alexa 780 (53-2.1). For ILC2s, the cells were stained with α-CD3-FITC (17A2), α-B220-PE (RA3-6B2), α-TCRgd-PE (eBioGL3), α-CD49b-PE (DX5), α-CD11c-PE (N418), α-CD11b-PE (M1/70), α-Ly6G-Alexa700 (RB6-8C5, Biolegend), α-CD127-APC-eFluor780 (A7R34), α-CD25-PECy7 (PC61, BD Biosciences), α-ST2- PerCP710

(RMST2-2), α -Thy1.2-eFluor450 (53-1.2), α -KLRG1-APC (2F1). For cytokine panel staining, in addition to the extracellular stains, we used α -IL-4-PE (11B11), α -IL-13-PE-Cy7 (ebio13A), α -IL-5-APC (TRFK5, BD Biosciences), α -IFN γ -BUV737 (XMG1.2, BD biosciences). Non-viable cells were excluded using fixable viability dye eFluor 780 or 506 reagents (Thermofisher). Data were acquired using a FACS Fortessa X-20 flow cytometer (BD Biosciences) and analyzed using FlowJo version 9 software (TreeStar). For the myeloid panel, we used α -CD45.2-APC-Cy7 (104), α -F4/80-PE-Cy7 (BM8), α -CD11c-PerCPCy5.5 (HL3), α -CD19-APC (eBio1D3), α -CD11b-PacificBlue (M1/70), α -Ly6G-Alexa700 (RB6-8C5, Biolegend), α -Siglec F-FITC (BB515, BD Biosciences), α -CD3-BUV737 (17A2, BD Biosciences), α -Arg1-PE (polyclonal, R&D Systems).

Single cell analysis. The scRNA-seq expression profile was obtained from publicly available Gene Expression Omnibus (GEO) with accession number GSE193816. While the downloaded data had already been analyzed and annotated, we did perform additional quality control. We used the Seurat (v4.3.0) R package to remove doublet and nonviable cells were removed by excluding cells expressing <200 or >3000 genes, >10% mitochondrial genes. The data was normalized, and variable features were identified before performing scaling (ScaleData) in Seurat. Next, we isolated data pre-annotated as CD4⁺ T cells and ran Principal Component Analysis (PCA). The minimal dimensionality used to run the Uniform Manifold Approximation and Projection (UMAP) clustering was established at 10 principal components at a resolution of 0.5. FeaturePlot was performed to plot the features ILRL1, FOXP3, IKZF2, IL4R, GATA3 and IL13. Expression of the features FOXP3, IL2RG, IL4R and GATA3 from cluster 4 were compared between cells from asthmatic and non-asthmatic patients generated after antigenic challenge. Finally, a heatmap of the top 10 genes that identify each cluster was generated using minimum log₂FC >0.25.

Statistical analysis. For all experiments, the mean and standard deviation are shown, unless otherwise stated. Multiple comparisons were tested using a two-way ANOVA with a Tukey post-test for comparison of all individual means within a figure or one-way ANOVA when required. For single comparisons, an unpaired Student T-test was used with the p-value expressed in the figure legend unless otherwise stated. All statistical analysis was performed with GraphPad Prism version 8 software (GraphPad Software).

FIGURE AND LEGENDS

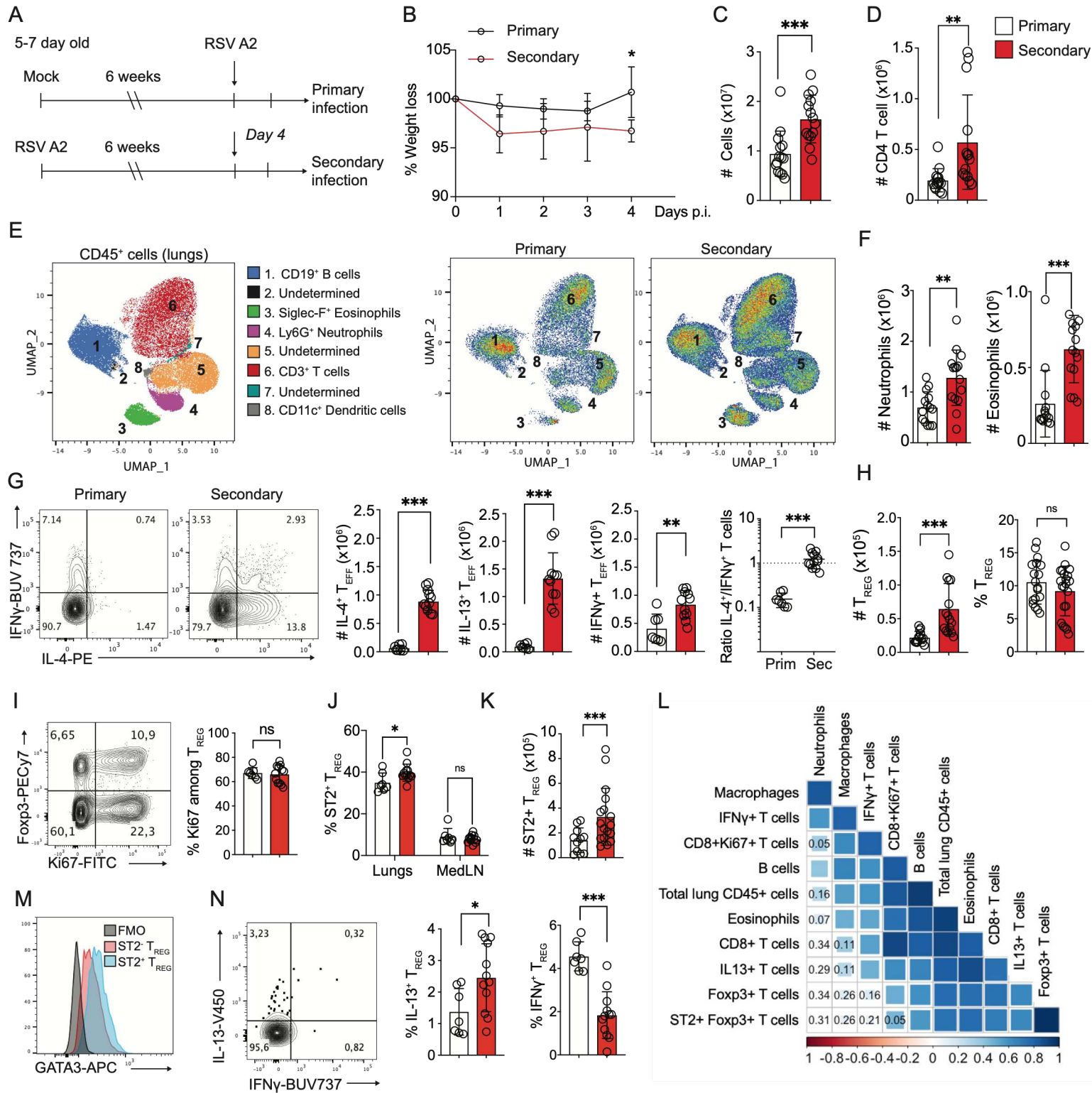


Figure 1

Figure 1. Neonatal RSV infection promotes expansion of ST2⁺ GATA3⁺ T_{REG} cells during dysregulated type 2 recall responses.

