ROLE OF THE IL-1 FAMILY OF ALARMINS ON THE FUNCTIONAL ADAPTATION OF REGULATORY T CELLS TO MUCOSAL INFECTIONS

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À Marie-Jeanne et Florence,

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Novel scientific knowledge requires the commitment of many. This is why this section is purposely placed at the very beginning, for without support from my family, friends, professors, colleagues and collaborators, none of this work would have been possible.

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Abstract

Regulatory T cells (T_{REG}) are a specialized subset of $CD4^+$ T cells that express the forkhead-box p3 (Foxp3) transcription factor, the master-regulator driving their suppressive phenotype. These cells can acquire tissue-specific adaptations that enable them to migrate and accumulate at mucosal surfaces where they play a critical, non-redundant, role in the maintenance of immune homeostasis. However, it remains to be understood how T_{REG} cells modulate their suppressive function during an infection, when an effective immune response is required. Using an unbiased screen to study the transcriptional changes that dictate the fate of T_{REG} cells in inflammation, we identified the differential expression of IL-1 alarmin receptors between T_{REG} cells that maintained or T_{REG} cells that lost Foxp3 expression. Alarmins are small molecules released upon cellular damage by structural and immune cells to communicate the level of danger to the surrounding milieu. Thus, we hypothesized that IL-1 alarmins dictate the functional adaptation of T_{REG} cells at mucosal surfaces.

In this work, we describe how IL-1β, IL-33 and IL-18, play critical roles in the local adaptation of T_{REG} cells to mucosal inflammation. First, we demonstrate using both T_H1 or T_H17-dominant viral and fungal infections that IL-33 and IL-1β signaling dictate the balance between suppressive (IL-33R/$ST2^+$) or permissive (IL-$1R1^+$) T_{REG} populations that orchestrates the immune response. Second, using a viral infection characterized by an exacerbated T_H2 response, we demonstrate that the pro-suppressive effect of IL-33 on $ST2^+$ T_{REG} cells is context dependent, as it is dampened by STAT6 signaling. Third, we uncovered that in the course of viral and parasitic infection a population of T_{REG} cells gain the ability to respond to IL-18 which, in turn, drives the proliferation and the expression of IFNγ in these cells and impairs their suppressive ability. Collectively, these results reveal that IL-1β, IL-33 and IL-18 provide a dynamic signal to distinct populations of mucosa-adapted T_{REG} cells that orchestrates the establishment of an effective and controlled adaptive response. Understanding how T_{REG} cells modulate their adaptation to local tissue environments is key towards developing targeted therapies in autoimmune, infectious and oncologic diseases.

Résumé

Les lymphocytes T régulateurs (T_{REG}) sont une population de lymphocytes T $CD4^+$ qui expriment forkhead-box P3 (Foxp3), un facteur de transcription qui joue un rôle clé dans l'expression des fonctions suppressives de la cellule. Ces cellules s'adaptent aux conditions inflammatoires pour pouvoir migrer et survivre en grand nombre dans les muqueuses où elles jouent un rôle critique dans le maintien de l'homéostase immunitaire. Toutefois, nous ne savons pas encore comment les T_{REG} modifient leur fonction suppressive lors d'une infection où une réponse immunitaire efficace est requise. En analysant des changements dans le programme transcriptionnel de T_{REG} lors d'inflammation, nous avons identifiés que des récepteurs d'alarmines de la famille de l'IL-1 étaient préférentiellement exprimés entre des T_{REG} qui ont maintenu et ceux qui perdu l'expression de Foxp3. Les alarmines, de petites molécules activées lors de dommage cellulaire, sont un moyen par lequel les cellules épithéliales et immunitaires communiquent le niveau de danger. Nous avons donc émis l'hypothèse que ces alarmines modulent différemment la fonction et le sort des cellules T_{REG} au fil de la réponse immunitaire.

À travers cette thèse, nous démontrons que l'IL-1β, l'IL-33 et l'IL-18, jouent un rôle critique dans l'adaptation des T_{REG}. En premier lieu, nous démontrons que l'IL-33 et l'IL-1β dictent la balance locale de T_{REG} suppressifs (IL-33R/$ST2^+$) ou permissifs ($IL\text{-}1R1^+$) qui sont essentiels à la réponse antivirale et antifungique orchestrée par des lymphocytes T_H1 et T_H17. En second lieu, nous démontrons que la fonction suppressive des T_{REG} $ST2^+$ dépend du contexte inflammatoire puisque lors d'une infection virale caractérisée par une réponse T_H2 elle est atténuée par un signal STAT6. Troisièmement, dans le cadre d'une infection virale et parasitaire, une population de cellules T_{REG} acquiert la capacité de répondre à l'IL-18, ce qui les mènent à produire de l'IFNγ et bloque leur capacité suppressive. Collectivement, ces résultats démontrent que l'IL-1β, l'IL-33 et l'IL-18 offrent un signal qui dicte l'adaptation des cellules T_{REG} à la muqueuse de manière à faciliter une réponse immunitaire efficace et contrôlée. Une connaissance des signaux qui dictent l'adaptation des cellules T_{REG} au site d'inflammation ouvre la voie au développement de thérapies ciblées dans le cadre de maladies auto-immunes, infectieuses et oncologiques.

Author contribution

The core of this thesis is the collection of three original manuscripts, one of which is published, another is in submission and one is currently being revised. The author contribution for the work presented in this thesis is as follows:

Chapter 1. General Introduction

Authors: I wrote the literature review

Chapter 2. The alarmins IL-1 and IL-33 differentially regulate the functional specialization of Foxp3+ regulatory T cells during mucosal inflammation

Authors: Fernando Alvarez, Roman Istomine, Mitra Shourian, Nils Pavey, Tho Al-Fakar Al-Aubodah, Salman Qureshi, Jörg H. Fritz, Ciriaco A. Piccirillo

I was responsible for the design of all the experiments (in collaboration with my supervisors Dr J. Fritz and Dr C. Piccirillo). I performed all the experiments and analysis of this study and wrote the paper. My colleagues Roman Istomine and Nils Pavey contributed to the *in vivo* Influenza experiments. Mitra Shourian and Dr S. Qureshi provided the model for the experiments involving *Cryptococcus neoformans*. My colleague Tho Al-Aubodah provided support for the colitis experiments. All authors provided valuable input throughout the study and the writing of the manuscript.

Chapter 3. Aberrant memory T_H2 responses against neonatal RSV infection impair the functional adaptation of $ST2^+$ T_{REG} cells through enhanced STAT6 signaling

Authors: Fernando Alvarez, Katherine Restori, Lydia Labrie, Roman Istomine, Elizabeth Fixman, Jörg H. Fritz, Ciriaco A. Piccirillo

I was responsible for the design of the experiments in collaboration with my supervisor Dr C. Piccirillo and co-supervisor Dr J Fritz. I performed all the experiments and analysis of this study and wrote the paper. Katerine Restori, Lydia Labrie and Dr Elizabeth Fixman provided the model of Respiratory Syncytial Virus (RSV) infection and contributed to the experiments. Roman Istomine contributed to the *in vivo* experiments. All authors provided valuable input throughout the study and the writing of the manuscript.

Chapter 4. The role of IL-18 in the functional adaptation of $Foxp3^+$ regulatory T cells in T_H1-driven mucosal infections

Authors: Fernando Alvarez, Roman Istomine, Alonso Da Silva Lira Filho, Tho Al-Fakar Al-Aubodah, Daniel Huang, Rakan Okde, Martin Olivier, Salman Qureshi, Jörg H. Fritz, Ciriaco A. Piccirillo

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I have also co-authored the following reviews

***Alvarez F**, Fritz JH, Piccirillo CA*. Pleiotropic Effects of IL-33 on CD4(+) T Cell Differentiation and Effector Functions. *Frontiers in immunology* 2019; **10:** 522.

Alvarez F, Aubodah T*, Yujian Y*, Piccirillo CA*. Mechanisms of T_{REG} cell adaptation to inflammation. *Journal of Leucocyte Biology.* 2020;1-13*. *These authors have contributed equally*

I am currently completing the manuscript on the following article

***Alvarez F**, Hendin H, Hodgins B, Pillet S, Fritz JH, Landry N, Ward B, Piccirillo, C*, Plant-derived H1-coated virus-like particles generate effective memory regulatory and effector T cell responses against Influenza A infection in both young and aged mice. *Manuscript in preparation.*

I have also contributed to the following articles

Bin Dhuban K, d'Hennezel E, Nagai Y, Xiao Y, Shao S, Istomine R, ***Alvarez F*** *et al.* Suppression by human FOXP3(+) regulatory T cells requires FOXP3-TIP60 interactions. Science immunology 2017; 2(12).

Istomine R, ***Alvarez F****, Almadani Y, Philip A, Piccirillo CA.* The Deubiquitinating Enzyme Ubiquitin-Specific Peptidase 11 Potentiates TGF-beta Signaling in CD4(+) T Cells to Facilitate Foxp3(+) Regulatory T and TH17 Cell Differentiation. *J Immunol 2019; 203(9): 2388-2400.*

Ayoub M, Shinde-Jadhav S, Mansure JJ, ***Alvarez F,*** *Connell T, Seuntjens J et al.* The immune mediated role of extracellular HMGB1 in a heterotopic model of bladder cancer radioresistance. *Scientific reports 2019;* ***9****(1)****:*** *6348.*

Robichaud N, Hsu BE, Istomine R, ***Alvarez F***, Blagih J, Ma EH *et al.* Translational control in the tumor microenvironment promotes lung metastasis: Phosphorylation of eIF4E in neutrophils. *Proceedings of the National Academy of Sciences of the United States of America* 2018; **115**(10)**:** E2202-e2209.

I, Fernando Alvarez, have read, understood and abided by all norms and regulations of academic integrity of McGill University

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CHAPTER 1 – General Introduction

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1. Immune homeostasis

Homeostasis is the dynamic process of maintaining a state of equilibrium in order to generate the optimal function of our organs. This process is critical for our survival as it is intimately linked with our ability to obtain nutrients and oxygen and release toxic waste. However, direct contact with the environment exposes us to a myriad of dangers. In order to respond to these threats and support homeostasis, the immune system has evolved a complex network of specialized cells and signalling molecules.

The vast majority of our immune cells are located at mucosal surfaces, at the forefront in our interaction with the environment. Here, structural cells play a critical part in communicating the level of danger, while immune cells provide the effector branch of the response. In order to maintain the integrity of the mucosa, immune cells, from both the innate and adaptive branch, develop strategies in order to suppress unwanted inflammation all-the-while facilitating potent and rapid effector responses when required. In particular, the adaptive immune system, composed notably of T and B cells, offers an especially specific and sensitive response, as it is capable of recognizing very low amounts of antigen and providing long-term protection against pathogens.

However, the effectiveness of the adaptive immune response comes at a cost, as it can get embroiled into a costly inflammatory spiral, in turn contributing to cellular damage, organ failure and death. As such, regulatory mechanisms have evolved to stop unwanted inflammatory responses, prevent prolonged immune responses and facilitate local repair mechanisms. These regulatory elements include central and peripheral tolerance mechanisms that prevent autoimmune injury, as well as unique regulatory elements at mucosal surfaces.

1.1 Mechanisms of central tolerance

Central tolerance occurs during the thymic development of T cells. During this process, precursor αβ T cell (Double negative, DN) from the bone-marrow enter the

thymus where they differentiate into $CD4^+CD8^+$ T cells through upregulation of both CD4 and CD8 co-receptors (Double positive, DP) and V(D)J recombination of their T cell receptor (TCR) locus through the recombination-activating genes RAG1 and RAG2. When the cells recognize the peptide-major histocompatibility complex (MHC) on cortical thymic epithelial cells (cTEC) they receive the signal for the conversion to single $CD4^+$ or $CD8^+$ T cell (Single positive, SP) according to their affinity to MHC-I or MHC-II complexes, failure of which drives anergy in faulty T cells that are later phagocytosed (1, 2). After this positive selection, the SP T cells migrate to the medullar region where they encounter medullary thymic epithelial cells (mTECs) and dendritic cells that express self-peptides on their MHC in order to select and drive the apoptosis of strongly self-reactive T clones (3). However, contrary to the belief that the thymic clonal deletion eliminates all self-reactive T cells, it does, in fact, "prune" the amount of self-reactive T cells (4), in order to avoid generating a gap in the T cell repertoire (5). This is consequential, as it is estimated that at least 4% of circulating T cell are self-reactive with generally low avidity (6). As such, peripheral control mechanisms are required in order to preserve immune tolerance.

1.2 Mechanisms of peripheral tolerance

There are multiple processes that restrict self-reactive T cells in the periphery in order to avoid lymphoproliferative autoimmune disease. These processes involve 1) peripheral deletion of chronically activated T cells, 2) activation-induced anergy, 3) antigenic ignorance, 4) inhibitory signals and 5) specialized regulatory cells.

Peripheral deletion of chronically activated T cells. Peripheral deletion through activation induced cell death (AICD) is a process by which T cells, that are chronically stimulated through repeated activation, are eliminated by apoptosis. This process is mediated through Fas-FasL ligation on T cells (7). Mice lacking Fas or FasL develop lymphadenopathies, while humans with functional mutations in these genes display an autoimmune lymphoproliferative syndrome (8), indicating that this process is key towards eliminating self-reactive T cells.

Activation-induced anergy. Another strategy that is observed to prevent further activation and clonal expansion of self-reactive T cells is through the induction of T cell anergy. Indeed, the process of T cell activation requires two main signals; the first through the engagement of the TCR with its cognate antigen and MHC, the second the ligation of the extracellular receptor CD28 to co-stimulatory receptors on the antigen-presenting cell (APC) (9). In the absence of sufficient co-stimulatory signal, T cells fail to activate and become anergic. Thus, successful activation requires that the APC display sufficient amounts of the co-stimulatory receptors. In the absence of inflammatory signals, APCs do not produce sufficient amounts of co-stimulatory receptors thereby promoting T cell anergy.

Antigenic ignorance. Since negative thymic selection eliminates T cells with the strongest avidity to self, the majority of the circulating self-reactive T cells possess a TCR with low avidity and thus, require high amounts of their cognate antigen for activation (10). In non-inflammatory conditions, self-antigens are rarely presented in the peripheral lymphatic system. As such, the majority of circulating - low avidity - self-reactive T cells will never encounter sufficient levels of cognate self-peptide for activation, so long as the tissue maintains its integrity (11).

Inhibitory signals. Inhibitory signals of T cell activation encompass both receptors and cytokines. Among the most well described receptors that lead to T cell inhibition are CTLA4 and PD1. The cytotoxic T-cell associated protein 4 (CTLA4) is a competitor of the co-stimulatory receptor CD28 for the CD80 and CD86 receptors on the surface of APCs. However, unlike CD28, CTLA4 ligation mediates a strong inhibitory signal for the T cell. To prevent continuous activation, large amounts of CTLA4 are stored in intracellular vesicles in the T cell and are released to the immunological synapse upon prolonged contact with the APC (12). Contrary to CTLA4 that acts on the co-stimulatory signal, ligation of the programmed-cell death 1 (PD1) receptor to the PD-1 ligand (PDL1 or PDL2) generates a signal that inhibits the TCR signalling pathway directly by de-phosphorylating Zap70 and PI3K (13). These inhibitory receptor-ligand processes are called immune checkpoints and are notably exploited by tumors to favor immune evasion (13). The

distinct mechanisms by which CTLA4 and PD1 inhibit T cell activation reveal that these processes are not redundant. Indeed, while the ligands of CTLA4, CD80/86, are mostly expressed by activated APCs during antigen presentation in lymph nodes, PDL1 is mostly found outside of the lymph nodes within target tissues and thus acts during the later phases of the immune response.

Inhibitory cytokines. Furthermore, soluble factors can provide an inhibitory signal to T cells. A range of specialized cell subsets produce cytokines like IL-10 and TGFβ, which, rather than directly inhibiting T cell activation, they 1) dampen antigen presentation by APCs (14), 2) inhibit T cell proliferation and cytotoxicity (15) and 3) skew the immune response towards specialized immunosuppressive and regulatory cell subsets (16, 17). Moreover, molecules like indoleamine-2,3-dioxygenase (18) and nitric oxide (NO) are produced by APCs and are able to directly inhibit T cell proliferation (19, 20).