- (A) 5–7-day old neonate mice were infected with a non-lethal 10⁶ TCID₅₀/gram of body weight inoculum of RSV A2. They were left to recuperate and were re-infected at 6 weeks of age with 10⁶ TCID₅₀/gram of body weight RSV A2 (Secondary). Control BALB/c mice received PBS as neonate and were infected with the same dose of RSV A2 (Primary). Pulmonary cells were isolated 4 days post-infection. Compiled or representative data of 3 distinct experiments.
- (B) Weight loss upon secondary infection. Representative of 3 distinct experiments.
- (C) Number of cells in the lungs in infected mice. Student T test. ***p<0.001
- (D) Number of CD4⁺ T cells (CD3⁺, CD4⁺) in the lungs of infected adult mice.
- (E) Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP v4.0.3; <https://arxiv.org/abs/1802.03426>) visualisation of the live CD45⁺ cells isolated from the lungs. The clusters were identified using an unbiased FlowSOM (v3.0.18; Grassen et al. 2015) clustering algorithm to visualise by Cluster Explorer (v1.7.6; Joseph Trotter, FlowJO Exchange).
- (F) Number of CD45⁺ CD3⁻ CD19⁻ Siglec F⁻ CD11b^{hi} Ly6G^{hi} (neutrophils) and CD45⁺ CD3⁻ CD19⁻ Siglec F⁺ CD11c^{low} Ly6G⁻ (eosinophils). Student T test. ***p<0.001
- (G) Representative flow cytometry of IL-4 and IFN γ in CD4⁺ T cells in the lungs of mice at day 4 post-infection and number of IL-4, IL-13 and IFN γ -producing CD4⁺ T cells (CD3⁺, CD4⁺) in the lungs of infected adult mice. Student T test. ***p<0.001. Ratio of IL-4⁺ over IFN γ ⁺ CD4⁺ T cell in the lungs of primary and secondary infected mice. Student T test. ***p<0.001.
- (H) Number of CD4⁺Foxp3⁺ T_{REG} cells and frequency of Foxp3⁺ among CD4⁺ T cells in the lungs at day 4. Student T test. ***p<0.001
- (I) Representative flow cytometry of Ki67 and Foxp3 among live CD45⁺CD4⁺ T cells and frequency of Ki67⁺ among CD4⁺Foxp3⁺ T_{REG} cells in the lungs at day 4. One-way ANOVA. Tukey post-hoc test. ***p<0.001.
- (J) Frequency of ST2⁺ cells among CD4⁺Foxp3⁺ T_{REG} cells in the lungs. Student T test. **p<0.01.
- (K) Number of ST2⁺ T_{REG} cells in the lungs of primarily or secondarily infected mice. Student T test. ***p<0.001.
- (L) Correlation matrix of the number of Eosinophils, Neutrophils, CD8⁺ T cells, Ki67⁺ CD8⁺ T cells, IL-13⁺ CD4⁺ T cells, IFN γ ⁺ CD4⁺ T cells, Foxp3⁺ T cells and ST2⁺ T_{REG} cells in the lungs of re-infected mice at day 4 performed using the Corrplot package in R from data obtained by flow cytometry.
- (M) Representative histogram of GATA3 expression among ST2⁺ (blue) and ST2⁻ (red) among T_{REG} cells from the lungs of re-infected (secondary) mice at day 4.
- (N) Frequency of IL-13⁺ and IFN γ ⁺ among CD4⁺ Foxp3⁺ T (T_{REG}) cells at day 4 post-infection. Student T test. *p<0.05 stimulated for 3 hours with PMA/ionomycin and in the presence of Golgi-Stop®.

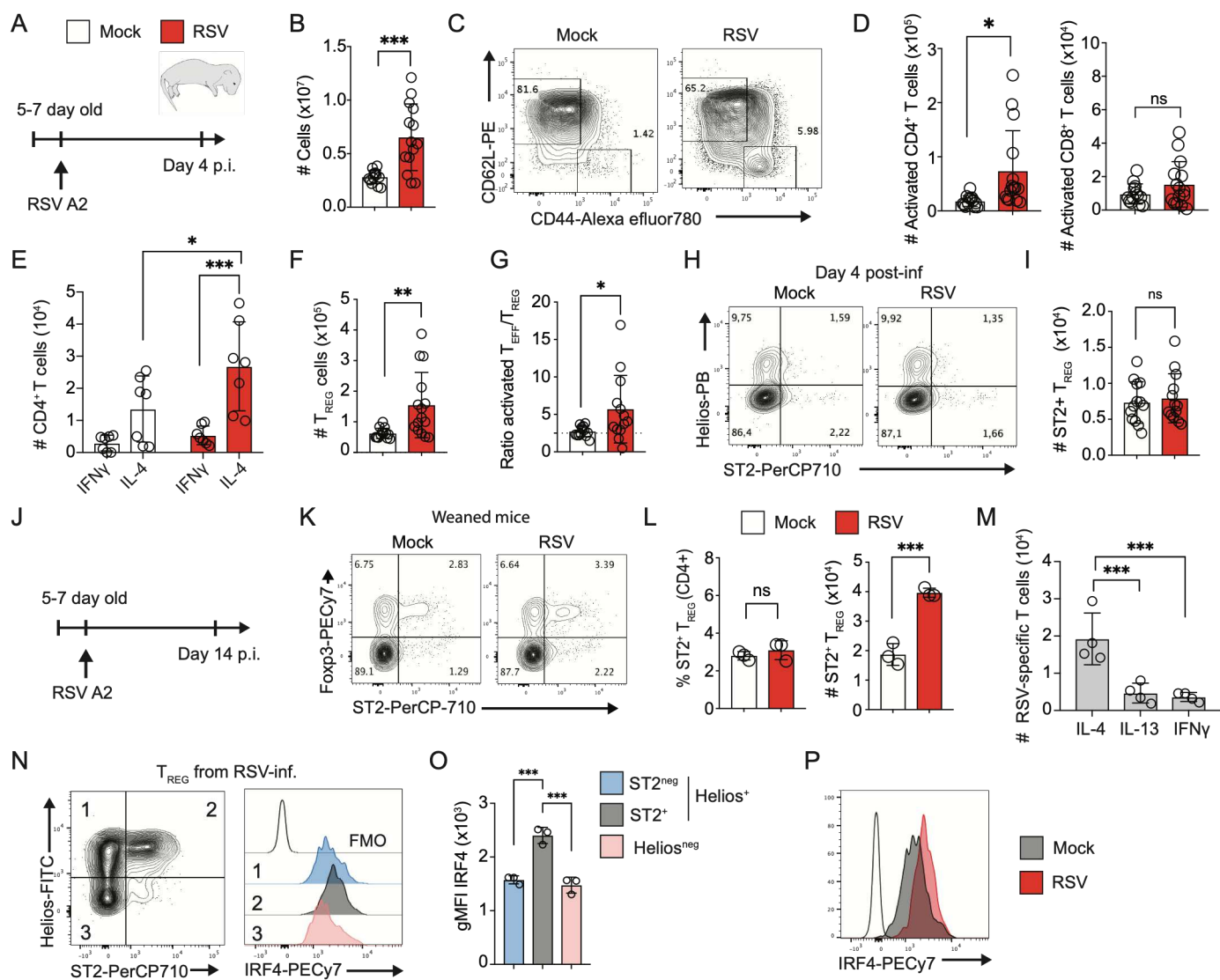


Figure 2

Figure 2. ST2⁺ T_{REG} cells accumulate late in the lungs of neonatal mice following RSV infection.

- (A) Neonate infection. 10⁶ TCID50/g of RSV A2 was instilled intra-nasally in 5–7-day-old BALB/c mice. The pups were sacrificed 4 days later, and the lungs were collected for cell analysis. Compiled data of 4 distinct experiments.
- (B) Cell numbers in the lungs at day 4 post-infection. Student T-test. ***p<0.001
- (C) Representative flow cytometry of activated effector T cells (CD62L^{lo}CD44^{hi}) in the lungs of infected neonate mice.
- (D) Number of CD62L^{lo}CD44^{hi} CD4⁺ T cells and CD8α⁺ T cells. Student T test. *p<0.05.
- (E) Numbers of IFNγ and IL-4 expressing CD4 T cells.
- (F) Number of CD4⁺Foxp3⁺ (T_{REG}) cells in the lungs. Student T-test. **p<0.01
- (G) Ratio of CD44^{hi} T_{EFF} over T_{REG} cells (# CD44^{hi}Foxp3^{neg}/# CD44^{hi}Foxp3⁺ CD4⁺ T cells) isolated from the lungs. Student T-test. *p<0.05
- (H) Representative flow cytometry of the expression of Helios and ST2 in pulmonary CD4⁺Foxp3⁺ T cells at day 4 p.i.
- (I) Number of ST2⁺ T_{REG} cells in the lungs at day 4 p.i.
- (J) 5-7-day-old mixed sex BALB/c mice were either mock infected or infected with RSV A2 (see methods) and were left to recuperate until weaning (14 days post-infection; 19-21 day old).
- (K) Representative flow cytometry of ST2 and Foxp3 expression among CD45⁺ CD4⁺.
- (L) Frequency among CD4⁺ and number of ST2⁺ T_{REG} cells in the lungs. (n=3/group) Student T test. ***p<0.001
- (M) Total lung cells were isolated and exposed 2 μg protein/ml of heat inactivated RSV A2 for 72 hours and in the presence of Golgi-stopTM for the last 6 hours. Numbers of IFNγ, IL-13 and IL-4 expressing antigen-specific CD4⁺ T cells calculated from RSV-infected pups. (n=4; individual stimulation/mice). One-way ANOVA. Tukey post-hoc. ***<0.001.
- (N) Representative flow cytometry of Helios and ST2 expression among CD45⁺ CD4⁺ Foxp3⁺ cells (left) and the relative expression of IRF4 in each quadrant (right).
- (O) Geometric mean fluorescence intensity (MFI) of IRF4 among Helios⁺ST2⁻ (blue), Helios⁺ST2⁺ (grey) and Helios⁻ST2⁻ (pink) T_{REG} cells. One-way ANOVA. ***p<0.001
- (P) Representative flow cytometry plot of the expression of IRF4 among CD4⁺Foxp3⁺ ST2⁺ cells from the lungs of mock(grey) and RSV A2 (red) infected BALB/c mice at day 14 p.i

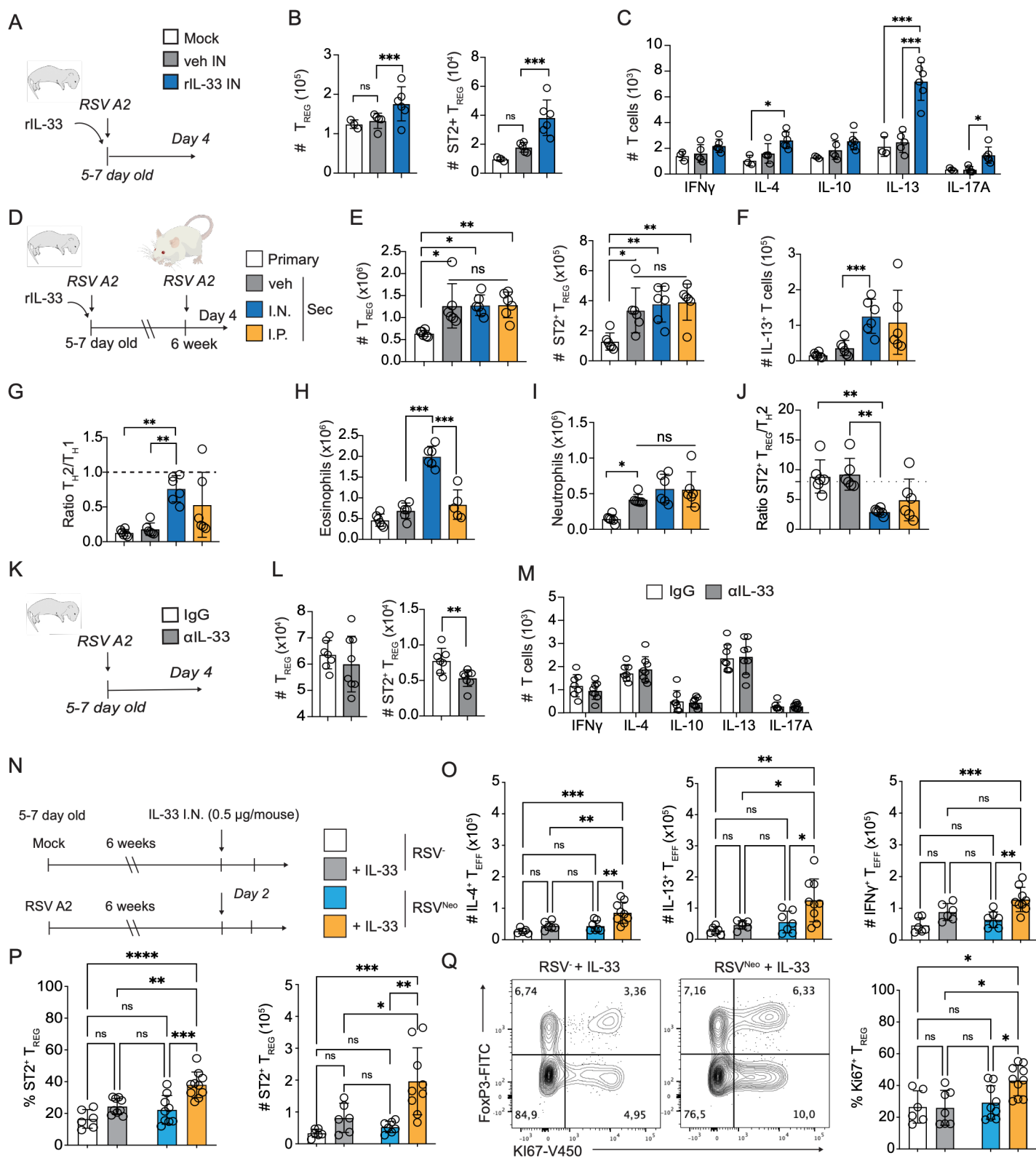


Figure 3

Figure 3. IL-33 favors the establishment of pathogenic T_H2 memory rather than durable $ST2^+$ T_{REG} responses following neonatal RSV infection.

- (A) To induce infection in neonates, 7-day-old mixed sex BALB/c mice were mock infected (uninfected) or infected with 10^6 TCID₅₀ of RSV A2/g of mice received 20 μ g/kg of rIL-33 administered intra-nasally (IN) or 0.0003% BSA IN (veh) and the lungs were collected at day 4 post-infection. One-way ANOVA. Tukey post-hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Compiled data of 2 distinct experiments.
- (B) Number of $CD45^+$ $CD4^+$ Foxp3 $^+$ (T_{REG}) cells in the lungs and $ST2^+$ T_{REG} cells in the lungs.
- (C) Number of IFN γ , IL-4, IL-13, IL-10 and IL17A producing $CD4^+$ T cells in the lungs.
- (D) Adult infection. 7-day-old pups were separated in 4 groups, a mock-infected group (Primary), a group that received the 0.0003% BSA (veh) i.n, a group that received rIL-33 i.n. and a group that received rIL-33 intra-peritoneally (I.P.) at the time of infection. The pups were left to recuperate and were re-infected with 10^6 TCID₅₀ of RSV A2/g at 6 weeks of age. The lungs were perfused and collected 4 days later. One-way ANOVA. Tukey post-hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Compiled data of 2 distinct experiments.
- (E) Number of $CD45^+$ $CD4^+$ Foxp3 $^+$ (T_{REG}) cells and $ST2^+$ T_{REG} .
- (F) Number of $CD45^+$ $CD4^+$ IL-13 $^+$ T cells.
- (G) Ratio of $CD45^+$ $CD4^+$ IL13 $^+$ (T_H2) / IFN γ^+ (T_H1) cells.
- (H) Number of $CD45^+$ $CD3^-$ $CD19^-$ Siglec F $^+$ $CD11c^{low}$ Ly6G $^-$ (eosinophils)
- (I) Number of $CD45^+$ $CD3^-$ $CD19^-$ Siglec F $^-$ $CD11b^{hi}$ Ly6G ^{hi} (neutrophils)
- (J) Ratio of the number of $ST2^+$ T_{REG} over the number of IL-13 $^+$ T_H2 cells.
- (K) IL-33 neutralisation. 7-day-old mixed sex BALB/c mice were infected with 10^6 TCID₅₀ of RSV A2/g of mice on the same day as receiving 5 μ g/200 μ L I.P. of either neutralizing α IL-33 or IgG2A (IgG). The lungs were collected 4 days later. Compiled data of 2 distinct experiments.
- (L) Number of $CD45^+$ $CD4^+$ Foxp3 $^+$ (T_{REG}) and $ST2^+$ T_{REG} . Student T-test. ** $p < 0.01$
- (M) Number of IFN γ , IL-4, IL-13, IL-10 and IL17A producing $CD4^+$ T cells.
- (N) 7-day-old mixed sex BALB/c mice were mock infected (Primary) or infected with 10^6 TCID₅₀ of RSV A2/g of weight of mice intra-nasally, left to recuperate for 6 weeks and challenged with 0.25 μ g of rIL-33 intra-nasally. Cells from the lungs were isolated at day 2 post-administration. Compiled data of 2 distinct experiments. One-way ANOVA. Tukey post-hoc. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$
- (O) Number of $CD4^+$ T cells in the lungs at day 2. Number of IL-4 $^+$, IL-13 $^+$ and IFN γ^+ $CD4^+$ T cells in the lungs at day 2. One-way ANOVA. Tukey post-hoc.
- (P) Frequency and number of $ST2^+$ among $CD4^+$ Foxp3 $^+$ (T_{REG}) in the lungs. One-way ANOVA. Tukey post-hoc.
- (Q) Representative flow cytometry and frequency of Ki67 $^+$ $CD4^+$ Foxp3 $^+$ (T_{REG}) in the lungs at day 2. One-way ANOVA. Tukey post-hoc.