Specialized regulatory cells. Various innate and adaptive immune cells are specialized to provide inhibitory signals to autoreactive T cells. These cells exploit the mechanisms described here to prevent autoreactive T cell activation or proliferation. Foxp3-expressing regulatory T cells (T_{REG}) are a subset of $CD4^+$ T cells that play a major role in the maintenance of peripheral tolerance. Indeed, absence of these cells leads to a severe and generalized lymphoproliferative autoimmune disease, in both mice and humans (21). However, other lymphocyte subsets, such as regulatory $CD8^+$ T cells (22), IL-10 expressing ($Foxp3^{NEG}$) $CD4^+$ T cells (Tr1) (23), and regulatory B cells (24) can directly promote immune tolerance at various stages of an immune response.

Importantly, these processes are also engaged at varying degrees at mucosal surfaces, where they act to prevent unwanted or prolonged inflammation and immuno-pathologies.

1.3 Mechanisms of mucosal homeostasis

Mucus membranes line various cavities of our body and cover the surface of internal organs in contact with the environment. All mucosal surfaces (including the eyes,

airways, gut, ears and mouth) are lined with their own specialized epithelial cells that are required for the host to interact with its environment. Although mucosal surfaces are composed of specialized cells, they share a basic composition. The outer layer is composed of one or more epithelial layers, protected by mucus, followed by loose connective tissue and a smooth muscle layer. These structures are highly vascularized by both blood and lymphatics that enable immune cell movement (25). In the outer layer of the airways, specialized cells such as pneumocytes facilitate the exchange of gases in the alveolar space of the lungs and enterocytes enable the absorption of nutrients and water in the lining of the gut.

However, these cells require protection as they are in direct contact with dangers from the external environment. These include highly evolved pathogens, such as viruses, bacteria, fungi and parasites as well as toxins and molecular allergens that disrupt the epithelial barrier's functions (26). To preserve epithelial integrity, fight off pathogens, facilitate tissue repair and preserve local homeostasis, both non-immune and immune components of the mucosa have developed a variety of strategies.

Importantly, understanding the mechanisms that drive immune homeostasis requires a thorough knowledge of a given specific tissue. Indeed, sterile, or near-sterile, microenvironments like the lower respiratory track or the bladder possess intrinsic mechanisms that differ from surfaces that are highly colonized by microbiota such as the skin and gut. These microbe-rich tissues have developed specialized strategies to adapt to the presence of microbes that are essential to ensure tissue homeostasis. Thus, we can define both general mechanisms, found at most sites, and specialized mechanisms that have evolved in a tissue-specific manner.

1.3.1 The role of epithelial cells in preserving mucosal homeostasis

The cells that compose the mucosal barrier do not rely solely on immune cells to maintain their function and integrity. Through tight junctions, the epithelial cells that line the mucus membrane form an efficacious barrier that prevents free passage from the environment to the underlying interstitial space. Moreover, mucus, produced by specialized goblet cells, plays an important role in controlling the local microbiota, by

preventing access to pathogens and environmental pollutants and hosting a variety of defense molecules. Finally, epithelial and endothelial cells can also release danger-associated molecular patterns (DAMPs) upon immune challenge, cell damage or death, conferring signals to immune cells thereby orchestrating both innate and adaptive immune responses (27, 28).

Apart from their function as a physical barrier, epithelial cells possess other mechanisms to prevent infection and inflammation and maintain tissue homeostasis. An important component in these processes is the release of antimicrobial peptides and proteins (AMPs) that exert direct microbicidal activities. AMPs comprise peptide families including defensins and cathelicidins, as well as proteins of the Reg3 family, that exert their bactericidal functions by targeting bacterial membranes. In addition, epithelial cells can also release enzymes like lysozyme or phospholipase A2 that facilitate bacterial cell lysis and promote fatty acid cleavage, respectively, thereby exerting antimicrobial and anti-inflammatory activities (29). In addition, cytokines produced locally by epithelial cells are a key line of communication with immune cells. Type I and type III Interferons (IFNs) are a key component in the response against viral pathogens and are readily produced and detected by epithelial cells (30). IL-10, a cytokine involved in tissue repair and immune suppression, can also be released locally by epithelial cells, providing a feedback mechanism between suppressive immune cells and the epithelia (31). Similarly, mucosal epithelial cells can produce TGF-β1, another critical mediator of immune homeostasis, and an important component of epithelial repair (32). Epithelial cells can also direct the differentiation of immune cells locally by releasing low levels of IL-25 and thymic stromal lymphopoietin (TSLP), which play a major role in facilitating immune cell recruitment, survival and differentiation (33-35). Finally, APRIL, a cytokine of the TNF family, released by intestinal epithelial cells facilitates the production of IgA by B cells (36). Collectively, epithelial cells possess both physical and chemical properties to support mucosal homeostasis. These elements enable epithelial cells to communicate with local immune cells and orchestrate targeted immune responses.

1.3.2 Innate cells in immune homeostasis

The innate immune system encompasses a large array of cells that possess the ability to recognize environmental dangers, process and present antigen to adaptive immune cells, and facilitate tissue homeostasis. As such, these cells can be located outside, between and inside the mucosal epithelial cell layers. Among these cells, some possess specific anti-inflammatory functions at the resting state, providing continuous support to maintain the integrity of the mucosal surface.

Macrophages. Among key examples found in the lungs is a subset of macrophages, cells specialized in phagocytosis and foreign antigen presentation, referred to as pulmonary alveolar macrophages (PAM). These specialized macrophages preserve pulmonary homeostasis by releasing low amounts of type I IFNs (37) that directly block T_H2 differentiation and facilitate lung repair. Deeper in the tissue membrane, interstitial macrophages promote angiogenesis and facilitate repair through the release of vascular endothelial growth factor A (VEGF-A) (38), fibroblast growth factor (FGF)-2 (39) and low amounts of TGF-β1 (40). Among these, a subset termed M_{REG} possesses the ability to suppress T cell proliferation in a cell contact-dependent manner (41).

Dendritic cells. Dendritic cells (DC) are also important mediators of immune homeostasis. There are many described subsets of DCs, that possess distinct abilities at mucosal surfaces. $CD103^+$ DCs, found in the intestines, lungs and skin, produce retinoic acid (RA) and TGFβ that drive T_{REG} cell differentiation *in situ (41, 42)*. These DCs contribute to local repair mechanisms and immune suppression by producing IL-10 and IL-27 (41). Other subsets of specialized DCs, including $CX3CR1^+$ DCs and plasmacytoid DCs, are also an integral part of the innate immune system through their roles in recognizing an immune challenge and efficiently priming T and B cell responses.

Innate lymphoid-like cells. Innate lymphoid cells (ILCs), that include group 1 ILC (ILC1), ILC2, ILC3 as well as natural killer (NK) cells, are abundant at mucosal sites. The lack of unique markers to define these cells has hindered the development of genetically

engineered mice to study their function. Nonetheless, there is ample evidence that ILCs contribute to immune homeostasis. For example, amphiregulin and IL-13 production by ILC2s facilitates lung repair during an Influenza A infection (43). While, in the gut, IL-22 by ILC3s mediates the release of anti-microbial peptides by epithelial cells (44).

These immune cells are some examples of innate cells that possess immunoregulatory properties and are part of a larger network that cooperate to promote tissue homeostasis. Moreover, innate cells are important for the priming and differentiation of the adaptive immune response. Finally, they can also directly contribute to the effector response through specialized inflammatory functions that facilitate pathogen clearance.

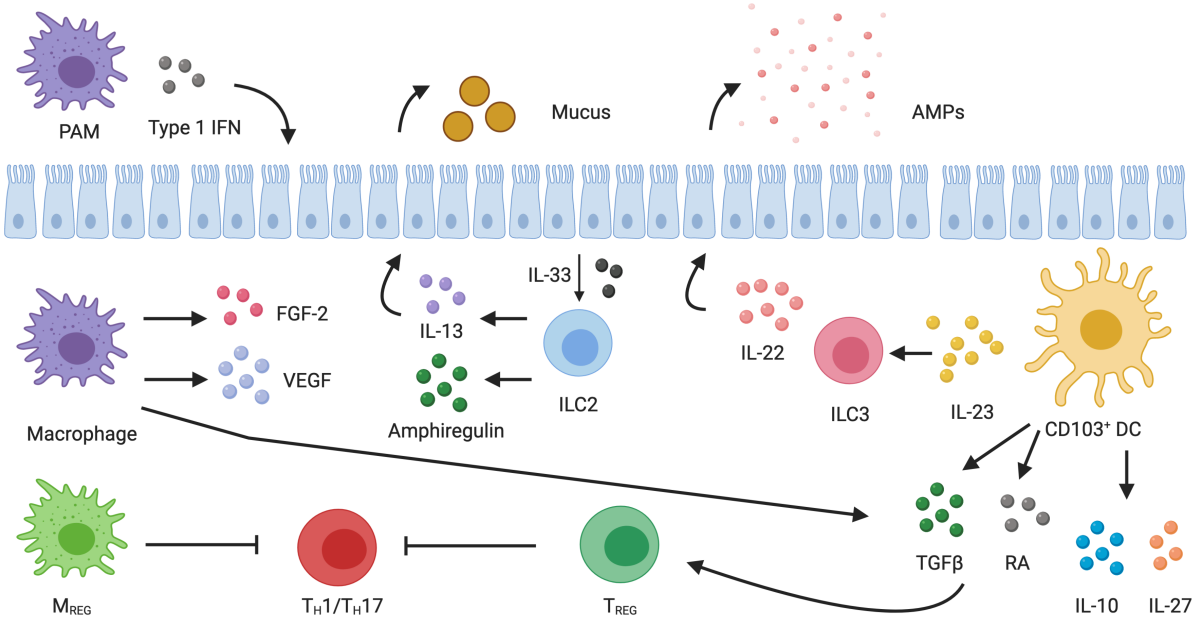

Figure 1. Anti-inflammatory properties of innate immune cells at mucosal surfaces. Innate immune cells prevent tissue damage by facilitating tissue repair, communicating with T_{REG} cells and preventing unwanted T cell effector responses. Pulmonary alveolar macrophages (PAM), anti-microbial peptides (AMPs), group 2 and group 3 innate-like cells (ILC2, ILC3), regulatory macrophages (M_{REG}), regulatory T cells (T_{REG}) and Dendritic cells (DC) contribute to the maintenance of immune homeostasis. Made using ©Biorender Software (biorender.com).

1.3.3 Adaptive immune cells in mucosal homeostasis

Adaptive immunity is characterized by its complexity and its high degree of molecular specificity. Contrary to the innate immune system which relies on an array of germline-encoded receptors to recognize microbial molecular patterns, the adaptive immune system can generate a wide diversity of antigen receptors (45). In turn, distinct cells remain in the body as long-lasting memory cells that rapidly eradicate pathogens upon re-challenge and prevent host damage. B cells and T cells are important members of the adaptive response that undergo somatic recombination and hypermutation of their respective receptor genes. In addition, B cells can further develop into plasma cells (PC) to release this receptor in the form of antibodies that can directly recognize and neutralize their target antigen.

On the other hand, the T cell response is characterized by the specialization of distinct subsets of helper T cells that orchestrate the immune response. Type 1 helper T cells (T_H1) and cytotoxic $CD8^+$ T cells are specialized to respond against intra-cellular pathogens. Type 2 helper T cell (T_H2) play a role in the defense against parasitic infections and the establishment of antibody-mediated immunity while type 17 helper T cells (T_H17) are specialized to orchestrate the response to fungal and bacterial infections. Moreover, follicular helper T cells (T_{FH}) play distinct roles in the generation germinal centers while type 9 helper T cell (T_H9) direct a wide array of the innate and adaptive cells functions. All these subsets are generated upon recognition with their cognate antigen on specialized APCs that provide the polarizing signals that drive their differentiation (46).

However, some specialized T cell subsets do not require the engagement of their TCR. For example, γδ T cells and mucosal-associated invariant T cells (MAIT) are among the first T cells to respond at mucosal sites, without the need for conventional antigen presentation (47, 48). Moreover, memory T and B cells can be readily activated without the need for antigen-recognition (49).

Collectively, the potent inflammatory functions of T cells reveal that while the adaptive immune system found in mammalians possesses a great ability to protect the host from a multitude of external and internal threats, this ability is a double-edged sword, as a delicate balance must be maintained to protect the host from a dysregulated immune

response that can lead to autoimmunity. In order to keep in check these pro-inflammatory cells, important regulatory elements among the adaptive immune system are required. Hence, distinct cell subsets of the adaptive effector arm have been shown to play crucial roles in orchestrating and suppressing inflammatory cells.

Regulatory CD4$^+$ T cells. Regulatory CD4$^+$ T cells are composed of unique T cell subsets (including Foxp3$^+$ T_{REG} and Foxp3^{NEG} Tr1 cells) that can directly suppress T cell responses or tolerize APCs at the site of inflammation (50). At the steady state, T_{REG} cells represent 8-15% of all circulating CD4$^+$ T cells and can reach 20-50% of all CD4$^+$ T cells in tissue (51, 52). There is clear evidence that these T cells are required for maintaining airway and oral tolerance (23, 53-55), preventing prolonged local inflammation (56) and promoting tissue repair (57). Selective depletion of these cells drives severe multiorgan autoimmunity and disrupts immune homeostasis in organs like the gut, skin and lungs (58), revealing that CD4$^+$ regulatory T cells are essential in maintaining peripheral tolerance and mucosal homeostasis.

Regulatory CD8$^+$ T cells. Regulatory CD8$^+$ T cells are a subset of CD8$^+$ T cells with a cytotoxic capacity and produce IL-10 (22), a potent anti-inflammatory cytokine. Indeed, through their ability to recognize antigens on MHC-I receptors, CD8$^+$ T_{REG} cells were shown to play an important role in peripheral T cell tolerance (22) and tumor evasion (59). Tracking and studying regulatory CD8$^+$ T cells is a challenging task as, apart from their ability to produce IL-10, there are currently no reliable markers ensuring their unequivocal identification. Although regulatory CD8$^+$ T cells are present in lungs upon immune challenge (22), little is known regarding their role at mucosal sites.

Double-negative T cells. CD4$^-$CD8$^-$ DN αβ T cells represent 1-3% of peripheral T cells and are readily found in the mucosa playing an active role in suppressing immune responses (60). These MHC-I restricted cells play a prominent part in tolerogenic responses during parasitic infection (61) and transplantation (62). Their presence and suppressive nature are likely important in mucosal homeostasis, but the lack of distinctive markers makes their study difficult.

Regulatory B cells. A group of B cells has also been shown to exert immunosuppressive functions during infection and inflammation and have since been termed regulatory B cells (4). They were shown to play prominent roles in attenuating colitis (63) and allergic disease (64) by producing high amounts of IL-10, IL-35 and TGFβ and facilitating the differentiation of regulatory $CD4^+$ T cells (65). Moreover, mice lacking IL-10-producing B cells have reduced numbers of $CD4^+$ T_{REG} cells (66), suggesting that these cells also contribute to $CD4^+$ T_{REG} cell homeostasis.

1.3.4 Immune deviation in mucosal homeostasis

Immune deviation is a process by which distinct specialized immune responses, notably type 1 (cellular) and type 2 (humoral) antagonize the differentiation and expansion of the other, in order to avoid an inflammatory escalation. Immune deviation can occur directly through the action of competing T_H cells. For example, IFNγ produced by T_H1 cells blocks the proliferation of T_H2 cells (67), while IL-4, produced by T_H2 cells, suppresses the migration and cytokine production of T_H1 cells (68). While this concept is not limited to adaptive immune cells (69) and can be exploited by pathogens to enhance their virulence (70), the antagonistic effect of type 1 and type 2 cytokines was shown to favor a quicker return to mucosal homeostasis after an infection (71, 72). Interestingly, both type 1 and type 2 immune responses are required to provide an effective response during viral, fungal and parasitic infections (73), revealing that, while some pathways are antagonistic, the overall response requires both branches. Moreover, this signaling network is complexified by the presence of other types of specialized responses, notably type 3 immunity raised against fungal and bacterial infections. Nevertheless, immune deviation is another mechanism by which the immune response is able to prevent an inflammatory escalation and favor a quick return to immune homeostasis.

2. Regulatory T cells in immune homeostasis

Several distinct subsets of $CD4^+$ regulatory T cells are known, the most abundant of them being the Forkhead-box p3 (Foxp3)-expressing T_{REG} cells. Contrary to other induced regulatory T cells; they are directly produced during thymic selection and are critical for both peripheral tolerance and mucosal homeostasis. However, $Foxp3^+$ T_{REG} cells are not unique in their ability to regulate T cell responses. Notably, a subset of $CD4^+$ T cells, named type 1 regulatory T cells (Tr1), can also suppress T cell responses at mucosal sites (23). Tr1 cells do not express Foxp3, and produce high amounts of IL-10, a cytokine involved in immune suppression. Tr1 cells are less abundant than $Foxp3^+$ T_{REG} cells, and do not possess the same arsenal of suppressive mechanisms. Interestingly, whereas $Foxp3^+$ T_{REG} cells have a prominent role in peripheral tolerance, Tr1 cells play a selective role in regulating local microenvironments (74). Moreover, contrary to $Foxp3^+$ T_{REG} cells, recent evidence suggest Tr1 cells are insufficient to confer long-term tolerogenic memory, suggesting their suppressive effect in the mucosa may be transient (75). Another regulatory population of $CD4^+$ T cells, type 3 helper T cells (T_H3) that produce high amounts of TGFβ, are also present in the periphery (76). However, the lack of unique markers limits our ability to deeply study these cells. Moreover, contrary to $Foxp3^+$ T_{REG} cells, it remains unclear if Tr1 and T_H3 represent stable or unique T cell lineages. As such, $Foxp3^+$ T_{REG} cells are recognized to play the major role in all aspects of immune homeostasis.

2.1 Discovery of $Foxp3^+$ Regulatory T cells

The presence of a T cell with regulatory properties was first suspected in 1969, when experiments with neonatal thymectomies in mice revealed that the timing of the procedure dictated the onset of severe autoimmunity. Mice that underwent a thymectomy at day 3, but not 1 or 7, of birth developed severe autoimmunity (77), suggesting that a specific suppressive T cell was released during that time window. These observations were confirmed in the 1970s, when Gershon et Kondo showed, using a xenotransplantation model with sheep erythrocytes, that thymic-derived cells possessed

the ability to promote immune tolerance to the transferred sheep cells (78). However, up until the 1990's, it was though that these suppressive T cells originated from conventional T cells that were primed in the periphery to express a plethora of suppressive and auto-regulatory cytokines, rather than being a distinct subset of specialized thymic-derived T cells (79). This clarified when, in 1993, two groups demonstrated that they could induce colitis in mice lacking T cells (BALB/c SCID) by transferring $CD45RB^{hi}$ T cells, but not $CD45RB^{low}$ or unfractionated T cells (80, 81), revealing that a unique population of suppressive T cells resided in the $CD45RB^{low}$ fraction. Shortly after, another group identified a distinct suppressive thymic-derived T cell subset characterized by expression of the IL-2 receptor alpha chain (CD25) (82). However, this surface receptor did not allow for the unequivocal identification of T_{REG} cells, since activated conventional T cells also expressed high levels of CD25 during inflammation (83). Finally, in 2001, simultaneous reports demonstrated that the transcription factor Foxp3, was responsible for the suppressive phenotype of T_{REG} cells (84, 85). The importance of Foxp3 in T_{REG} cell function was substantiated by the findings that the absence of Foxp3 or a functional mutation in the Foxp3 gene leads to a severe autoimmune disease and an early death in both mice (*scurfy*) and humans (Immune dysregulation, poly-endocrinopathy, enteropathy, X-linked syndrome or IPEX) (84, 85). An important breakthrough came in 2003, when it was shown that Foxp3 was the master transcription factor that drives the T_{REG} suppressive transcriptional program (86-88), revealing that $Foxp3^+$ T_{REG} cells originate from thymic selection, rather than being exclusively induced in the periphery like other helper T cell subsets. Moreover, the identification of Foxp3 provided a consistent internal marker for T_{REG} subsets of mice. In humans, however, activated $CD4^+$ T_{EFF} cells transiently express Foxp3 – much like CD25 – and thus Foxp3 would not allow for the clear identification of human T_{REG} cells (89).

2.2 Importance of the Foxp3 transcription factor

T_{REG} cells possess a unique transcriptional and translational program. Unequivocally, Foxp3 is the master transcription factor that dictates this program, as it either promotes or represses gene expression in T_{REG} cells. Foxp3 is a 421 amino acid

long member of the forkhead/winged-helix transcriptional regulators (85) that can directly 1) bind promoter or enhancer regions in the genome where it recruits either a repressor or an activator protein complex and 2) form complexes with other transcription factors in order to sequester them, localize to other regions of the genome or control its own activity. Importantly, TCR activation is required to fully observe the range of Foxp3 activity (90). Some key components of what constitutes a T_{REG} "signature" have been described. For example, Foxp3 is an active repressor of the pro-inflammatory cytokines *il2*, *ifng* and *il17* while promoting the expression of *ctla4* and *il2ra* (91, 92).

Moreover, Foxp3 binds to multiple transcriptional partners forming higher molecular complexes, which expands the range of genes targeted by Foxp3 in T_{REG} cells. These partners include NFAT, AML-1/Runx1, NF-kB, ROR-α, as well as a number of histone acetyl transferase (HAT) and histone deacetyl transferase (HDAC) enzymes (93). Through these complexes, Foxp3 can be either an active promoter or a repressor of transcription. For example, when the complexes include RELA, HELIOS and KAT5, Foxp3 is an active promoter, whereas if Foxp3 is bound to EZH2, YY1 and IKZF3, it is a repressor (94). As such, although the transcriptomic and proteomic profile of T_{REG} cells is not uniquely defined by Foxp3, it remains at the heart of the T_{REG} cell program. To add to the complexity, single-cell sequencing has demonstrated that T_{REG} cells "adapt" their transcriptional profile to non-lymphoid tissues, expanding the possibilities of Foxp3 interaction with other transcription factors thereby influencing the transcriptional profile of tissue-localized T_{REG} cells (95).

2.3 Transcriptional regulation of Foxp3 expression

In order to preserve self-tolerance, the stability of Foxp3 expression and function in T_{REG} cells must be durable. This is particularly important in T_{REG} cells upon activation and expansion (115), when they are subjected to many inflammatory signals, large-scale metabolic changes and cellular exhaustion. As such, the transcription of *foxp3* is tightly regulated by the epigenetic landscape of the locus and the interaction of Foxp3 with transcriptional partners.

Enhancer regions. The *foxp3* locus possesses a Satb1-dependent super enhancer in a highly conserved non-coding sequence (CNS), termed CNS 0, which is critical for the development of T_{REG} cells within the thymus (96). Satb1 expression is progressively down regulated in T_{REG} cells as they complete their maturation in the thymus (96), suggesting that its role is primarily required for the initial expression of Foxp3.

Several other CNS regions present in introns (CNS 1, 2,3) of the *foxp3* gene have been shown to play additional roles in the formation of multimeric enhancers that promotes *foxp3* transcription. Specific deletions of these CNS regions (97) demonstrate that these sites are unique in enhancing *foxp3* transcription. For example, the CNS 1 is a critical site for the binding of SMAD3, downstream of TGFβ signaling (98) while the CNS 2 region is more complex, as it is composed of specific cytosine–phosphate–guanine (CpG) motifs that are highly demethylated, a region called the T_{REG}-specific demethylated region (TSDR). Progressive methylation of this site hinders the binding of enhancer complexes formed by Foxp3, c-REL, NFAT, STAT5, RunX1-CBFβ and Est1 (99). Finally, the CNS 3 region is recognized by an NF-κB complex containing c-REL that facilitates the formation of an enhanceosome (100).

Promoter regions. The promoter region of *foxp3* is recognized by several distinct transcription factors such as STAT5, GATA3, FOXO, AP-1, NFAT, RunX and SP-1, which dictate the transcription of Foxp3 (101). In turn, these proteins can form multimeric complexes with other nuclear factors that regulate their ability to activate or suppress the expression of the gene. Although the majority of these factors promote Foxp3 expression, GATA3 and Stat6, through IL-4, were shown to repress *foxp3* transcription (102). Similarly, STAT3, downstream of IL-6 signalling, recognizes the CNS 2 region and competes with STAT5 to repress *foxp3* transcription (103). Binding of TGF-β to its heterodimeric receptor formed by TGF-βRI and TGF-βRII triggers a serine/threonine kinase activity, which leads to the phosphorylation and heterodimerization of the SMAD2/3 complex and subsequent binding to SMAD4. SMAD2/3 has multiple known targets in the genome and plays a key role in the promoting Foxp3 transcription by binding to the CNS1 site on the foxp3 locus (98) and, while forming an “enhanceosome” complex with NFAT, p65, CREB and c-Rel, binds the promoter of Foxp3 (104) (**Figure 2**). As

such, TGF-β signalling leads to the *de-novo* induction of Foxp3 from conventional T cells in the periphery (105, 106). Moreover, TGF-β is an important signal for the expression of another master transcription factor, RORγT. Foxp3 has been shown to directly bind RORγT in order to prevent its transcriptional activity (107). In turn, RORγT has been demonstrated to bind the promoter of foxp3 in order to repress its transcription (108), generating a tug-of-war that ultimately defines the phenotypic fate of the polarized T cell. Collectively, these observations illustrate that extracellular signals can dictate the transcription of *foxp3* and the stability of the Foxp3^{+} T_{REG} cell transcriptional signature (**Figure 2**).

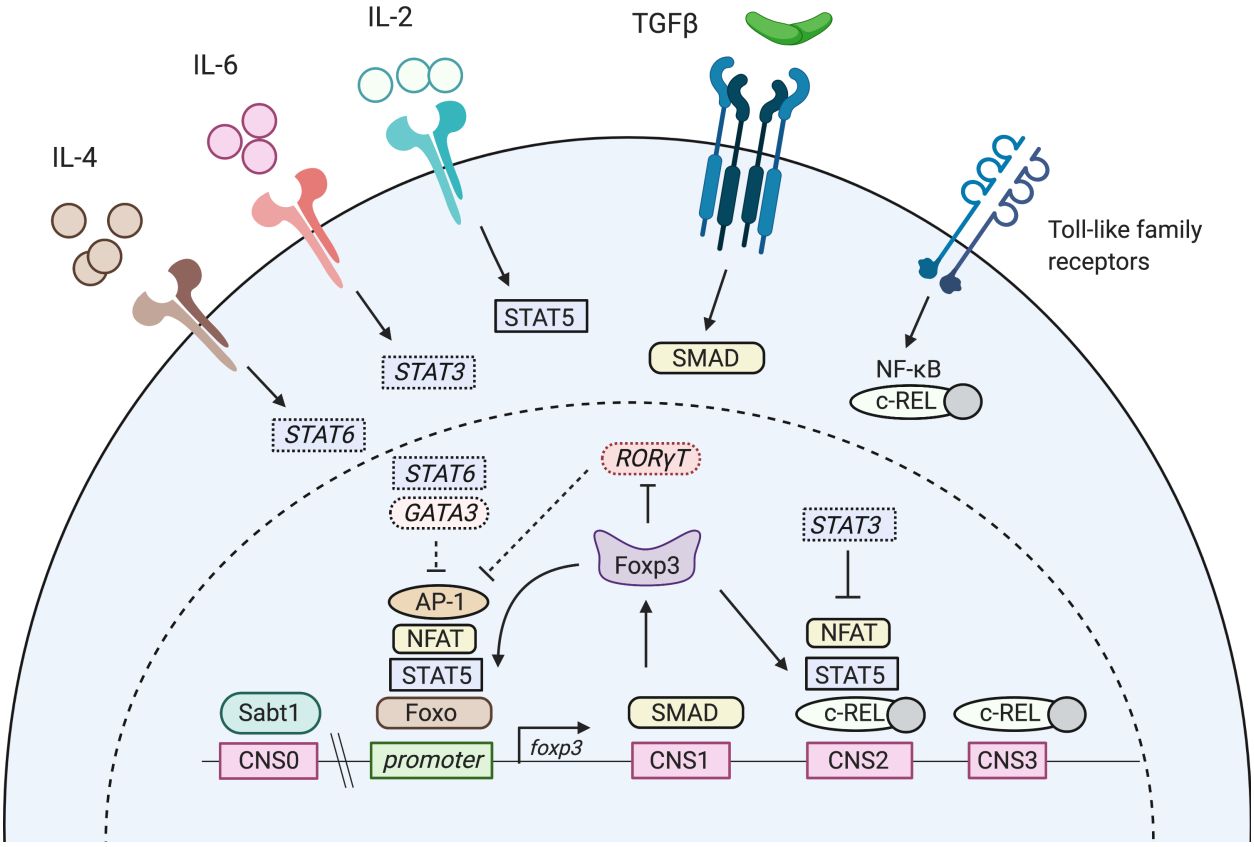

Figure 2. Important regulatory elements of *foxp3* transcription involve external signals. Known external stimuli that lead to pathways that either promote or negatively regulate foxp3 transcription. These competing signals can dictate the stability of foxp3 expression during inflammation. Made using ©Biorender Software (biorender.com).

2.4 Ontogeny of Foxp3$^+$ T_{REG} cells at mucosal surfaces

Mucosal surfaces require that strong and effective immune responses be directed towards pathogens while immune tolerance be established towards harmless antigens, such as food-antigens encountered upon nutrient consumption or self-antigens that arise through tissue damage. Two main subsets of Foxp3$^+$ T_{REG} cells are readily found at these sites: T_{REG} cells that originate from the negative selection process in the thymus (tT_{REG} cells) and T_{REG} cells that arise from the induction of Foxp3 in naïve T cells in the periphery (pT_{REG}). These two T_{REG} cells were found to play non-redundant roles in the maintenance of immune homeostasis, as both are necessary to provide efficient control of inflammation at mucosal surfaces.

2.4.1 Development of peripherally induced T_{REG} cells

Contrary to tT_{REG} cells, pT_{REG} cells originate from the polarization of naïve T cells outside the thymus. Naïve T cells exposed to TGF-β, IL-2 and retinoic acid, produced locally by resident macrophages or DCs, are important factors in the conversion of conventional naïve T cells to Foxp3-expressing T_{REG} cells (56, 109). As such, neutralizing TGF-β in the lungs blocks pT_{REG} generation (110). This is explained by the fact that the TGF-β pathway leads to the phosphorylation of Smad2 and Smad3 and the binding of Smad4 that recognize specific binding sites on the Foxp3 promoter and the conserved non-coding enhancer 1 (CNS 1) (97). Thus, CNS 1-deficient mice, which specifically lack Foxp3$^+$ pT_{REG}, develop inflammation of the mucosal epithelia in the lungs (111). IL-2 is also an important signal required for this process, and plays a key role is driving Foxp3 expression over RORγT, especially in a competitive tissue microenvironment like the gut (112). Specifically, IL-2 signaling is important for the induction and stabilization of Foxp3 as it 1) it inhibits a DNA methyltransferase that disrupts the efficient expression of Foxp3 (113) and 2) binds the conserved non-coding region 2 of *foxp3* (114).