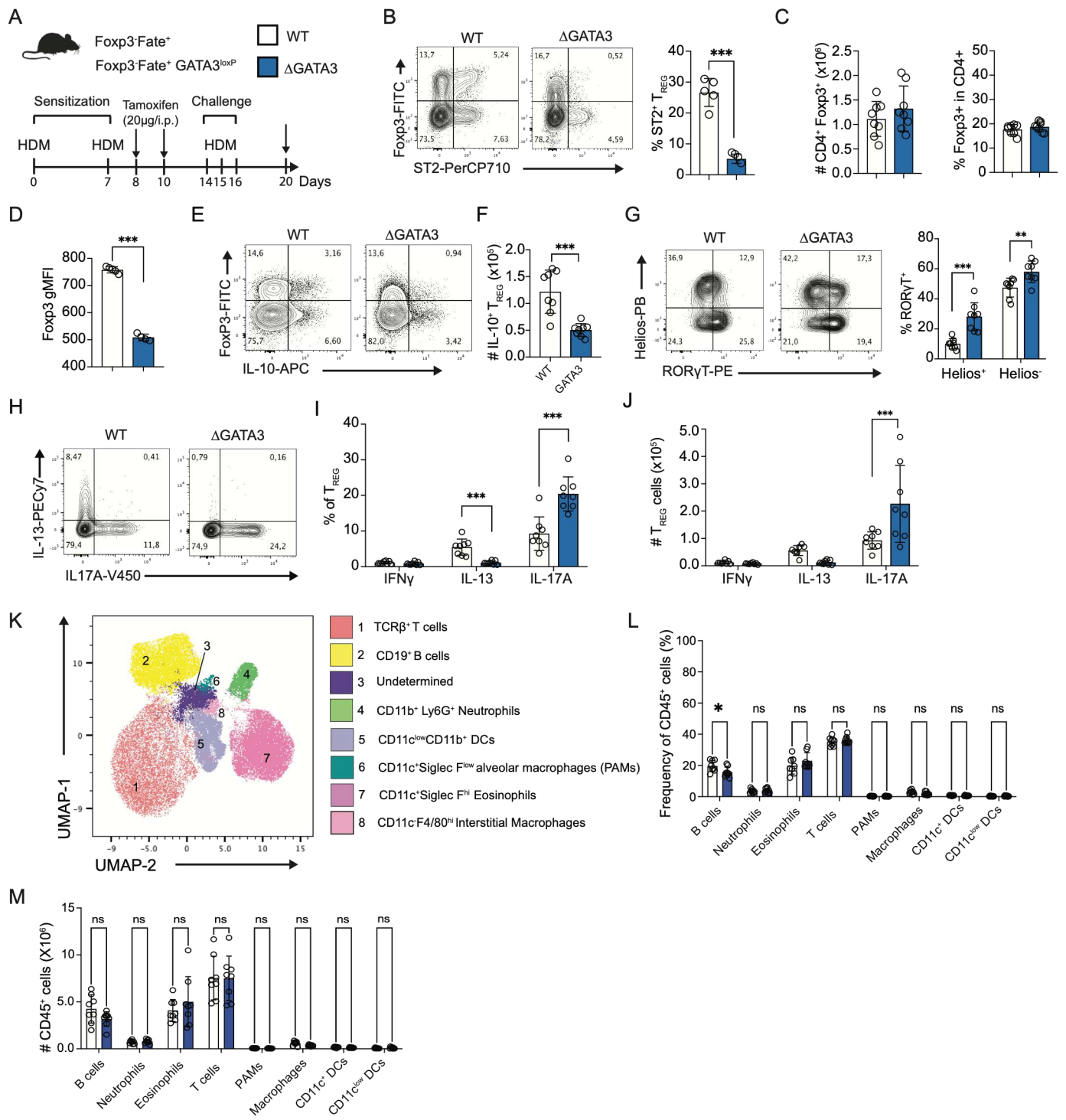


Figure 4

Figure 4. GATA3 is required to maintain the Type 2 adapted phenotype of pulmonary T_{REG} cells during allergen-driven inflammation in adult mice.

- (A) 8–10-week-old female Fate⁺ T_{REG}^{WT} (WT) or T_{REG}^{ΔGATA3} (ΔGATA3) mice were sensitized with 10 μg/mouse of HDM (*Dermatophagoides pteronyssinus extract*) with the protocol described in Fig 2. Compiled data from 2 independent experiments.
- (B) Representative flow cytometry of the expression of Foxp3 and ST2 in CD4⁺ T cells isolated from the lungs at day 20. Frequency of ST2⁺ among pulmonary T_{REG} cells. Student T test. ***p<0.001
- (C) Number and frequencies of live CD45⁺ CD4⁺ Foxp3⁺ (T_{REG}) in the lungs.
- (D) Geometric mean fluorescence intensity (gMFI) of Foxp3 in CD45⁺ CD4⁺ Foxp3⁺ cells. Student T test. ***p<0.001
- (E) Representative flow cytometry of the expression of Foxp3 and IL-10 in CD4⁺ T cells activated for 3 hours in PMA + ionomycin + Golgi-Stop® at day 20.
- (F) Number of IL-10⁺ CD4⁺ Foxp3⁺ (T_{REG}) in the lungs. Student T test. p<0.001
- (G) Representative flow cytometry of the expression of Helios and RORγT in CD4⁺ Foxp3⁺ T cells and frequency of RORγT⁺ among Helios⁺ and Helios⁻ pulmonary T_{REG} cells. Two-way ANOVA. **p<0.01; *p<0.05
- (H) Representative flow cytometry of the expression of IL-13 and IL-17A in CD4⁺ Foxp3⁺ T cells isolated from the lungs at day 20.
- (I) Frequencies of IFNγ⁺, IL-13⁺ and IL-17A⁺ among pulmonary T_{REG} cells after a 3 hour stimulation in PMA + ionomycin + Golgi-Stop®. Two-way ANOVA. **p<0.01; *p<0.05
- (J) Number of IFNγ⁺, IL-13⁺ and IL-17A⁺ T_{REG} cells isolated from the lungs at day 20.
- (K) Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP v4.0.3; <https://arxiv.org/abs/1802.03426>) visualisation of the live CD45⁺ cells isolated from the lungs. The cluster were identified using an unbiased FlowSOM (v3.0.18; Grassen et al. 2015) clustering algorithm to visualise by Cluster Explorer (v1.7.6; Joseph Trotter, FlowJO Exchange).
- (L) Frequency of each subset identified in (B) among CD45⁺ cells. Two-way ANOVA. Tukey's test. *p<0.05
- (M) Cell counts of each subset identified in (B) in the lungs at day 20. One-way ANOVA. Tukey's test.