2.4.2 Roles of tT_{REG} and pT_{REG} cells

As discussed earlier, tT_{REG} cells are crucial in the process of peripheral tolerance. However, they also play a role in mucosal homeostasis. Some of the seminal work on establishing the functional roles of pT_{REG} and tT_{REG} cells in the mucosa was obtained by experiments with the *scurfy* mouse, a genetically engineered mouse that lacks the *foxp3* allele and dies of a severe T-cell-dependent autoimmune response (85). To understand the relative importance of pT_{REG} and tT_{REG} in mediating peripheral and mucosal tolerance in these mice, $CD4^+Foxp3\text{-}GFP^+$ ($Foxp3^{GFPki}$) T_{REG} and $CD4^+Foxp3^{DTR}$ T_{EFF} ($Foxp3^-$) cells were adoptively transferred in order to generate two populations of T_{REG} cells: 1) a thymic T_{REG} population, and 2) a diphtheria-toxin (due to the expression of the diphteria-toxin receptor (DTR)) sensitive pT_{REG} cell that arise from the transferred $Foxp3^-$ T cells ($Foxp3^{DTR}$). In this model, the selective depletion of the diptheria-sensitive pT_{REG} cells leads to increased inflammation in both the lungs and the gut (115), revealing an essential role of these *in vivo* generated pT_{REG} cells in maintaining mucosal homeostasis at the steady state. Similarly, in a murine model of inflammatory bowel disease (IBD) induced by injecting $CD4^+$ naïve T cells into lymphopenic mice ($RAG^{-/-}$), splenic T_{REG} cells were shown to be able to dampen disease (116). Intriguingly, this rescue does not occur when T_{EFF} cells from *scurfy* ($Foxp3^{-/-}$) mice are transferred (so that they can't express Foxp3 to become pT_{REG} cells), revealing that locally induced pT_{REG} cells also play a part in the rescue of this pathology (117). In fact, these results suggest that thymic T_{REG} cells contribute to the development of pT_{REG} cells in this model either through the production of TGF-β or through their modulation of local antigen-presenting cells (115). Collectively, these experiments reveal the complementary roles of both tT_{REG} and pT_{REG} in maintaining mucosal homeostasis.

2.4.3 TCR repertoire of pT_{REG} and tT_{REG} cells

T_{REG} cells possess a TCR repertoire with very little overlap with the TCR repertoire of conventional ($Foxp3^-$) T cells (118), suggesting they occupy distinct niches in the immune response. Moreover, the functional specialization of tT_{REG} and pT_{REG} cells

subsets can also be appreciated on the basis of this repertoire. Indeed, while tT_{REG} cells possess a TCR repertoire largely skewed towards self-derived peptides (118) pT_{REG} possess a non-self reactive TCR repertoire with little overlap with their thymic counterparts (119, 120), revealing a "division of labor" between T_{REG} cell subsets. It remains unclear if a specific TCR repertoire predisposes naïve T cells to express Foxp3 in the periphery or if the engagement of the TCR is timed to a tolerogenic environment (for example with the release of TGFβ) that in turn favors Foxp3 expression. We know, however, that if a specific, pT_{REG}-isolated, antimicrobial TCR sequence is constitutively introduced in $CD4^+$ T cells in the thymus it does not favor the selection of antimicrobial tT_{REG} cells, suggesting that pT_{REG} cells arise from an antigenic-driven process at the mucosal surface (121).

The localisation and TCR repertoire of pT_{REG} cells make them important to establish a tolerance to oral and aero-antigens (111). In a murine model of oral allergy, ovalbumin-specific TCR (D011.10 TCR) transgenic mice generate OVA-specific pT_{REG} cells in the gut mucosa that are critical to suppress the OVA-specific T cell response upon ovalbumin consumption (110, 122). Moreover, airway administration of OVA leads to a similar accumulation of OVA-specific pT_{REG} cells in the lungs (123). However, pT_{REG} cells remain, by themselves, insufficient to prevent the onset of inflammation (123), highlighting the complementary role of both tT_{REG} and pT_{REG} cells in controlling peripheral inflammation during allergy. As such, the acquisition of a broad TCR pool by distinct subsets of T_{REG} cells at the mucosa provides a significant advantage to support local homeostasis. Moreover, while thymic T_{REG} cells are present in neonates as soon as 3 days after birth, peripherally induced T_{REG} cells accumulate slowly with time (124), highlighting how pT_{REG} induction is conditioned by environmental exposure.

2.4.4 Markers of tT_{REG} and pT_{REG} cells

Although many markers were proposed to phenotypically differentiate pT_{REG} from tT_{REG} cells, none of these strategies have yielded absolute and exclusive distinction of these two subsets. Indeed, both subsets share key suppressive functions, express Foxp3 and display a highly similar T_{REG} cell transcriptional signature at mucosal surfaces.

A specific region, labelled the TSDR (T_{REG}-specific demethylated region) in the CNS 2 region of the *foxp3* gene, was shown to be highly demethylated in tT_{REG} cells (125) and has been proposed to differentiate recent thymic emigrants from peripheral induced T_{REG} cells. The selective demethylation of this region allows for the formation of an enhancer complex that facilitates the transcription of *foxp3*. Importantly, pT_{REG} cells isolated from mucosal surfaces display a higher degree of methylation of the TSDR (125, 126). Nonetheless, the degree of TSDR demethylation does not adequately differentiate tT_{REG} from pT_{REG} cells as there is a high level of heterogeneity in methylation status among T_{REG} cells found outside the thymus (125), suggesting that as thymic T_{REG} cell are activated and polarized, the TSDR is progressively methylated.

Neuropiliin-1 (Nrp-1), an extracellular membrane receptor in T_{REG} cells, is frequently cited as a marker for tT_{REG} cells (127). However, Nrp1 does not provide for the identification of all tT_{REG} cells, as it was shown to be expressed only later in T_{REG} cell development and do not encompass all tT_{REG} cells (128). Finally, the transcription factor Helios has been proposed as a marker of tT_{REG} cells, as pT_{REG} cells were shown to lack its expression (129). However, it was recently shown that splenic-isolated Helios-negative T_{REG} cells can gain Helios expression during homeostatic expansion (130), revealing that Helios expression can be modulated during tT_{REG} cell activation and polarization.

The inability to fully distinguish pT_{REG} from tT_{REG} cells remains an obstacle to the interpretation of results. Combining new mice models and cellular markers will help in distinguishing between these two subsets and understanding their biological roles.

2.5 Understanding the role of the transcription factor Helios in T_{REG} cells

Helios is a zinc-finger transcription factor of the Ikaros family that is tightly associated with $Foxp3^+$ T_{REG} cells (129). It is expressed by a subset of T_{REG} cells in both mice and humans (129), as well as many other mammalian species (131, 132), revealing an evolutionary conserved mechanism of expression in T_{REG} cells. Unfortunately, little emphasis has been made on the effect of Helios on T_{REG} cell function, although there is clear evidence that it plays an important role in these cells.

Indeed, when T_{REG} cells lack Helios expression ($Foxp3^{CRE}$ $Helios^{fl/fl}$) the animals develop chronic inflammation by six months of age as they fail to control T follicular helper and T_H1 effector responses (133). This highlights that Helios expression by T_{REG} cells is key for their suppressive function and the stability of their transcriptional program. Similarly, unlike murine splenic $Helios^+$, $Helios^-$ T_{REG} cells express increased levels of the T_H17 associated genes *rorc, il6ra* and *il23r*, revealing a close relationship between $Helios^-$ T_{REG} cells and T_H17 cells, and suggests a mechanism by which $Helios^-$ T_{REG} cells are more prone to become pro-inflammatory (130). Nonetheless, little is known regarding the molecular pathways dictating Helios expression in T_{REG} cells, nor do we fully understand its role in T_{REG} cell function and transcriptional stability.

3. Mechanisms of suppression by T_{REG} cells

Regardless of their developmental origin, all T_{REG} cells possess the ability to suppress a wide variety of cells types. To do so, they employ both contact-dependent and contact-independent mechanisms. The variety of suppressive mechanisms used by T_{REG} cells reflects their high level of specialization. Interestingly, because of the redundancy between these suppressive functions, it remains difficult to assess the relative importance of one over the other in tissue.

3.1 Inhibitory cytokines

T_{REG} cells produce a wide array of inhibitory cytokines. Although not exclusive to these cells, T_{REG} cells are strong producers of IL-10. IL-10 signals through the IL-10 receptor complex composed of a dimer of two IL-10R1 and two IL-10R2 chains. The extracellular binding of IL-10 leads to the phosphorylation of Jak1 and Tyk2 and the subsequent transactivation of the signal transducers STAT1, STAT3 and STAT5 (134, 135). IL-10 is able to inhibit inflammatory cytokine production and block the production of co-stimulatory signals by antigen presenting cells (14). Importantly, although IL-10 inhibits T cell activation, it also skews the immune response by inhibiting T_H1 differentiation while not affecting T_H2 differentiation and function (136, 137). Concomitantly, IL-10 favors the

maturation of B cells, while preventing their apoptosis in the later developmental stages (138), suggesting that IL-10 skews the immune response toward humoral immunity. However, IL-10 was also shown to increase granzyme B production by T_H2 cell and promote their apoptosis both *in vitro* and *in vivo* (139), while recent evidence suggests that IL-10 producing T_{REG} cells have a suppressive effect on T_H2 cells in models of asthma and allergy (140). Thus, more investigation is required in order to fully understand the role of IL-10 for the control of T_H2-driven inflammation. On the other hand, in a model of Th17-driven intestinal inflammation, it was shown that IL-10 production by T_{REG} cells was required to control disease progression (141) by specifically targeting T_H17 cells (142). Thus, IL-10 remains an important suppressive mechanism of T_{REG} cells.

Another important cytokine T_{REG} cell can produce is TGF-β, which plays a key role in maintaining T cell tolerance. Indeed, T cell specific deficiency of the TGF-β receptor II (TGFBRII) leads a severe multifocal autoimmunity in 3-5 week-old mice (143, 144). Three isoforms of TGF-β (β1, β2 and β3) can be expressed by several innate and adaptive immune cells at mucosal surfaces, suggesting that TGF-β1 production by T_{REG} cells is less critical in the control of mucosal inflammation. However, although TGF-β1 was shown not to be necessary for T_{REG} cells to suppress T cell expansion *in vitro* (145), TGF-β1 production by T_{REG} cells has been reported to control T_H1-mediated colitis in mice (146). As such, the TGF-β1 produced by T_{REG} cells may indirectly suppress T cell responses *in vivo* by targeting other aspects of the immune response (reviewed in (147)).

Finally, it was discovered that both murine and human T_{REG} cells can produce IL-35, a member of the IL-12 family of cytokines with potent immunosuppressive functions (148, 149). In mice, IL-35 production by T_{REG} cells depends on Foxp3 expression (150) and can suppress T cell expansion *in vitro* (148). While human T_{REG} cells do not constitutively express IL-35 (151), human rhinovirus infection induces IL-35-producing T_{REG} cells (152), suggesting that unknown polarizing factors drive IL-35 production by T_{REG} cells. As such, more investigation is required in order to understand the role and development and role of IL-35 producing T_{REG} cells in human disease.

3.2 Cytolysis

T_{REG} cells can directly kill effector T cells through the production of granzyme B and perforin similar to cytolytic $CD8^+$ T cells (153). However, T_{REG}-induced cytolysis was shown to require granzyme B but not perforin to kill responder T cells *in vitro* (154). Nonetheless, both perforin and granzyme B production by T_{REG} cells are necessary for T_{REG} cell-mediated suppression *in vivo* (155). Finally, recent evidence suggests that granzyme B production by T_{REG} cells can inflict self-damage upon release (156), suggesting a mechanism by which T_{REG} cell populations undergo self-inflicted apoptosis.

3.3 Targeting of antigen presenting cells

T_{REG} cells can also regulate the strength of the immune response is by controlling the interaction between T cells and APCs. On one hand, T_{REG} cell constitutively express the inhibitory receptor CTLA4, which binds with higher affinity to the co-stimulatory receptor CD80 and CD86 expressed by APCs, thereby competing with CD28 expressed by T effector cells. This binding leads APCs to down regulate a wide variety of stimulatory receptors on their surface, strips CD80 and CD86 from their surface and promotes the production of indoleamine 2,3-deoxygenase (IDO) (18) in DCs (18), an enzyme that alters the fitness of nearby T cells through the conversion of tryptophan to kynurenine (157).

On the other hand, T_{REG} cells can also express the receptor LAG3, which bind MHC-II in dendritic cells, altering their maturation. Blockade of LAG3 *in vitro* and *in vivo* was shown to reduce the suppressive ability of T_{REG} cells (158), but also reduced T_{REG} cells proliferation and survival (159). Nonetheless, LAG3 expression by T_{REG} cells was shown to play a critical role in controlling the development of experimental acute encephalomyelitis (EAE), likely through their specialized ability to suppress IL-23 production by $CX3CR1^+$ macrophages (160), revealing a critical non-redundant role for the production of LAG3 by T_{REG} cells.

3.4 Metabolic and signaling disruption

T_{REG} cells possess the ability to control the concentration of critical molecules in their vicinity in order to impair the expansion, differentiation and activation of other immune cells.

IL-2 is an essential cytokine for the expansion of antigen-receptor activated T cells (161, 162). Exogenous IL-2 supplementation abrogates T_{REG}-mediated *in vitro* suppression by facilitating the expansion of the responder T cells, while knock-down of the IL-2 receptor alpha (CD25) on T_{REG} cells does not abrogate suppression (163), illustrating that this is not the only suppressive mechanism of T_{REG} cells. Nonetheless, through the constitutive expression of CD25, T_{REG} cells act as an IL-2 sink *in vivo* to slow T cell expansion (164). Moreover, it was found that, by monopolizing IL-2, follicular T_{REG} cells favor $BCL6^{+}$ follicular helper T cell (T_{FH}) differentiation during Influenza (165), revealing that this mechanism can also orchestrate the nature of the immune response. Finally, T_{REG} cells can also inhibit IL-2 production by activated effector T cells, further dropping the availability of local IL-2 during inflammation (166).

Similarly, T_{REG} cells can control immune activation through the hydrolysis of the extracellular ATP that is released upon cellular damage and hypoxic conditions (reviewed in (167)). T_{REG} cells express the ectonucleotidases CD39 and CD73 on their surface that convert extracellular ATP to ADP. In turn, ADP binds to the adenosine receptors (P1 receptors) on the surface of immune cell types and blocks their expansion. CD39-deficient T_{REG} cells have reduced suppressive ability both *in vitro* (168) and *in vivo* (169) while CD73-deficient T_{REG} cells fail to control inflammatory responses in mice (170). Collectively, these observations demonstrate how T_{REG} cells possess the ability to utilize and modulate extracellular metabolites to suppress the immune response.

4. Contribution of T_{REG} cells to the immune response against infections

4.1 General contribution of T_{REG} to infections

The role of T_{REG} cells in preventing or dampening autoimmune disease and rejection of organ transplantation is well established. In addition, T_{REG} cells also play important roles in the control of chronic inflammatory diseases like IBD. However, although T_{REG} cells have now been identified for more than 25 years, their roles during the course of infection are starting to be appreciated. This issue likely stems from the fact that generalized T_{REG} cell depletion was a strategy used to study the role of T_{REG} cells in the course of infection. Indeed, in recent years, a better characterization of tissue localized T_{REG} cells demonstrated that they form a heterogeneous population whose subsets play distinct roles on the outcome of the immune response.

4.1.1 Parasite infections

Some of the early work involving T_{REG} cells in infectious disease stem from studies of parasitic infections in rodent models. Parasitic infections are recognized by their generally chronic nature, with important phases of disease involving parasite entry (promastigotes), maturation, reproduction and release. During this process, T_{REG} cells play an important role in regulating the memory response to parasites. Indeed, in a mouse model of sub-cutaneous infection with the parasite *Leishmania major*, T_{REG} cells are necessary for the generation of a robust and long-lasting T cell memory response (171, 172). Depletion of $CD25^+$ T_{REG} cells before the injection of promastigotes leads to a rapid control of the early infection but leaves the host susceptible to re-infection (172). This was shown to be due to both IL-10-dependent and independent suppression of T cell responses targeted against the parasite. Indeed, it was shown that, while T_{REG} cells favor the establishment of a chronic infection, they, in turn, allow for the establishment of a memory T cell response that can target future infections (172).

Similarly, the recruitment and accumulation of T_{REG} cells at the site of chronic parasitic infection is documented for other parasites such as *Toxoplasma gondii* (173),

Plasmodium (174) *and Schistosoma* (175). In these infections, generalized depletion of T_{REG} cells leads to a better immune outcome, suggesting that active suppression by these cells is detrimental to an effective T cell response (**Table 1**). However, these models are not as well characterized as the *Leishmania major* murine model, and further investigation may reveal that T_{REG} cells play a similar role.

4.1.2 Bacterial infections

Foxp3$^+$ T_{REG} cells were also found to accumulate during the course of bacterial infections with *Helicobacter sp.* (176, 177), *Salmonella enterica* (178) and *Citrobacter rodentium* (179). Depletion of T_{REG} cells during the early phase of Helicobacter and Salmonella infections accelerates the clearance of the pathogen. However, late depletion of T_{REG} cells does not influence the course of disease (178), suggesting the still abundant T_{REG} cells dampen their suppressive capacity in the late phases of infections. Moreover, during a bacterial *Citrobacter rodentium* infection, T_{REG} cells help to prime the generation the generation of a protective T_H17 cell response, facilitating the clearance of the pathogen (179). Indeed, the ability of T_{REG} cells to orchestrate the nature of the immune response is not fully understood and may play a prominent role in many infectious diseases.

4.1.3 Viral infections

In models of viral infection, ablation of T_{REG} cells revealed that they were generally beneficial, as it prevented acute immunopathology (180). Beneficial effects of T_{REG} cells were notably observed for infection with the West Nile virus (180), Herpesvirus simplex virus (HSV) (181-183) and lympho-choriomeningitis virus (LCMV) (184) (**Table 1**). However, this was not the case in models with chronic infections induced by the human immunodeficiency virus (HIV) or Hepatis C virus (HCV) where the presence of T_{REG} cells was found to be detrimental to the host as they suppress the establishment of anti-viral CD4$^+$ and CD8$^+$ T cell responses (185, 186). Interestingly, characterisation of the anti-viral host response revealed that the role of T_{REG} cells for suppressing systemic and

tissue-specific immunity differed. During chronic and systemic cytomegalovirus (CMV) infection, the targeted depletion of T_{REG} cells enabled the generation of potent CMV-specific $CD4^+$ and $CD8^+$ T cell responses in the spleen, but facilitated viral replication in salivary glands (187), revealing the complex roles of T_{REG} cells.

There is evidence that T_{REG} cells influence the outcome of pulmonary viral infections. In particular, Influenza A virus (IAV) and the Respiratory Syncytial Virus (RSV) pulmonary infections in mice have revealed that T_{REG} cells drive the immune response through diverse mechanisms.

Influenza A virus. Influenza viruses of the *Orthomyxoviridae* family infect airway and alveolar epithelial cells as well as macrophages, cause acute injury and trigger the innate and adaptive immune response (reviewed in (188)). In order to clear the virus, an efficient adaptive immune response, including both humoral and cellular immunity, is required (189). Early studies revealed that IAV infected mice show a robust induction of $CD25^+$ T cells and that partial depletion of these cells with anti-CD25 antibodies prior to infection did not alter the course of disease (190). However, analysis of broncho-alveolar lavage fluid of IAV infected mice revealed that $CD4^+Foxp3^+$ T cells are recruited to the lungs prior to $CD4^+Foxp3^-$ T cells (191), suggesting that T_{REG} cells are not redundant bystanders during infection. Notably, thymic T_{REG} cell also display direct antigenic recognition of Influenza-derived antigens and are specifically recruited to the lungs (192), suggesting that tT_{REG} cells are active players in the local pulmonary immune response. These locally recruited T_{REG} cells were shown to express high levels of Tbet, highlighting the capacity of tT_{REG} cells to adapt to a T_H1-driven Influenza-specific response (192).

During IAV, T_{REG} cells delay weight loss and prolong survival (193). Notably, they respond to inflammatory signals by releasing amphiregulin, a cytokine involved in cell repair (57) and facilitate the contraction of the T cell response after viral clearance (194). Moreover, they contribute to the development of influenza-specific germinal centers (GCs) in the draining mediastinal lymph nodes (MedLN) by monopolizing IL-2 (IL-2 sink) locally, in turn blocking the STAT5-dependent loss of Bcl6 in follicular helper T cells (T_{FH}) and contributing to their differentiation (165). Although many aspects of T_{REG} cell function during Influenza remain to be understood, these examples illustrate that T_{REG} cells play

both a role in orchestrating the effector arm of the immune response and limiting prolonged inflammatory damage (195).

Respiratory Syncytial Virus (RSV). Infection with the Respiratory Syncytial Virus (RSV), a single-strand RNA of the *Pneumoviridae* family, remains the leading cause of infant bronchitis and causes high morbidity and mortality in the elderly (196, 197). The infection is characterized by a high replicating phase in the nasopharynx that last 5 days which spread to the lower respiratory track where it leads to acute bronchitis and pneumonia (198). An important component of the RSV-induced pathology is the recruitment of neutrophils and eosinophils in the respiratory track in the early phase of disease before the immune system is able to mount an effective cytotoxic T cell response required to clear the virus (199), leading to acute respiratory symptoms. In infants, T_{REG} cells, are depleted in the peripheral blood for three weeks following infection, suggesting either active recruitment to the lungs or targeted depletion during the infection (200). Incidentally, in a mouse model of RSV, systemic depletion of $CD25^+$ T_{REG} cells with anti-CD25 (PC61) slows the anti-viral $CD8^+$ T cell in the lungs, suggesting local T_{REG} cells favor the anti-viral response (201). In this model, the lungs had increased neutrophil and NK cell infiltration, higher levels of IL-6, IFNγ and TNFα and delayed infiltration of $CD8^+$ T cells (201). Moreover, T_{REG} cells contribute to suppress the deleterious RSV-specific type 2 immune response, characterized by IL-13 expressing $CD4^+$ T cells and enhanced expression of the T_H2-defining transcription factor GATA3 in the airways (202). These results reveal that T_{REG} cells contribute to the anti-viral response by suppressing the T_H2 response over the more advantageous T_H1. Furthermore, the timing of depletion of T_{REG} cells is critical, as targeting of T_{REG} cells after the establishment of an RSV infection (day 5) can increase the severity of the disease while not affecting viral clearance, confirming the role of T_{REG} cells in facilitating immune contraction (203). Collectively, these results reveal that T_{REG} cells are not passive in the course of RSV infection.

Further characterisation of tissue localized T_{REG} cells during both Influenza A and RSV infections is required to understand why their role is so complex and dynamic.

Infection	Pathogen	T_{REG} cells	*References*
Parasitic	*Leishmania major*	♦ Accumulate at the site of infection; ♦ Important for the establishment of a memory response	*Belkaid et al. Nature. 2002 (172)*
	Leishmania braziliensis	♦ Accumulate at the site of infection; ♦ Detrimental to the T cell response	*Campanelli et al. J Infect Dis. 2006 (204)*
	Plasmodium falciparum	♦ Accumulate at the site of infection; ♦ Detrimental to the T cell response	*Walther et al. Immunity. 2005 (205)*
	Schisostoma mansoni	♦ Facilitate the reproduction of the parasite; ♦ Detrimental to the T cell response	*Baumgart et al. J Immunol. 2006; (175)*
Bacterial	*Helicobacter sp.*	♦ Facilitates *Helicobacter* persistence	*Kao et al. Gastroenterology. 2009 (176)*
	Citrobacter rodentium	♦ Facilitates the generation of a protective T_H17 response	*Wang et al. Mucosal Immunol, 2014 (179)*
	Salmonella enterica	♦ Early depletion leads to a quicker clearance of the pathogen	*Johanns et al. PLoS Patho, 2010 (178)*
Viral	*Herpes simplex virus (HSV)*	♦ Control the severity of disease	*Suvas et al. J Immunol. 2004 (183)*
	Human Immunodeficiency virus (HIV)	♦ Control the expansion of HIV^+ T cells	*Weiss et al. Blood. 2004 (206)*
	Hepatitis C virus (HCV)	♦ Increase in blood ♦ Suppress T cell expansion in an antigen-specific manner	*Cabrera et al. Hepatology. 2004 (186)*
	Influenza virus (IAV)	♦ Accumulate in the lungs in an antigen-specific manner ♦ Engage repair mechanisms ♦ Control exacerbated responses by CD4 and CD8+ memory T cell ♦ Facilitate T_{FH} development in the lymph node	*Brincks et al. J Immunol. 2013 (185); Moser et al. PLoS Patho, 2014 (194); Bedoya et al. J Immunol, 2013 (192)*
	Respiratory syncytial virus (RSV)	♦ Accumulate in the lungs; ♦ Accelerate CTL responses; ♦ Control lung immunopathology and contribute to reduced morbidity	*Fulton et al. J Immunol. 2010 (207); Loebbermann et al. Mucosal Immunol, 2012 (203)*
	West Nile Virus (WNV)	♦ Protect against exacerbated encephalitis	*Lanteri MC et al. J Clin Invest 2009 (180)*

Table 1. Examples of the involvement of T_{REG} cells in infectious disease.

4.2 Gaps in our understanding of the role of T_{REG} cells during infections

The role of T_{REG} cells during mucosal and systemic infections remains to be understood. Indeed, the strong suppressive abilities of T_{REG} cells is incompatible with the fact that effective clearance of a pathogen requires an expanding inflammatory immune response. There is increasing evidence that tissue localized T_{REG} cells are important for the repair of lung alveolar epithelium, the contraction of the adaptive immune response and the establishment of memory T cell pools (194, 208, 209). Yet, T_{REG} cells, being an intricate part of the immune response, accompany the adaptive immune migration during all phases of inflammation. Their continued presence throughout pathogen-driven inflammation imply evolutionary mechanisms that modulate their suppressive ability in the early expansion phase of the immune response (210, 211).

Disregarded aspects of inflammation may explain this knowledge gap when observing T_{REG} cell function and fate in infectious studies. Indeed, the timing of the inflammation, the localization and the phenotypic characteristics of T_{REG} cells during infection are key towards studying the nature of their function in the course of the response. Specifically, the effect of the **dynamic inflammatory signals** released during mucosal inflammation are largely unknown.

Technological advances in the field of flow cytometry, genetically engineered mouse models and single-cell sequencing (212) have reveal that tissue-localized $Foxp3^+$ T_{REG} cells can display unique transcriptional and translational programs as they respond to external signals in the course of disease. This process, labelled tissue “adaptation”, is key towards understanding the role and fate of T_{REG} cells during infectious disease and suggests that external inflammatory signals dictate the function of these cells.

5. Regulatory T cell "adaptation"

The mucosal Foxp3$^+$ T_{REG} cell pool is comprised of multiple, diverse subtypes with distinct phenotypes and specialized functions. Under mostly unknown signals, Foxp3$^+$ T_{REG} cells were shown to acquire a transcriptional signature similar to their T_H1, T_H2, T_{FH} or T_H17 counterparts in order to selectively regulate T_{EFF} cell responses and control inflammation at defined anatomical sites (213, 214); a phenomenon that has been originally labelled as "functional specialization". Furthermore, recent advances in *in vivo* cell tracking of CD4$^+$Foxp3$^+$ T_{REG} cells have confirmed the existence of T cells that have lost Foxp3 expression (*ex*Foxp3) in otherwise healthy mice (215), suggesting that the loss of Foxp3 expression phenomenon can even occur outside of an inflammatory milieu. The role of the different subsets of regulatory T cells, and the existence of *ex*Foxp3 cells suggests the possibility of an epigenetic reprogramming in these cells. Thus, the possibility that T_{REG} cells can display a transcriptional "plasticity", entailing their transient conversion into a pro-inflammatory T cell, is actively debated. Collectively, it remains to be demonstrated if T_{REG} cells can transiently lose Foxp3 to promote local T cell responses and re-acquire Foxp3 expression and its transcriptional program in later stages (216).

On the other hand, there are examples that *ex*Foxp3$^+$ T cells can be spontaneously generated during inflammation. In a mouse model of rheumatoid arthritis (RA), it was shown that T_{REG} cells lost Foxp3 expression and converted to T_H17 cells in order to contribute to the inflammatory response (217). Similarly, adoptive transfer of T_{REG} cells into TCRβ$^{-/-}$ mice (lacking circulating T cells) during a skin infection with *Leishmania major* leads to the conversion of T_{REG} cells to potent T_H17-like cells (218). In these conditions, *ex*Foxp3 cells are actively generated at the site of infection and start producing high amounts of IL-17A. These results suggested a transcriptional promiscuity between T_H17 and T_{REG} cells whose balance may depend on specific inflammatory conditions. Specificities of the transcriptional signature of *ex*Foxp3 have been proposed (219), but with the caveat that different experimental models and time points results in a variety of profiles. Of particular interest is the need to detect both phenotypic and epigenetic modifications in T_{REG} cells as it happens during the course of inflammation.

To avoid further confusion, we propose the following definitions:

Reprogramming: Ability of a T cell to modify its epigenetic landscape in order to accommodate novel gene expression while silencing other genes.

Functional adaptation: Sum of the effects of reprogramming on the phenotype and function of T_{REG} cells during immune challenge.

5.1 How do T_{REG} cells "adapt"?

Adaptation requires a T_{REG} cell to accumulate sufficient epigenetic modifications (reprogram) to significantly modify its original transcriptional and translational program in order to provide a functional advantage to the global immune response during immune challenge. In other words, **efficacious adaptation is the sum of all the changes that occur in T_{REG} cells aimed at a phenotype and function that confers an advantage to the immune response.**

There are clear examples that T_{REG} cells undergo tissue adaptation. Among these examples, T_{REG} cells expressing the T_H1-associated transcription factor T-bet were shown to co-express CXCR3 and migrate to an inflammatory site where they would suppress T_H1 cells (220, 221). Furthermore, Foxp3-specific knockout of STAT3 ($Foxp3^{Cre}STAT3^{lox/lox}$) leads to the inability of $Foxp3^+$ T cells to supress T_H17 responses in the gut (213, 222). Finally, expression of GATA3 in T_{REG} cells residing at mucosal sites plays a key role in enhancing Foxp3 expression and proliferation in order to maintain mucosal homeostasis (221, 223). In extreme cases, it has been reported that T_{REG} cells can completely lose Foxp3 expression and acquire a pro-inflammatory phenotype (215, 218). All these experiments reveal that specific signals are needed to drive the specialization of T_{REG} cells to allow for their adaptation to the tissue during immune challenge. Traditionally, in order to understand the steps that lead to the tissue-adaptation of T_{REG} cells, many studies focused on key signals involved in the epigenetic transformation of T_{REG} cells. However, to understand the consequences of this epigenetic adaptation on the function and fate of T_{REG} cell during inflammation, three components

must be considered: 1) How the polarizing signals influence their transcriptional program; 2) what signals modulate of Foxp3 expression in these cells; 3) what signals impact the suppressive functions of adapted T_{REG} cells.

5.2 Known signals that influence T_{REG} cell adaptation

An important concept of adaptation is the ability of the local inflammatory environment to dictate the transcriptional program of T_{REG} cells. Some of the most well described signals that modulate the polarization of T_{REG} cells include cytokines that signals through the JAK/STAT pathway and SMAD pathways.

5.2.1 The JAK/STAT and SMAD signalling pathways in T_{REG} cells

Polarizing cytokines that signal through the signal transducer and activator of transcription (STAT) are among the best-documented elements that can drive T_{REG} cell differentiation, modulate their function and influence their migration. The technical complexity linked to the isolation, activation and expansion of T_{REG} cells has initially delayed the identification of these processes when compared to conventional T cells. Here we describe the effect of some of the described cytokines that signal through STATs in T_{REG} cells.

IL-2. The cytokine IL-2, which signals through STAT5, is the first described cytokine involved in the differentiation, survival and proliferation of T_{REG} cells. When compared to conventional T_{EFF}, T_{REG} cells express high amounts of IL2Rα (CD25) on their surface at resting state (224). Although T_{REG} cells actively suppress IL-2 production through a Foxp3-dependent mechanism (225), T_{REG} cells are dependent on exogenous IL-2 for their proliferation and survival *in vivo* and *in vitro* (226). As discussed above, IL-2 plays a key role in maintaining the transcriptional program of suppressive T_{REG} cells (227). Moreover, IL-2 is required for the differentiation of peripherally induced $Foxp3^+$ T cells (pT_{REG}) (228). In addition, it was recently described that IL-2 is required for the differentiation and function of $GATA3^+$ T_{REG} cells that respond to IL-33 stimulation, predominantly found at

mucosal surfaces (223, 229, 230). This suggests that IL-2 is also a defining factor for the adaptation of T_{REG} cells to mucosal surfaces. In contrast, a subset of splenic $CD25^{lo}$ $Foxp3^{+}$ T_{REG} cells, a population that remains to be characterized in depth, was shown not to require IL-2 to maintain Foxp3 expression, demonstrating that other signals are able to compensate for the absence of IL-2 (231).