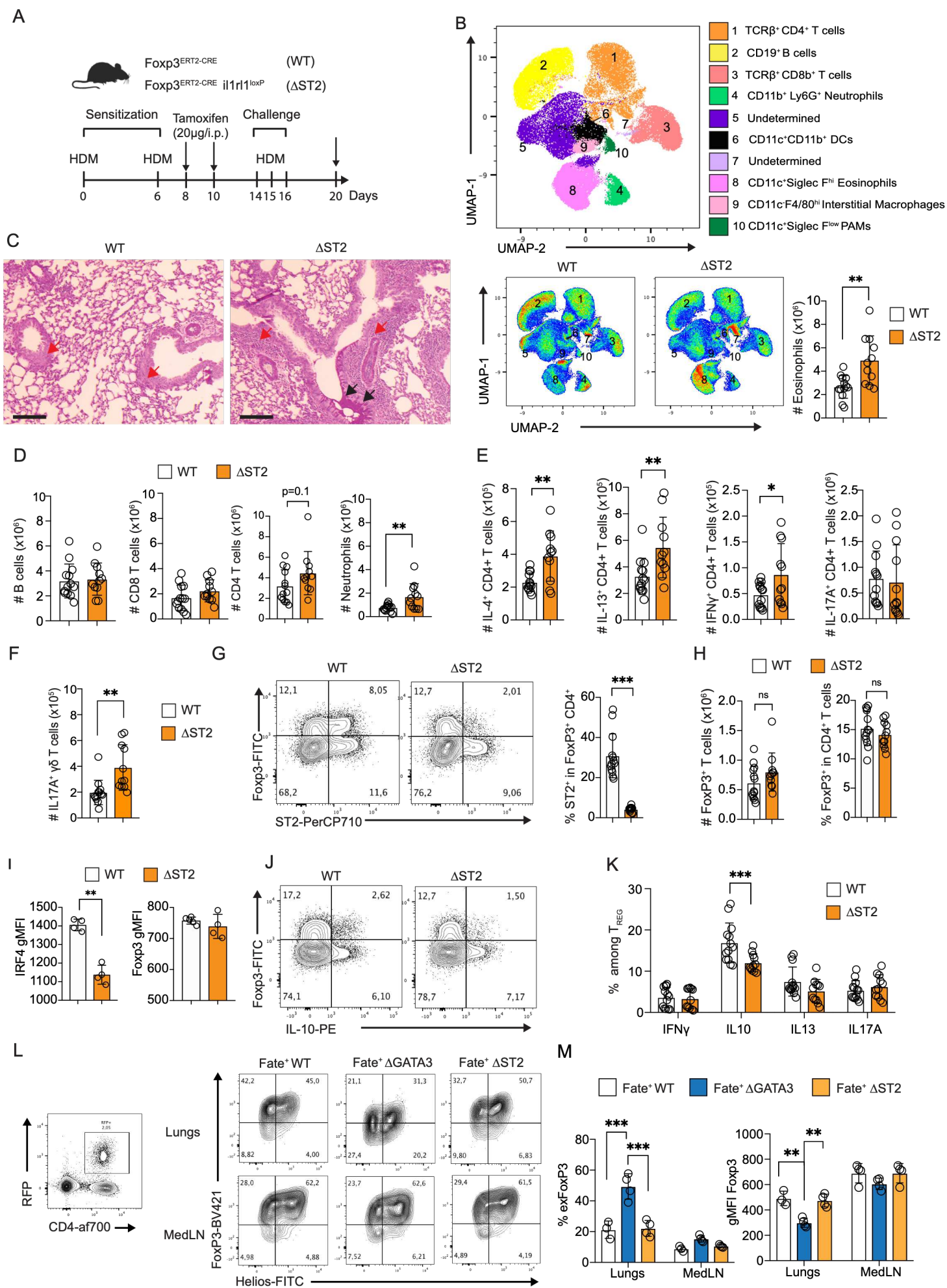


Figure 4

Figure 5. IL-33 is required for pulmonary T_{REG} cells to suppress allergen-driven innate and adaptive immune responses in the lungs of adult mice.

- (A) 8-10 weeks old Foxp3^{ERT2-CRE} (WT) or *il1rl1*^{loxP} (T_{REG}^{ΔST2}; ΔST2) female mice were sensitized with 10 μg/mouse of HDM (*Dermatophagoides pteronyssinus* extract; Greer Laboratories) at days 0 and 7. In between the sensitization and the challenge, the mice received 20 μg of tamoxifen in corn oil i.p. (days 8 and 10), before 3 consecutive days of HDM challenge and the lungs were perfused and collected at day 20. Compiled data from 3 independent experiments (n=3-4/group; N=3).
- (B) Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP v4.0.3; <https://arxiv.org/abs/1802.03426>) visualisation of the live CD45⁺ cells isolated from the lungs. The cluster were identified using an unbiased FlowSOM (v3.0.18; Grassen et al. 2015) clustering algorithm to visualise by Cluster Explorer (v1.7.6; Joseph Trotter, FlowJO Exchange). (bottom) Representative UMAP plot of the total live CD45⁺ cells isolated from 1 mouse from each group and counts of CD11c⁺Siglec-F^{hi}Ly6G⁻ eosinophils in the lungs. Student T test. **p<0.01.
- (C) Representative histology (Periodic acid-Schiff stain) of the lungs from one mouse per group at day 20. Cellular infiltrates (red arrows) and mucus deposition (black arrows) highlighted. Scale at 100μm.
- (D) Number of CD19⁺ B cells, CD8b⁺ T cells, CD4⁺ T cells and CD11b⁺Ly6G⁺ neutrophils in the lungs at day 20. Student T test. **p<0.01
- (E) Number of IL-4, IL-13, IFNγ and IL17A expressing CD4⁺ T cells isolated from the lungs and activated for 3 hours in PMA + ionomycin + GolgiStop™ at day 20. Student T test. *p<0.05; **p<0.01
- (F) Number of IL17A expressing γδ TCR⁺ T cells isolated from the lungs and activated for 3 hours in PMA + ionomycin + GolgiStop™ at day 20. Student T test. **p<0.01
- (G) Representative flow cytometry of the expression of Foxp3 and ST2 in CD4⁺ T cells and frequency of ST2⁺ among CD4⁺Foxp3⁺ T cells isolated from the lungs at day 20. Student T test. ***p<0.001
- (H) Number of CD4⁺ Foxp3⁺ T cells and frequency of Foxp3⁺ among CD4⁺ T cells in the lungs at day 20. Student T test. ***p<0.001
- (I) Geometric mean fluorescence intensity of IRF4 and Foxp3 among Foxp3⁺ CD4⁺ T cells at day 20. Representative of 3 distinct experiments. Student T test. ***p<0.001
- (J) Representative flow cytometry of IL-10 and Foxp3 expression among CD4⁺ T cells isolated from the lungs at day 20 and stimulated for 3 hours with PMA + ionomycin + Golgi Stop™.
- (K) Frequencies of IFNγ, IL-10, IL-13 and IL17A among CD4⁺ Foxp3⁺ T cells isolated from the lungs and activated for 3 hours in PMA + ionomycin + GolgiStop™ at day 20. Two-way ANOVA. ***p<0.001.
- (L-M) 8–10-week-old female T_{REG}-Fate⁺ (Fate⁺ WT), *gata3*^{loxP} (Fate⁺ΔGATA3) mice and *il1rl1*^{loxP} (Fate⁺ΔST2) were sensitized with 10 μg/mouse of HDM (*Dermatophagoides pteronyssinus* extract) using the same methodology as in (A).
- (L) Representative flow cytometry of the expression of Foxp3 and Helios in RFP⁺CD4⁺ T cells (representative gating in left indent) in the lungs or mediastinal lymph node (MedLN) of the mice.
- (M) Percent of Foxp3⁻ (exT_{REG}) from the lungs or mediastinal lymph nodes (MedLN) at day 20 and geometric MFI (gMFI) of Foxp3 among RFP⁺ CD4⁺ T cells. Two-way ANOVA. ***p<0.001, **p<0.01