IL-4. The IL-4 cytokine was described to induce T_H2 cell differentiation (232). While IL-4 signals through STAT6, leading to the up regulation of the transcription factor GATA3 in T_H2 cells, the accumulation of $GATA3^{+}$ T_{REG} cells in mucosal tissues at the steady-state does not depend on IL-4 or STAT6 (223). Moreover, contrary to IL-2 (227), IL-4 has been shown to disrupt Foxp3 expression, leading to the accumulation of *ex*Foxp3 T_{REG} cells *in vivo* (233) and to block Foxp3 induction *in vitro* (234). Moreover, IL-4 impairs the suppressive ability of T_{REG} cells *in vitro* by promoting the proliferation of T_H2 cells (235). Similarly, a gain-of-function mutation in the IL4Rα was shown to decrease the suppressive ability of T_{REG} cells (236). However, IL-4 is also an important signal for the proliferation and suppressive ability of a subset of T_{REG} cells *in vivo* (237). Indeed, T_{REG} cells from IL-4-deficient mice fail to efficiently suppress autologous T cells, while low amounts of exogenous IL-4 restores this ability (237). Similarly, human T_{REG} cells respond to low amounts of IL-4 by increasing their suppressive ability (238). Finally, Foxp3-specific knock-down of the IL-4Rα ($Foxp3^{CRE}$ $IL4R\alpha^{fl/fl}$) impairs the ability of T_{REG} cells to suppress T_H2-driven inflammation in a model of helminth infection *in vivo* (239), revealing that IL-4 is required for the tissue-adaptation of T_{REG} cells.

IL-6. IL-6 signals through IL-6Rα and the signal-transducing β-subunit gp130 (CD130), that leads to the phosphorylation of STAT3 (240). *In situ*, high concentrations of IL-6 drive the polarization of T_H17 cells over $Foxp3^{+}$ pT_{REG} in the presence of TGF-β (241). IL-6 signaling also promotes Foxp3 degradation (242) and inhibits Smad3 binding to the *foxp3* locus, thereby interfering with the transcription and function of Foxp3 (243). As such, IL-6 signalling was shown to impair T_{REG} cell-mediated suppression *in vitro* and *in vivo* (97, 238, 244). However, IL-6 does not impair $Helios^{+}$ T_{REG} cells accumulation and their *ex-vivo* suppressive ability (245), suggesting that IL-6 selectively inhibits the suppressive

function of distinct subsets of T_{REG} cells. Finally, IL-6, in combination with TGFβ, promotes RORγT expression in T_{REG} cells, leading to the expression of T_H17-associated genes in T_{REG} cells (246). As such, also further investigation is required, there is evidence that IL-6 plays a role in regulating the tissue-adaptation of T_H17-like T_{REG} cells at mucosal surfaces.

IL-10. While T_{REG} cells are strong producers of IL-10 (141, 247), they are also found to express a functional IL-10 receptor, composed of two subunits of IL-10Rα and two subunits of IL-10Rβ each, that phosphorylate STAT3 (142). IL-10-mediated signalling, but not IL-6 or IL-23, potentiates the suppressive ability of T_{REG} cells in T_H17-driven inflammation (142, 248). Although it was suggested that IL-10 contributes to the maintenance of Foxp3 expression in T_{REG} cells (249), this effect remains debated (142). A such, the role of IL-10 on the tissue-adaptation of T_{REG} cells remains unexplored.

IL-12. IL-12 binds to the heterodimer formed by the IL-12Rβ1 and β2 chains which in turn phosphorylate STAT4 (250). In T_{REG} cells, IL-12 was described to block T_{REG}-mediated suppression *in vitro* (251)*,* and induce IFNγ production in these cells (252-254). Moreover, IL-12 drives the conversion of T_{REG} cells to T_H1-like T cells by blocking *de novo* Foxp3 production (251). As such, T_{REG} cells respond to IL-12 by activating a T_H1-associated transcriptional profile that may lead to the loss of Foxp3 expression and the conversion to T_H1 cells. In all, IL-12 is one of the better characterized cytokines involved in the polarization of T_{REG} cells. However, it remains to be understood how this process occurs at the molecular level.

While the effect of other STAT-activating cytokines has also been, to varying degrees, investigated, these examples illustrate how these polarizing signals can facilitate the tissue-adaptation of T_{REG} cells. Furthermore, it remains to be understood when and where that process occurs. In conventional T cells, differentiation occurs at the time of TCR engagement with an antigen-presenting cell, where the immediate vicinity provides these polarizing cytokines (255, 256) and a recent study suggest it is also the case in T_{REG} cells (257). Collectively, these examples demonstrate that T_{REG} cells can differentiate and adapt to inflammatory conditions during immune challenge.

5.2.2 TGF-β and the SMAD pathway

TGF-β is one of the most studied cytokines driving T_{REG} cell differentiation and maintenance in the periphery (105). In addition to directly promoting Foxp3 expression, TGF-β is an important signal for the upregulation of the transcription factor RORγT in T_{REG} cells (107), leading to RORγT^{+}Foxp3^{+} co-expressing T cells that display T_H17-like characteristics while maintaining highly suppressive functions *ex vivo* (258). As such, TGF-β exerts key roles in the adaptation of T_{REG} cells to mucosal inflammation and favors T_{REG} cell homeostasis. It remains to be understood if distinct adapted subsets of T_{REG} cells respond differently to TGF-β signaling.

5.3 Known roles of "adapted" T_{REG} cells

Once a Foxp3^{+} T_{REG} cells acquires sufficient signals to up regulate another master transcription factor, they acquire novel capacities to migrate, survive and suppress in the inflamed tissue. Some of the best described transcription factors involve Tbet, GATA3, RORγT and BCL6.

5.3.1 Control of T_H1 immunity: Tbet^{+} T_{REG} cells

The master transcription factor Tbet plays a key role in regulating the transcriptional program of T_H1 cells (259). However, the expression of Tbet is not limited to CD4^{+} T cells, as it plays important functions in other lymphoid and myeloid immune cells including CD8^{+} T cells (260), B cells (261), NK and ILC1 cells (262) as well as macrophages and DCs (263). T_{REG} cells expressing Tbet were originally identified in a model of *Leishmania major* infection in mice (220). In this model, Tbet expression in T_{REG} cells was necessary for the co-expression of CXCR3, a chemokine that enabled the T_{REG} cells to migrate to the inflamed tissue. Similarly, removal of Tbet^{+} T_{REG} cells leads to a T_H1-driven autoimmune disease in mice (264), suggesting that the expression of the transcription factor Tbet is required for the maintenance of immune homeostasis.

However, Tbet-deficiency in $CD4^+$ T cells leads to increased $Foxp3^+$ T cells (265), suggesting that expression of this transcription factor by T_{REG} cells may lead to the loss of Foxp3 expression. IL-12, through STAT4-mediated signaling, and IFNγ through STAT1 activation, were both suggested to drive Tbet expression in T_{REG} cells (266). Mechanistically, IFNγ signaling has been suggested to act primarily by promoting the expression of the IL12Rβ2 on T_{REG} cells (266), underlining the concerted action of IL-12 and IFNγ for Tbet regulation. However, this signaling pathway remains to be confirmed.

$Tbet^+$ T_{REG} cells were also identified during an Influenza A infection, where $Tbet^+$ T_{REG} cells accumulate in the lungs and its draining mediastinal lymph nodes following viral infection (192). These T_{REG} cells displayed markers of activation (ICOS, GITR, CTLA4 CD44 and CD69), expressed an antigen-specific TCR specific and suppressed $CD4^+$ and $CD8^+$ T cells responses after adoptive transfer into infected hosts (192). Finally, $Tbet^+$ T_{REG} cells were also identified in isolated human memory T_{REG} cells ($CD45RO^+CD127^{low}CD25^{high}$) and expressed CXCR3 and low amounts of IFNγ (267). Although the consequences of acquiring Tbet expression for T_{REG} cells remain unknown, Tbet expression facilitates the migration and suppressive function of T_{REG} cells during T_H1-driven immune responses.

5.3.2 Control of T_H2 immunity: $GATA3^+$ T_{REG} cells

GATA3 is a major transcription factor associated type 2 helper T (T_H2) cells. The expression of GATA3 in T_{REG} cells was first demonstrated in *in vitro* assays after T_{REG} cell activation (223, 268). GATA3 was shown to regulate the expression of the chemokine receptor CCR8, as well as the expression of the alarmin receptor ST2 (IL-33R) (269). Knock down of GATA3 in T_{REG} cells results in a spontaneous inflammatory disorder in mice, characterized notably by T cell infiltration in the lungs and gut, and a T_H17-like conversion of T_{REG} cells (223, 268), illustrating that GATA3 plays a key role in maintaining the suppressive phenotype of T_{REG} cells. Similarly, $CCR8^+$ T_{REG} cells were shown to play a role in suppressing the immune response during experimental acute encephalomyelitis (EAE) (270). GATA3 was shown to directly bind to the CNS2 of the Foxp3 locus, promoting Foxp3 transcription (271). Moreover, co-immunoprecipitation assays revealed

that GATA3 formed a complex with Foxp3 (272), suggesting a role for GATA3 in modulating Foxp3-mediated gene regulation. Collectively, these results suggest GATA3 is an important transcription factor for the tissue-adaptation of locally suppressive T_{REG} cells.

5.3.3 Control of T_H17 immunity: RORγT$^+$ T_{REG} cells

T_{REG} cell expressing the T_H17-associated master transcription factor RORγT were originally identified in models of gut and kidney disease where T_H17 responses predominate (273). Interestingly, Foxp3 directly binds RORγT through a peptide expressed in the exon 2 of Foxp3 (274). In turn, the Foxp3-RORγT complex localizes to the nucleus, where it promotes or supresses gene transcription (274). In particular, this complex blocks *il17a/f* transcription, but does not alter the suppression of *il2* and *ifng* transcription by Foxp3 (274). Interestingly, IL-6, which is required for the upregulation of RORγT in T cells (275), potentiates the expression of T_H17-associated genes in T_{REG} cells in a dose-dependent manner (217). Thus, a minimum of two signals are required for the upregulation of RORγT in T_{REG} cells, SMAD2/3, through TGFβ, and STAT3, through IL-6 or IL-23 (276). Moreover, IL-1β, an alarmin, was also shown to potentiate the conversion of Foxp3$^+$ into Foxp3$^+$RORγT$^+$ T_{REG} cells *in vitro* (277), although the effect and role of this cytokine on T_{REG} cells remains to be fully understood. RORγT$^+$ T_{REG} cells were shown to be highly suppressive *ex vivo* and through adoptive transfer in vivo, were shown to control of T_H17-driven inflammation (274). In remains to be understood how RORγT$^+$ T_{REG} cells respond to inflammatory signals, but these results revealed that T_{REG} cells can adapt their transcriptional signature to acquire RORγT-associated genes (274).

5.3.4 Other lineage-defining transcription factors in T_{REG} cells

Bcl6 is a transcription factor associated with follicular helper T cell (T_{FH}) differentiation and function(278). Interestingly, follicular regulatory T (T_{FR}) cells expressing Bcl6 modulate T_{FH} cell proliferation and function by adopting some of their target cell's characteristics (279, 280). However, the specific signals required for the full

differentiation Bcl6$^+$ T_{REG} cells remain elusive. In T_{FH} cells, IL-6, IL-21 and ICOS signalling were all shown to play important roles in the up regulation of Bcl6 (281, 282). Among those cytokines, IL-21 blocked the expansion of expansion of T_{FR} through reducing IL-2 responsiveness (280), suggesting that the signals that leads to the upregulation of Bcl6 in T_{REG} cells may differ from T_{FH} cells. Interestingly, Bcl6 expression by T_{REG} cells was also shown to directly inhibit the expression of GATA3 (283), suggesting internal mechanisms that regulate the differentiation of T_{REG} cell subsets. Moreover, IRF4$^+$ T_{REG} cells were also shown to play a key role in the control of T_H2 responses (284). However, little is known about the factors that regulate the expression of IRF4 in T_{REG} cells.

Collectively, T_{REG} cells possess the ability to recognize and respond to polarizing signals and adapt their transcriptional program accordingly. However, little is known as to how these transcriptionally polarized T_{REG} cells respond to inflammatory signals and how they function in the tissue during an effective immune response.

6. Alarmins and their effects on T_{REG} cells

Mucosal barriers do not play an inert role during exposure to dangers. Epithelial and endothelial cells, as well as many local innate immune cells, generate molecules as a way of communicating danger to the rest of the immune system. These danger-associated molecular patterns (DAMPs) dictate the nature and amplitude of the immune response. Since DAMPs encompass a large array of molecules, some specialized endogenous proteins specifically released upon cellular damage have previously been re-defined as a group of "alarmins".

6.1 Definition of alarmins and known effects on T_{REG} cells

Alarmins are a relatively new term in immunology that emerged from a proposal by Dr Joost Oppenheim at the *EMBO Workshop on Innate Danger Signals and HMGB1* in February 2006 (285). It has been suggested that alarmins encompass endogenous proteins that possess intrinsic biological activity on the immune system. This proposed description classifies "alarmins" as molecules that **1)** are released upon non-programmed cells death; **2)** can be produced by immune cells without dying; **3)** can recruit and activate receptor-expressing immune cells; and **4)** can contribute to the restoration of immune homeostasis and epithelial repair mechanisms (285). In recent years, several examples of dysregulated expression or activities of alarmins were associated with immune-related pathologies of many distinct diseases. Thus, alarmins can play both pro-inflammatory and regulatory roles at the site of inflammation. Importantly, T_{REG} cells were found to be able to respond to locally released alarmins, although the specific effects of these signals on their function and fate remain ill defined (**Table 2**).

Alarmin		*Receptor*	*Effect*	*Reference*
HMGB1	Reduced	RAGE	Increased suppression, Increased proliferation	*Wild et al, Int Immunol, 2012 (286)*
	disulfide-bond	TLR4	Shift from Foxp3 to RORγT expression	*Li et al. J Viral Hepat, 2014 (287);* *Xu et al. Clin Immunol, 2015 (288)*
HSP	HSP60	TLR2	Increased suppression	*Kolinski et al., Cent Eur J Immunol, 2016 (289);* *Zanin-Zhorov, J Clin Invest, 2006 (290)*
	HSP70	unknown	Increased suppression, Production of IL-10, TGFβ	*Wachstein et al., PloS One, 2012 (291)*
Defensins	α	unknown	unknown	
	β	CCR6, CCR2, TLRs	Increased suppression, Increased proliferation	*Bruhs et al. J Inv Dermato, 2016 (292);* *Navid et al. J Immuno, 2012 (293)*
S100	A/B	RAGE, TLR4	unknown	*Xia et al. Front Immunol, 2018 (294)*

Table 2. Known receptors and effects of alarmins on T_{REG} cells. Many receptors for alarmins have been identified on the surface of $Foxp3^+$ T_{REG} cells, although the functional role of these alarmins remains largely unknown.

6.2 The IL-1 family of alarmins

This section has been adapted from our recent publication (295). A prominent family of alarmin introduced early in this classification is the IL-1 family comprised of 11 members. IL-1 family members include IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ and IL-37 which possess agonist properties and IL-1RA, IL-36RA and IL-38, which possess antagonist properties through their respective receptors (296). Two isoforms of IL-1 are readily found at mucosal surfaces, IL-1α and IL-1β, with the former being constitutively active, and the latter requiring protease-mediated cleavage to allow IL-1R-mediated signaling (296). IL-1 was shown to act directly on naïve T cells to promote their proliferation and survival (297). However, IL-1 was later shown to play a critical role on the expansion and function of T_H17 cells (298), and was shown to skew the differentiation of T_H17 cells over $Foxp3^+$ T cells in a TGF-β competing environment (299). Finally, contrary to murine T cells, IL-1β was shown to play a key role in the differentiation of human T_H17 cells (300). The particular role of IL-1α in this process remains largely unknown, although this cytokine shares the same receptors (IL1R1/IL1RAcP) on the surface of T cells.