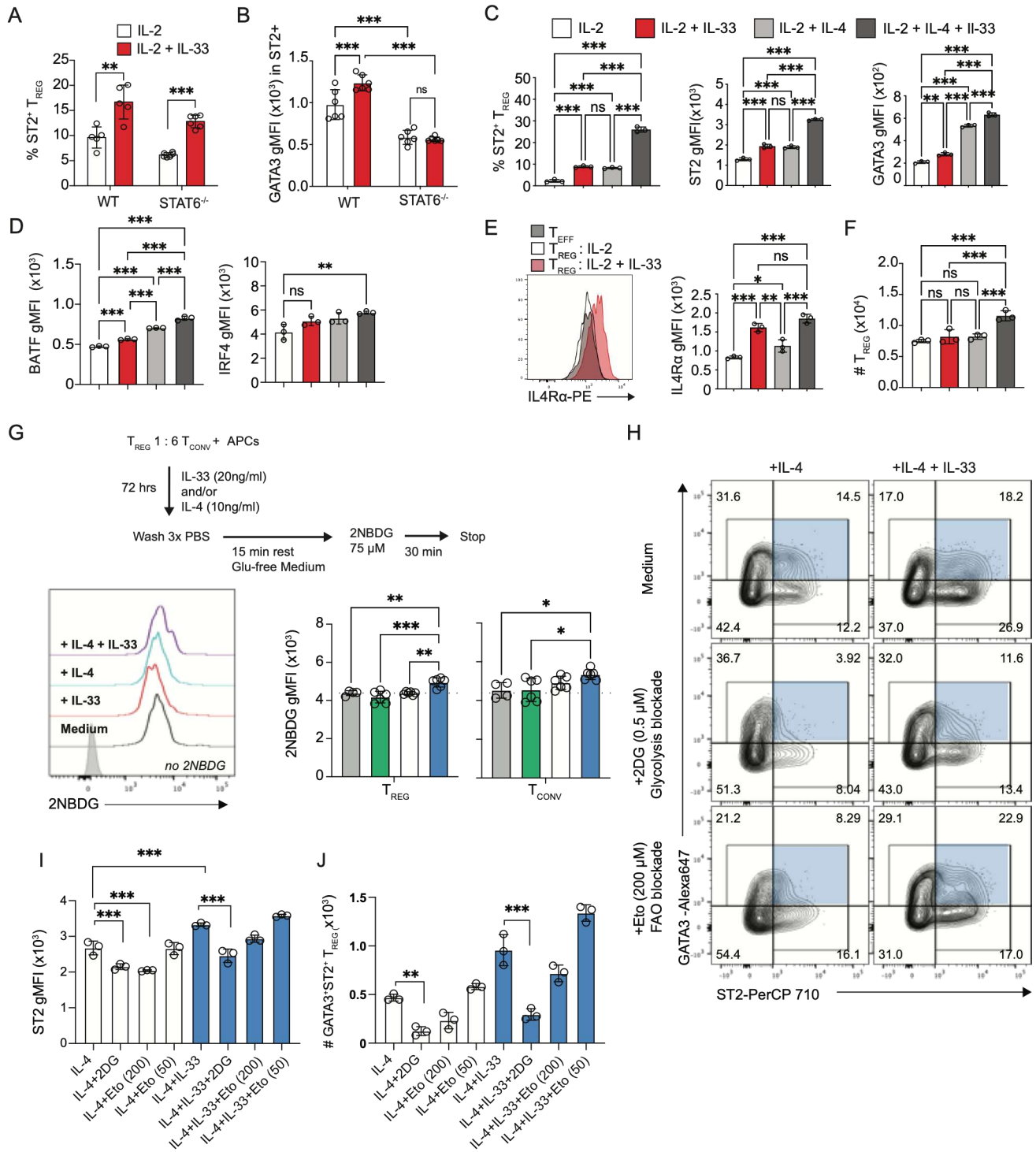


Figure 6

Figure 6. IL-33 promotes the generation and expansion of specialized IL4R α ^{hi} GATA3⁺ ST2⁺ T_{REG} cells through a glycolysis-dependent program.

- (A) MACs sorted CD4⁺ T cells from BALB/c or Δ STAT6 mice were activated *in vitro* in the presence of IL-2 (100U/ml) and IL-33 (10ng/ml) for 72 hours. Representative of 2 independent experiments. Frequency of ST2⁺ T_{REG} among live cells. One-way ANOVA. Tukey post-hoc test. ***p<0.001, **p<0.01, *p<0.05
- (B) Geometric mean fluorescence intensity (gMFI) of GATA3 in CD4⁺Foxp3⁺ ST2⁺ cells from Δ STAT6 or WT and Two-way ANOVA. Tukey post-hoc test. ***p<0.001
- (C) CD4⁺GFP⁺ T cells were isolated from the spleen of B6.Foxp3^{GFP^{ki}} mice and sorted as T_{REG}. The cells were activated by plated α CD3 (5ug/ml) and α CD28 in the presence of IL-2 (200U/ml) (Medium) with added IL-33 (10ng/ml), IL-4 (50ng/ml) or a combination of both. Representative of 3 independent experiments. Frequency of ST2⁺ T_{REG} cell and geometric mean fluorescence intensity (MFI) of ST2 and GATA3 at 72 hours. One-way ANOVA. Tukey post-hoc test. ***p<0.001, **p<0.01
- (D) Geometric mean fluorescence intensity (gMFI) of the expression of BATF and IRF4 in T_{REG} cells at 72 hours. One-way ANOVA. Tukey post-hoc test. ***p<0.001, **p<0.01
- (E) Representative histogram of IL4R α expression on CD4⁺Foxp3⁺ cells and geometric mean fluorescence intensity (MFI) of IL4R α on T_{REG}.
- (F) Number of live CD4⁺ Foxp3⁺ cells at 72 hours. One-way ANOVA. Tukey post-hoc test. ***p<0.001
- (G) GFP⁺ CD4⁺ T cells (T_{REG}) from the spleen of B6.Foxp3^{GFP} reporter mice sorted by FACs Aria® were TCR activated in the presence of CTV-stained conventional Foxp3-GFP^{neg}CD4⁺ T cells (T_{CONV}) at a ratio of 1:6 in the presence of mitomycin-treated CD4⁺ cells (APCs) from the same donor. After 72 hours of proliferation in the presence of IL-33 (20ng/ml) and/or IL-4 (10ng/ml), the media was washed with PBS, left to rest for 15 minutes in fresh temperature-controlled glucose-free medium and exposed to glucose-free medium containing 2NBDG for another 30 minutes. Representative histogram of the intracellular absorption of 2NBDG in CTV^{neg} Foxp3⁺CD4⁺ T cells in different conditions and geometric mean fluorescence intensity (gMFI) of 2NBDG amongst live CTV^{neg} Foxp3⁺CD4⁺ T (left) and live CTV⁺Foxp3^{neg}CD4⁺ T cells (T_{CONV}).
- (H) Representative flow cytometry of ST2 and GATA3 expression in Foxp3-GFP⁺ CD4⁺ T cells (T_{REG}) following the same methodology, but in the presence of 2DG (0.5 μ M) or Etomixir (Eto; 50 or 200 μ M) at the start of the culture (time 0hr). Representative of 3 independent experiments.
- (I) Geometric mean fluorescence intensity (gMFI) of ST2 on the surface of Foxp3-GFP⁺ CD4⁺ T cells (T_{REG}) at 72 hrs.in the presence of 10 ng/ml IL-4 (white bars) or in combination with 20 ng/ml of IL-33 (blue bars). One-way ANOVA. Tukey post-hoc test. ***p<0.001.
- (J) Number of live ST2⁺GATA3⁺ CD4⁺ Foxp3⁺ (T_{REG}) cells at 72 hours. One-way ANOVA. Tukey post-hoc test. ***p<0.001.