A unique feature of the IL-1 family, with the exception of IL1RA, is their capacity to accumulate as pro-cytokines and possess enzymatic cleavage sites in their sequence. However, cleavage is not always required for these pro-cytokines to bind and activate their receptors. For example, while caspase 1 and caspase 8 are required for the activation of IL-1β and IL-18, pro-IL-33 does not require enzymatic processing to exert its biological activity (301). However, processing by neutrophils proteases, notably cathepsin G and elastase, as well as proteases by airway allergens were shown to enhance pro-IL-33 cleavage, enhancing IL-33 activity (302). This peculiarity reveals that IL-33, as opposed to IL-1β or IL-18, exerts most of its effect in a caspase-independent manner during homeostatic conditions. Thus, IL-1, IL-18 and IL-33 possess intrinsic biomolecular specificities that dictate their dynamic at mucosal sites and their role on the innate and adaptive immune system.

6.2.1 IL-1, IL-33 and IL-18 receptors

The IL-1 receptor family utilizes the MyD88-dependent downstream signaling pathway in T cells. Moreover, some members of this family share an accessory receptor that together with the binding receptor forms the active heterodimer on the surface of T cells (296).

ST2 (IL1RL1) is the receptor for IL-33 and was found in four distinct isoforms: 1) the membrane-bound form of ST2 (ST2L or ST2), which instructs MyD88-dependent signaling; 2) the soluble form of ST2 (sST2) - that originates from another promoter region of the *il1rl1* gene and lacks the transmembrane and cytoplasmic domains of ST2, thereby acting as a decoy for IL-33 (303), and is notably used as a biological marker of cardiac injury (304); 3) ST2V, a splice variant identified in a tumor cells line (305); and 4) ST2LV, a splice variant found in chicken, which lacks the transmembrane domain of ST2 (306). However, the role and biological relevance of ST2V and ST2LV remain ill-defined. The membrane-bound ST2 receptor requires the formation of a heterodimer with the ubiquitously expressed IL-1R accessory protein (IL-1RAcP) at the cell surface in order to bind IL-33 and to instruct downstream signaling (296).

IL-18R1 is the binding receptor of IL-18 and requires the presence of the IL-18RAcP (accessory protein) to elicit downstream signaling (307). The biological action of IL-18 is further controlled by the action of the IL-18 binding protein (IL-18BP) that can recognize and sequester extracellular IL-18 and act as a negative feedback loop (307) but was also suggested to act as a carrier (308). The biological action of IL-18BP is further explained by a difference in its affinity threshold for IL-18 when compared to the IL-18R1. While the affinity of IL-18BP for IL-18 is around 0.4nM (309), IL-18R1 was reported, at its lowest, to be of 3 nM (310). The role of the IL-18RAcP is particularly important in that aspect, as when the heterodimer IL-18R1/IL-18RAcP is formed, the affinity of the receptor is lowered to 0.3-0.4 nM (311). In all, both IL-18R1 and IL-18RAcP are critical to provide sufficient binding for IL-18 and to initiate the downstream MyD88 signalling cascade.

IL-1R1 is the receptor for IL-1α, IL-1β and the IL-1R antagonist (IL-1RA). Similar to the ST2 and IL-18R complex, IL-1R1 requires the binding of an accessory receptor, IL-1RAcP, to elicit intracellular signaling (312). IL-1R signaling is controlled by both, a

receptor antagonist (IL-1RA), that binds the IL-1R1 with 30 times higher affinity than IL-1β (313), and the IL-1R2 decoy receptor that enables IL-1β binding but does not possess an intracellular TIR domain to activate the signalling cascade (314) (**Figure 3**).

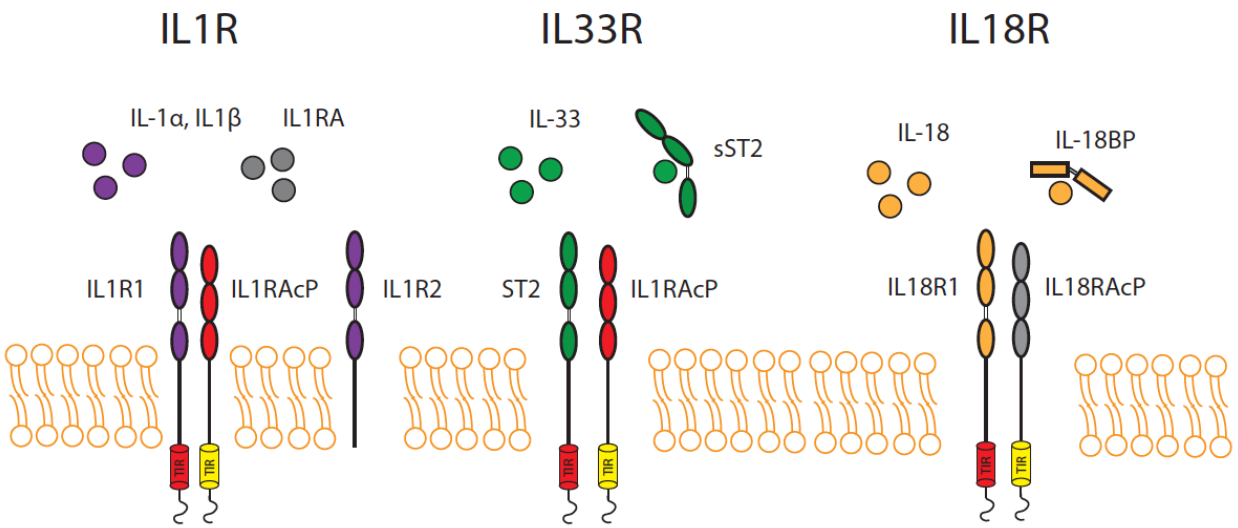

Figure 3. IL-1, IL-33 and IL-18 receptor complexes and associated control mechanisms. The IL-1R, IL-33R(ST2) and IL18R complexes are composed of heterodimers formed of their cytokine-binding dimer (IL-1R1, ST2 and IL18R1, respectively) and their respective accessory receptor (IL1RAcP, IL18RAcP) in order to provide a TIR-dependent downstream signal. Made using Adobe Illustrator® Software.

6.2.2 Signaling pathway of IL-1, IL-18 and IL-33

Binding of IL-1, IL-18 and IL-33 to their respective heterodimeric receptor leads to the dimerization of the TIR domain in the receptor with MyD88. Subsequently, the N-terminal death domain 28 of MyD88 then recruits the IL-1R-associated kinase 1 (IRAK1) (315) and IRAK4 (316). The IRAK1/4 complex then activates the downstream mitogen-activated protein kinase (MAPK) through the TNF receptor-associated factor 6 (TRAF6) that does not possess enzymatic activity but plays a critical role as an ubiquitin E3 ligase (317). As such, TRAF6 is required for the induction of NF-kB, JNK, p38 and PI3K (318). Interestingly, there are examples that the alarmins of the IL-1 family can activate ERK even in TRAF6-deficient cells, indicating a parallel activation cascade upon signaling

(317). Specifically, IL-33 was found to induce the expression of ST2L in TRAF6-deficient embryonic fibroblasts (317), indicating the presence of distinct pathways in the IL-33R-mediated signaling cascade. However, most of these studies were performed in cells other than T cells (319, 320). As such additional investigations are needed to detail signaling mechanisms in primary T cells.

TRAF6 activation in T cells. TRAF6 regulates TCR signaling in T cells via ubiquitination of the LAT adapter and phosphorylation of the IKK/NEMO complex (321). As such, TRAF6 is required for the maintenance of peripheral tolerance and control of T cell hyperreactivity. TRAF6 deficiency leads to a hyperactivation of the PI3K-AKT pathway in T cells and, interestingly, to enhanced T_H2 polarization in mice (322). In $Foxp3^+$ T_{REG} cells, TRAF6 is essential for their survival, proliferation and suppressive function (323, 324). This combination of T cell hyperactivation and impaired T_{REG} cell activity leads to severe multiorgan inflammation in mice deficient in TRAF6 (323).

The downstream targets of TRAF6 include the phosphorylation of JNK1/2 and p38 (317) (**Figure 4**). The lack of JNK in T cells results in decreased inflammatory cytokine production, while proliferation and IL-2 production remained unaltered (325). Similarly, the p38-MAPK pathway engages effector cytokine production in by T cells (326). Importantly, TRAF6, while being a key component of the TCR signaling pathway, is a downstream target of the alarmins of the IL-1 family (317, 327). Thus, although further characterization is required, alarmins of the IL-1 family can provide additional TRAF6 activation that signals for the survival, proliferation and cytokine production of T_{REG} cells during immune challenge.

ERK activation in T cells. Biochemical dissection of signalling pathways induced by IL-1 family members in mammalian cell lines was performed using data mining through an extensive survey of the literature (320). While this model includes the phosphorylation and activation of ERK1/2, JNK1/2, p38 and PI3K/AKT signaling pathways, the underlying epigenetic and transcriptional processes affected by these signals remain largely unknown. This is likely due to the large heterogeneity of the cells studied. In general, enhanced ERK activity in T cells leads to a reduction in the TCR activation threshold, as

it delays the binding of the inhibitory protein SHP-1 to the TCR complex, thereby potentiating the activation of T cells that receive suboptimal antigenic stimulation (328). ERK1 is particularly required for the proliferation of T_H2 but not T_H1 cells and, as such, plays a major role in the exacerbation of asthma (329). On the other hand, ERK2 is specifically required for optimal T_H1 and T_H17 cell differentiation and function (330, 331). This was shown to occur notably through the control of the master transcription factors of these subsets, as ERK2 suppresses the transcription of Foxp3 (T_{REG}) and GATA3 (T_H2) and favors the expression of Tbet (T_H1) (330). Importantly, the lack of either ERK1 or ERK2 does not hinder the suppressive ability of T_{REG} cells (330) and favors the TGF-β-mediated induction of Foxp3 (331). Thus, ERK1/2 activation is a major pathway involved in the control of the function of T helper cell subsets at mucosal sites. Further investigations into the T cell-intrinsic modulation of ERK1 and ERK2 by alarmins of the IL-1 family are needed to decipher their role for shaping Th subset-specific responses.

The signaling unit p38, composed of four known members (α,β,γ,δ) exert key roles in T cell activation and proliferation. Constitutive activation of p38α and p38β (p38αβY323F) was shown to skew T cell differentiation towards T_H1 and T_H17 responses (332). In contrast, knock-down of p38 α/β leads to an increase in T_{REG} cells (333). Interestingly, the p38 pathway has been shown to be directly involved in the function of IL-33 -responding T_H2 cells, as inhibition of p38, but not JNK or PI3K, resulted in a lack of IL-5 production by T_H2 cells under IL-33 signalling (326). Finally, although we know little about the role of JNK activation by IL-1 alarmins on T cells, JNK1/2 was shown to play a critical role in T cell function, but not particularly in their activation (325).

While some signalling pathways downstream of IL-1, IL-18 and IL-33 are known, the transcriptional targets depend largely on the epigenetic and transcriptional state of the recipient T cell and its environmental context.

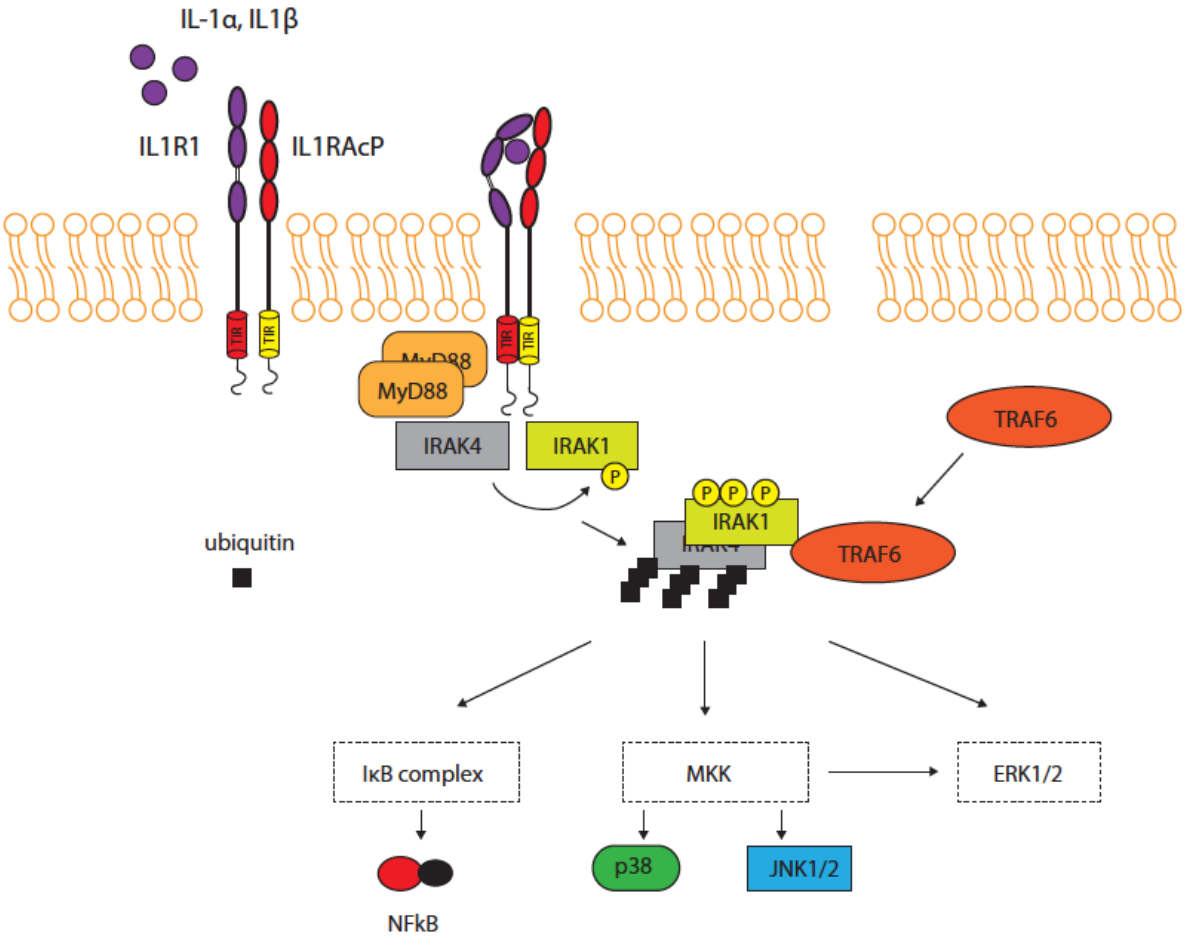

Figure 4. IL-1 signaling activates the ERK1/2, NFκB, p38 and JNK1/2 pathways. The IL-1 family of alarmins share a signaling pathway that involves the dimerization of TIR domains on the cytoplasmic tail of their respective heterodimeric receptors. In turn, Myd88 recruits IRAK1-4 that, upon phosphorylation, facilitates the recruitment of TRAF6. The formed IRAK1/4/TRAF6 complex dissociates from the membrane and expands the signal into a downstream cascade that leads to the activation of NFκB, MAP kinases (MKK) and ERK1/2. Made using Adobe Illustrator® Software.

6.2.3 Know effects on T_{REG} cell function

The role of the IL-1 family of cytokines has been extensively studied in helper T cells (334), and it was shown that they play important roles in defining the function of distinct T_H1, T_H2 and T_H17 cells (**Figure 5**). However, these effects remain largely ill-defined in T_{REG} cells. Nonetheless, there is sufficient evidence that T_{REG} cell can respond to these alarmins and that these signals are responsible in defining their tissue adaptation *in vivo*.