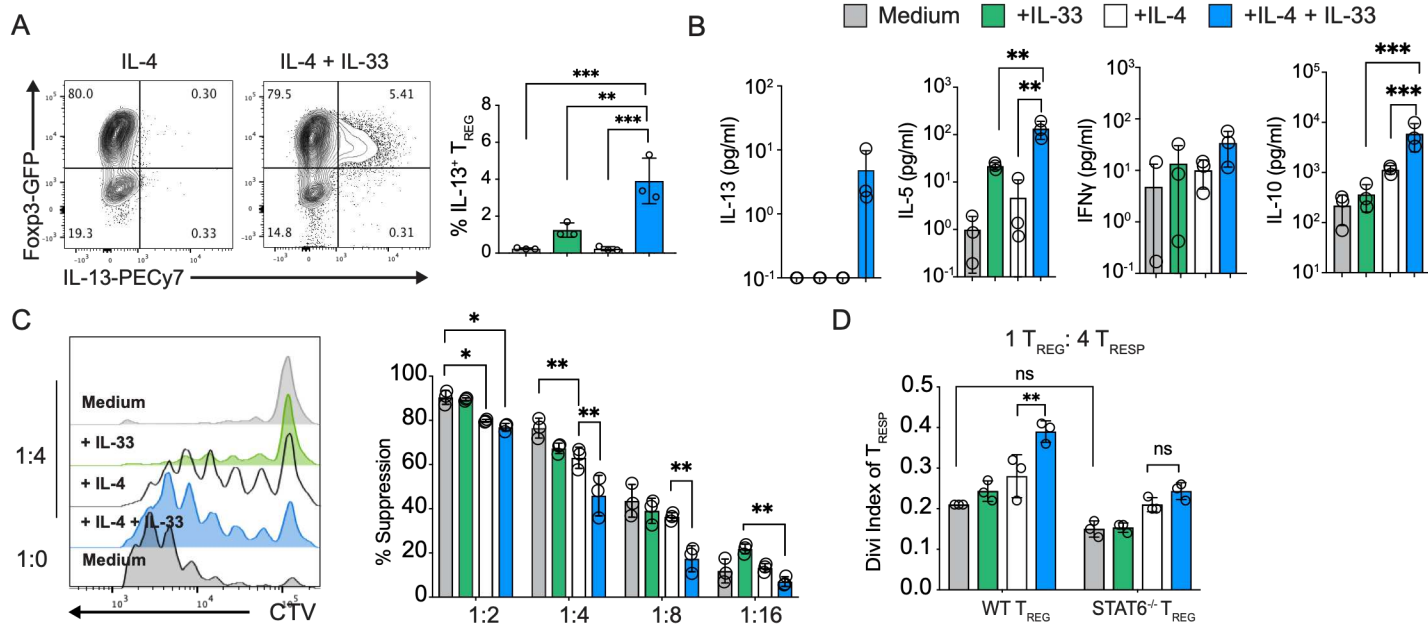


Figure 7

Figure 7. IL-33 and IL-4 synergize to promote T_H2 cytokine production and dysregulates T_{REG} cell-mediated suppression.

- (A) CD4⁺GFP⁺ T cells were isolated from the spleen of B6.Foxp3^{GFPki} mice and sorted as T_{REG}. The cells were activated by plated α CD3 (5 μ g/ml) and α CD28 in the presence of IL-2 (200U/ml) (Medium) with added IL-33 (10ng/ml), IL-4 (50ng/ml) or a combination of both. After 72 hours, the T_{REG} cells were exposed to PMA + ionomycin + Golgi-Stop® for 3 hours. Representative flow cytometry plot of the expression of IL-13 and Foxp3 in live cells and frequency of IL-13⁺ CD4⁺ Foxp3⁺ (T_{REG}) at 72 hours. Representative of 3 distinct experiments.
- (B) Multiplex ELISA of the expression of IL-10, IL-13, IL-5 and IFN γ obtained from the supernatant from (A) prior to the PMA + ionomycin + Golgi-Stop® stimuli. Each point represents 1 distinct experiment, technical triplicates were combined in a single sample (N=3/ n=3). The quantities (pg/ml) were normalized per 10 000 cells. One-way ANOVA. Tukey correction. *p<0.05; **p<0.01; ***p<0.001.
- (C) CD4⁺GFP⁺ (T_{REG}) and CD4⁺GFP⁻ (T_{RESP}) cells (B6.Foxp3^{GFPki}) were sorted and the T_{REG} were co-cultured at 1:2 (25 x10³) 1:4 (12.5 x10³) or 1:8 (6.75 x10³) with 50 x10³ CTV-labelled T_{RESP} cells and activated with α CD3 and irradiated APCs for 72 hours. Representative histogram of the proliferation of CTV-labelled T_{RESP} cells in distinct conditions at 72 hours and % suppression (1- Div. index/ Div.index 1:0) *100). Two-way ANOVA. *p<0.05; **p<0.01; ***p<0.001. Representative of 3 distinct experiments.
- (D) CD4⁺CD25^{hi} (T_{REG}) and CD25⁻ (T_{RESP}) cells were sorted from BALB/C (WT) and STAT6^{-/-} mice. T_{RESP} cells from WT mice were CTV labelled and cultured at a 1 T_{REG} to 4 T_{RESP} (1:4 ratio) in the presence of irradiated APCs (CD4⁻ fraction) for 72 hours. Division index of the expansion of T_{RESP} cells. Two-way ANOVA. **p<0.01. Representative of 2 distinct experiments.

REFERENCES

1. Wu P, Hartert TV. Evidence for a causal relationship between respiratory syncytial virus infection and asthma. *Expert Rev Anti Infect Ther.* 2011;9(9):731-45.
2. Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, et al. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. *Lancet.* 1999;354(9178):541-5.
3. Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. *Clin Microbiol Rev.* 2010;23(1):74-98.
4. Basha S, Surendran N, Pichichero M. Immune responses in neonates. *Expert Rev Clin Immunol.* 2014;10(9):1171-84.
5. Roux X, Remot A, Petit-Camurdan A, Nahori MA, Kiefer-Biasizzo H, Marchal G, et al. Neonatal lung immune responses show a shift of cytokines and transcription factors toward Th2 and a deficit in conventional and plasmacytoid dendritic cells. *Eur J Immunol.* 2011;41(10):2852-61.
6. Singh R, Alape D, de Lima A, Ascanio J, Majid A, Gangadharan SP. Regulatory T Cells in Respiratory Health and Diseases. *Pulm Med.* 2019;2019:1907807.
7. Cormier SA, You D, Honnegowda S. The use of a neonatal mouse model to study respiratory syncytial virus infections. *Expert Rev Anti Infect Ther.* 2010;8(12):1371-80.
8. Durant LR, Makris S, Voorburg CM, Loebbermann J, Johansson C, Openshaw PJ. Regulatory T cells prevent Th2 immune responses and pulmonary eosinophilia during respiratory syncytial virus infection in mice. *J Virol.* 2013;87(20):10946-54.
9. Comas-Garcia A, Lopez-Pacheco CP, Garcia-Zepeda EA, Soldevila G, Ramos-Martinez P, Ramos-Castaneda J. Neonatal respiratory syncytial virus infection has an effect on lung

inflammation and the CD4(+) CD25(+) T cell subpopulation during ovalbumin sensitization in adult mice. *Clin Exp Immunol*. 2016;185(2):190-201.

10. Yang J, Zou M, Chu X, Floess S, Li Y, Delacher M, et al. Inflammatory perturbations in early life long-lastingly shape the transcriptome and TCR repertoire of the first wave of regulatory T cells. *Front Immunol*. 2022;13:991671.

11. Han Y, Ouyang X, Chen Y, Lai S, Sun H, Wu N, et al. scRNA-seq profiling of neonatal and adult thymus-derived CD4+ T cells by a T cell origin-time tracing model. *J Mol Cell Biol*. 2023;14(12).

12. Alvarez F, Istomine R, Shourian M, Pavey N, Al-Aubodah TA, Qureshi S, et al. The alarmins IL-1 and IL-33 differentially regulate the functional specialisation of Foxp3(+) regulatory T cells during mucosal inflammation. *Mucosal Immunol*. 2019;12(3):746-60.

13. Arpaia N, Green JA, Molledo B, Arvey A, Hemmers S, Yuan S, et al. A Distinct Function of Regulatory T Cells in Tissue Protection. *Cell*. 2015;162(5):1078-89.

14. Liu Q, Dwyer GK, Zhao Y, Li H, Mathews LR, Chakka AB, et al. IL-33-mediated IL-13 secretion by ST2+ Tregs controls inflammation after lung injury. *JCI Insight*. 2019;4(6).

15. Schiering C, Krausgruber T, Chomka A, Frohlich A, Adelmann K, Wohlfert EA, et al. The alarmin IL-33 promotes regulatory T-cell function in the intestine. *Nature*. 2014;513(7519):564-8.

16. Faustino LD, Griffith JW, Rahimi RA, Nepal K, Hamilos DL, Cho JL, et al. Interleukin-33 activates regulatory T cells to suppress innate gammadelta T cell responses in the lung. *Nat Immunol*. 2020;21(11):1371-83.

17. Saikumar Jayalatha AK, Hesse L, Ketelaar ME, Koppelman GH, Nawijn MC. The central role of IL-33/IL-1RL1 pathway in asthma: From pathogenesis to intervention. *Pharmacol Ther*. 2021;225:107847.

18. Momen T, Ahanchian H, Reisi M, Shamsdin SA, Shahsanai A, Keivanfar M. Comparison of Interleukin-33 Serum Levels in Asthmatic Patients with a Control Group and Relation with the Severity of the Disease. *Int J Prev Med*. 2017;8:65.

19. Sjoberg LC, Nilsson AZ, Lei Y, Gregory JA, Adner M, Nilsson GP. Interleukin 33 exacerbates antigen driven airway hyperresponsiveness, inflammation and remodeling in a mouse model of asthma. *Sci Rep*. 2017;7(1):4219.

20. Chen CC, Kobayashi T, Iijima K, Hsu FC, Kita H. IL-33 dysregulates regulatory T cells and impairs established immunologic tolerance in the lungs. *J Allergy Clin Immunol*. 2017;140(5):1351-63 e7.

21. Tregoning JS, Yamaguchi Y, Harker J, Wang B, Openshaw PJ. The role of T cells in the enhancement of respiratory syncytial virus infection severity during adult reinfection of neonatally sensitized mice. *J Virol*. 2008;82(8):4115-24.

22. Hassan J, Reen DJ. Reduced primary antigen-specific T-cell precursor frequencies in neonates is associated with deficient interleukin-2 production. *Immunology*. 1996;87(4):604-8.

23. Guo L, Wei G, Zhu J, Liao W, Leonard WJ, Zhao K, et al. IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells. *Proc Natl Acad Sci U S A*. 2009;106(32):13463-8.

24. Harker JA, Yamaguchi Y, Culley FJ, Tregoning JS, Openshaw PJ. Delayed sequelae of neonatal respiratory syncytial virus infection are dependent on cells of the innate immune system. *J Virol*. 2014;88(1):604-11.

25. Alvisi G, Brummelman J, Puccio S, Mazza EM, Tomada EP, Losurdo A, et al. IRF4 instructs effector Treg differentiation and immune suppression in human cancer. *J Clin Invest.* 2020;130(6):3137-50.
26. Cretney E, Xin A, Shi W, Minnich M, Masson F, Miasari M, et al. The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. *Nat Immunol.* 2011;12(4):304-11.
27. Voss LC, Schmidt C, Harberts A, Spohn M, Bradtke P, Borchers A, et al. Interferon Regulatory Factor 4 dose-dependently controls peripheral Treg cell differentiation and homeostasis by modulating chromatin accessibility in mice. *Front Immunol.* 2025;16:1604888.
28. Yu W, Ji N, Gu C, Yao J, Ding M, Zhou D, et al. IRF4 is Correlated with the Conversion to a Th17-Like Phenotype in Regulatory T Cells from the Malignant Pleural Effusion. *Int J Gen Med.* 2021;14:6009-19.
29. Saravia J, You D, Shrestha B, Jaligama S, Siefker D, Lee GI, et al. Respiratory Syncytial Virus Disease Is Mediated by Age-Variable IL-33. *PLoS Pathog.* 2015;11(10):e1005217.
30. Iijima K, Kobayashi T, Matsumoto K, Ohara K, Kita H, Drake LY. Transient IL-33 upregulation in neonatal mouse lung promotes acute but not chronic type 2 immune responses induced by allergen later in life. *PLoS One.* 2021;16(5):e0252199.
31. Hayakawa M, Yanagisawa K, Aoki S, Hayakawa H, Takezako N, Tominaga S. T-helper type 2 cell-specific expression of the ST2 gene is regulated by transcription factor GATA-3. *Biochim Biophys Acta.* 2005;1728(1-2):53-64.
32. Alladina J, Smith NP, Kooistra T, Slowikowski K, Kernin JJ, Deguine J, et al. A human model of asthma exacerbation reveals transcriptional programs and cell circuits specific to allergic asthma. *Sci Immunol.* 2023;8(83):eabq6352.
33. Alvarez F, Liu Z, Bay A, Piccirillo CA. Deciphering the developmental trajectory of tissue-resident Foxp3(+) regulatory T cells. *Front Immunol.* 2024;15:1331846.
34. Delacher M, Imbusch CD, Weichenhan D, Breiling A, Hotz-Wagenblatt A, Trager U, et al. Genome-wide DNA-methylation landscape defines specialization of regulatory T cells in tissues. *Nat Immunol.* 2017;18(10):1160-72.
35. Khatun A, Wu X, Qi F, Gai K, Kharel A, Kudek MR, et al. BATF is Required for Treg Homeostasis and Stability to Prevent Autoimmune Pathology. *Adv Sci (Weinh).* 2023;10(28):e2206692.
36. Collision LW, Vignali DA. In vitro Treg suppression assays. *Methods Mol Biol.* 2011;707:21-37.
37. Zaghouani H, Hoeman CM, Adkins B. Neonatal immunity: faulty T-helpers and the shortcomings of dendritic cells. *Trends Immunol.* 2009;30(12):585-91.
38. Wohlfert EA, Grainger JR, Bouladoux N, Konkel JE, Oldenhove G, Ribeiro CH, et al. GATA3 controls Foxp3(+) regulatory T cell fate during inflammation in mice. *J Clin Invest.* 2011;121(11):4503-15.
39. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, et al. A large-scale, consortium-based genomewide association study of asthma. *N Engl J Med.* 2010;363(13):1211-21.
40. Srinivasa BT, Restori KH, Shan J, Cyr L, Xing L, Lee S, et al. STAT6 inhibitory peptide given during RSV infection of neonatal mice reduces exacerbated airway responses upon adult reinfection. *J Leukoc Biol.* 2017;101(2):519-29.

41. Weiss K, Wanner N, Queisser K, Frimel M, Nunn T, Myshrall T, et al. Barrier Housing and Gender Effects on Allergic Airway Disease in a Murine House Dust Mite Model. *Immunohorizons*. 2021;5(1):33-47.
42. Lytle CD, Sagripanti JL. Predicted inactivation of viruses of relevance to biodefense by solar radiation. *Journal of virology*. 2005;79(22):14244-52.
43. Sun Y, López CB. Preparation of Respiratory Syncytial Virus with High or Low Content of Defective Viral Particles and Their Purification from Viral Stocks. *Bio Protoc*. 2016;6(10).
44. REED LJ, MUENCH H. A SIMPLE METHOD OF ESTIMATING FIFTY PER CENT ENDPOINTS¹². *American Journal of Epidemiology*. 1938;27(3):493-7.
45. Nakada EM, Shan J, Kinyanjui MW, Fixman ED. Adjuvant-dependent regulation of interleukin-17 expressing $\gamma\delta$ T cells and inhibition of Th2 responses in allergic airways disease. *Respir Res*. 2014;15(1):90.
46. Dominguez-Villar M, Hafler DA. Regulatory T cells in autoimmune disease. *Nat Immunol*. 2018;19(7):665-73.

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