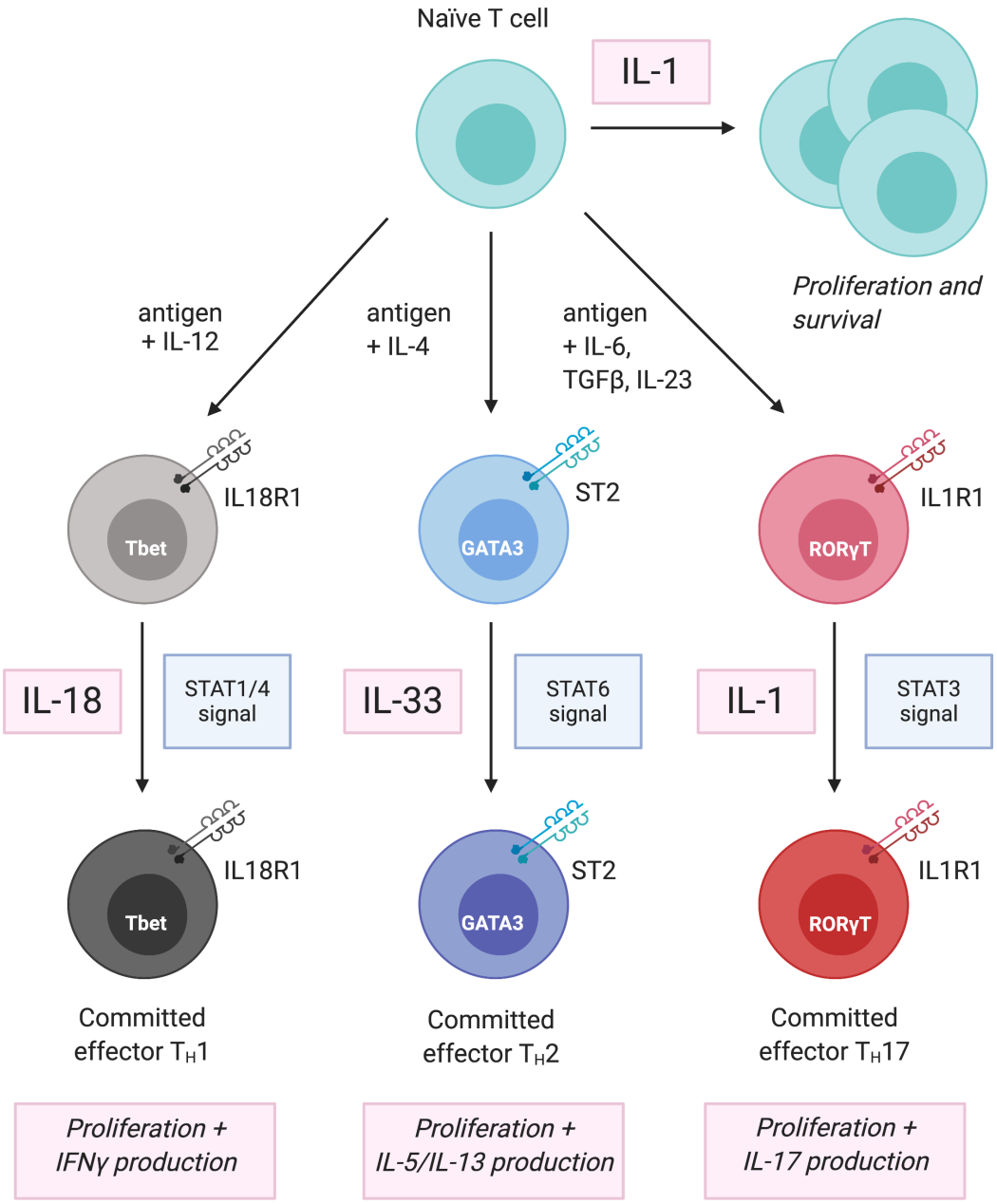

Figure 5. Known effect of IL-1 family of cytokines on effector T cells. (Adapted from (334)). This simplification of the polarization of T helper subsets involves TCR engagement through antigen recognition (antigen) and a corresponding STAT signal. The commonly described pathway of T_H1, T_H2 and T_H17 differentiation involve STAT1/4 (IFNγ, IL-12), STAT6 (IL-4), STAT3 (IL-6, IL-23) and SMAD (TGFβ) signals, while the IL-1 family alarmins potentiate this differentiation through enhanced proliferation and effector cytokine production. Made using ©Biorender Software (biorender.com).

IL-1 and T_{REG} cells. Although IL-1 was shown to induce the expansion of naïve T cells and facilitate the differentiation of murine and human T_H17 cells (298), it is only in recent years that the effects of IL-1 on T_{REG} cells have been explored. A significant portion of human memory ($CD45RO^+$) T_{REG} cells were shown to readily express IL-1R1 on their surface (335), suggesting that "experienced" T_{REG} cells can acquire IL-1R1 expression. However, the mechanisms leading to IL-1R1 expression remained to be understood, as ectopic Foxp3 expression in conventional T cells does not induce the expression of IL-1R1 (336). Functionally, IL-1β was shown to facilitate the production of IL-17A by murine $CD4^+$ $Foxp3^+$ T cells *in vitro* (277). Similarly, is has been demonstrated that IL-1β is critical for the *in vitro* differentiation of human $CD4^+CD25^{high}CD127^{low}$ $Foxp3^+$ T_{REG} cells into IL-17 producing cells (337). The definitive role of IL-1 on $ROR\gamma T^+$ T_{REG} cells remains to be fully understood, as it was shown that IL-1 facilitates the proliferation and survival, while hindering suppressive functions *in vitro* and *in vivo* (338, 339). Interestingly, T_{REG} cells also express high levels of the decoy receptor IL-1R2, which has been suggested to act as another suppressive mechanism by T_{REG} cells to neutralize IL-1 signalling (335, 336). However, the definitive role of IL-1 on T_{REG} cell adaptation in an inflammatory milieu, its link with RORγT expression, and its effects on defining the fate of T_{REG} cells at mucosal surfaces remains to be understood in depth.

IL-18 and T_{REG} cells. Very little is known regarding role of IL-18 on T_{REG} cells. Recent reports highlighted that IL-18 enhances the production of amphiregulin in T_{REG} cells *in vitro* and *in vivo*, thereby facilitating tissue restoration after pulmonary Influenza A infection (57). Similarly, IL-18 was shown to play a key role in the control of T_H1 and T_H17 responses in the gut as $IL18R1^{ko}$ T_{REG} cells failed to control the onset of a T cell-mediated colitis (340). Finally, in an experimental model of ovalbumin-induced asthma, transferred WT but not IL18R1-deficient T_{REG} cells successfully prevented the onset of disease (341). In these reports, IL-18 by itself did not induce the differentiation of IL18R1+ T_{REG} cells (340, 341). Hence, it has been suggested that IL-18 signalling enhanced the survival and suppressive ability of T_{REG} cells at mucosal barriers. Although the mechanisms remain ill

defined, these results revealed the importance of IL-18 signalling for the homeostasis of T_{REG} cells at mucosal surfaces. Thus, the origin of IL-18R$^+$ T_{REG} cells, their role in inflammation and their fate remains to be investigated.

IL-33 and T_{REG} cells. IL-33 has been the focus of recent research in T_{REG} cell biology (reviewed in (295, 342). The receptor ST2 is expressed by murine T_{REG} cells through the combined action of IL-2 and IL-33 at the time of TCR activation (343), suggesting that ST2 is expressed at undetectable levels by T_{REG} cells prior to the stimuli. Importantly, it has been shown that ST2-expressing T_{REG} cells co-express GATA3 (343, 344), which was shown to promote the expression of the *il1rl1* (ST2) gene (345). Thus, IL-2-mediated GATA3 expression in T_{REG} cells may lead to the low expression levels of ST2. Functionally, IL-33-responding T_{REG} cells are suggested to be highly suppressive (344) and play a key role in maintaining intestinal homeostasis (343) and suppressing anti-tumor immune responses (346-348). Moreover, IL-33 facilitates the proliferation of T_{REG} cells (229). However, IL-33 can also drive the production of the T_H2 associated cytokines IL-5 and IL-13 in T_{REG} cells (344, 349, 350). Moreover, ST2$^+$ T_{REG} cells accumulate in the lungs during allergen-driven allergy and fail to control the exacerbated T_H2 response (350). As such, the effect of IL-33 on the functional adaptation of T_{REG} cells remains to be understood.

6.3 IL-1 alarmins and T_{REG} cells in infectious disease

To study the relative contribution of the members of the IL-1 family on T_{REG} cell adaptation, we must first understand how they are released in the course of disease and their relative importance in disease progression. As previously stated, the activity of the distinct members of the IL-1 family is, with the notable exception of pro-IL-33, intimately linked to the presence of extracellular caspases which correlates with the level of membrane damage. Interestingly, IL-33 is described to play a role in promoting regulatory functions of immune cells rather than promoting inflammation (351), suggesting that the constitutive release of IL-33 is important for immune homeostasis. However, overexpression IL-33 leads to diffuse inflammation and multi-organ infiltration of myeloid

cells (352), while the protease-cleaved form of IL-33 in the lungs drive T_H2 responses (353), revealing that the concentration and activity of IL-33 orchestrates the pro- or anti-inflammatory nature of the immune response. Such examples reveal the complexity of the alarmin network. Moreover, the timing of the release of these alarmins are dependent on the disease. For example, during acute viral diseases such an Influenza A infection, peak production of IL-33 and IL-1β are attained at day 3 of infection, corresponding to peak viral production but before the peak of the adaptive immune response (354), while in chronic diseases such as *C. neoformans*, extracellular higher IL-33 (355) is achieved prior to the peak of IL-1α/β (356), in turn defining the nature the adaptive immune response in the lungs.

As such, alarmins play important roles in defining the outcome of disease. For example, absence of IL-1 signaling (*il1r1*-deficient mice) during Influenza leads to reduced immunopathology and compromise viral clearance and increase mortality (357). Similarly, a lack IL-1 signalling in the course of *Cryptococcus neoformans* infection in the lungs leads to increased death, as the mice fail to control the infection and display a T_H2-skewed immune response (356). On the other hand, while little is known of the role of IL-33 during Influenza, lack of IL-33 signalling (T1/ST2-deficient mice) facilitates the clearance of *C. neoformans* and increases the survival of infected mice (355). Similarly, IL-18 potentiates anti-viral responses during Influenza in both mice and human (358, 359).

Importantly, while the general roles of IL-1 alarmins have been investigated in various infectious diseases, it remains to be understood how they direct the function and fate of T_{REG} cells specifically. Moreover, little is known on the consequences of their simultaneous signal on T_{REG} cells. Indeed, while some studies focused on the effect of a single IL-1 alarmin, we have yet to decipher the competitive and/or synergistic roles of alarmins on T_{REG} cells that occur during infection. Collectively, it remains to be understood how IL-1 alarmins influence T_{REG} cell adaptation in the course of infectious disease.

7. Rationale

The requirement for an effective immune response during an active infection requires a refined balance between effector and suppressor responses. Indeed, while a pro-inflammatory response is required to clear infection, a potent suppressive environment is required to avoid immune-mediated pathology. Foxp3$^+$ T_{REG} cells are at the heart of this process as they possess the ability to suppress the immune response and promote tissue healing. Importantly, T_{REG} cells have been shown to expand in the inflamed tissue early in the course of infectious disease. However, their presence during the rapid expansion of pro-inflammatory T cells is incompatible with their potent immunosuppressive functions that could hinder and delay pathogen clearance. Thus, it remains to be understood how T_{REG} cells adapt their suppressive ability during the early phase of disease.

T_{REG} cells have been shown to adopt distinct transformations that enable them to acquire characteristics assigned to pro-inflammatory T_H cells subsets (e.g. T_H1, T_H2, T_H17). This process, referred to as reprogramming, enables T_{REG} cells with the ability to express master transcription factors other than Foxp3, (e.g. Tbet, GATA3 or RORγT) and acquire the expression of chemokines and cytokines that enable them to migrate to the challenged tissue. Nonetheless, it remains to be understood how these transformations influence the ability of T_{REG} cells to respond to local inflammatory signals.

In recent years, it has been shown that alarmins produced specifically during infectious disease modulate the activity of T cells in the tissue. Specifically, the IL-1 family of cytokines, including IL-1, IL-18 and IL-33, have been shown to potentiate distinct T_H cell responses while suppressing others, revealing that these alarmins might orchestrate many other features of the immune response, including the functional adaptation of T_{REG} cells. As such, **this work examined the general hypothesis that T_{REG} cells respond to locally produced IL-1 alarmins in order to adapt their function and fate in the course of infectious disease.**

8. General Objectives

The hypothesis has been tested by experiments based on the following three objectives:

1. Investigate the roles of IL-33 and IL-1 on the function and fate of Foxp3$^+$ regulatory T cells during the course of mucosal infections.
   (Chapter 2, published in Mucosal Immunology(360))

2. Investigate the effect of IL-33 on Foxp3$^+$ regulatory T cells in the course of a respiratory syncytial virus infection.
   (Chapter 3, manuscript in preparation)

3. Investigate the impact of IL-18 on the fate and function of tissue-resident Foxp3$^+$ regulatory T cells upon mucosal immune challenge.
   (Chapter 4, manuscript in preparation)

CHAPTER 2 - The alarmins IL-1 and IL-33 differentially regulate the functional specialization of Foxp3+ regulatory T cells during mucosal inflammation

Chapter 2- The alarmins IL-1 and IL-33 differentially regulate the functional specialization of Foxp3+ regulatory T cells during mucosal inflammation

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2.1 Bridging statements for Chapter 2

Acute infection models are highly informative to study the effect of local inflammatory signals on local CD4$^+$ T_{REG} cells that undergo functional plasticity in order to acquire specialized effector functions *in situ* and adapt to the evolving nature of immune responses. T_{REG} cells are found at all mucosal sites, including the lung, where there is strong evidence that they promote local homeostasis (1). Growing evidence indicates that Foxp3$^+$ T_{REG} cells acquire tissue-specific adaptations that promote their local homeostasis and functions (2, 3) Our lab has previously shown that lymphopenia and chronic inflammation favour lineage and functional plasticity of Foxp3$^+$ T_{REG} cells. This leads to the downregulation of Foxp3 expression, compromised suppressive function and the reprogramming into pro-inflammatory cytokine-producing (e.g. IFNγ, IL17) *ex*Foxp3$^+$ T cells, which in turn promote potent effector immune responses of the host (4).

Using a similar approach, we isolated and characterized CD4$^+$ T cells that maintained Foxp3 (stable T_{REG}) and cells that lost Foxp3 (exT_{REG}) in order to uncover the mechanistic events leading to the destabilization of Foxp3 expression in T_{REG} cells. In doing so, we identified a unique role of the IL-1 family of cytokines for the reprogramming of T_{REG} cells during inflammation. Through the use of distinct infection models, we demonstrate that two alarmins, IL-33 and IL-1β, provide antagonizing signals to T_{REG} cells to promote or destabilize Foxp3 expression, thereby regulating the adaptation of T_{REG} cells during immune challenge. This is the first demonstration of how distinct alarmins of the IL-1 family exert antagonizing effects in driving the specialization of T_{REG} cells at mucosal surfaces.

2.2 Summary

$CD4^+Foxp3^+$ regulatory T (T_{REG}) cells are critical mediators of peripheral tolerance and modulators of immune responses. Functional adaptation of T_{REG} cells, through acquisition of secondary transcription factors is critical for their effector differentiation towards local inflammatory stimuli including infections. The drivers and consequences of this adaptation of T_{REG} cell function remain largely unknown. Using an unbiased screen, we identified receptors of the IL-1 family controlling the adaptation of T_{REG} cells. Through respiratory infection models, we show that the IL-33 receptor (ST2) and the IL-1 receptor (IL1R1) selectively identify stable and unstable T_{REG} cells at mucosal surfaces, respectively. IL-33, not IL-1, is specifically required for maintaining the suppressive function of T_{REG} cells. In the absence of ST2, T_{REG} cells are prone to lose Foxp3 expression and acquire RORγT and IL1R1, while, in the absence of IL-1R1, they maintain Foxp3 expression and resist the acquisition of a Th17 phenotype. Finally, lack of IL-1 signaling enhances the accumulation of $ST2^+$ T_{REG} over pro-inflammatory T_{REG} cells in a *Cryptococcus neoformans* infection. These observations show that IL-1 and IL-33 exert opposing functions in controlling the functional adaptation of T_{REG} cells, ultimately dictating the dynamics of adaptive immunity to pathogens.

2.3 Results

1. Differential IL-33R (ST2) and IL1R1 expression distinguishes functionally stable from unstable T_{REG} cells.

To delineate the mechanistic basis of functional plasticity of T_{REG} cells *in vivo*, we exploited a Foxp3$^+$ T_{REG} cell transfer model in lymphopenic hosts that we previously described (3). Specifically, Foxp3$^+$ T_{REG} (GFP$^+$) cells from Ly5.1$^+$ Foxp3^{GFPki} reporter mice were FACS-purified and transferred into TCRβ$^{-/-}$ Ly5.2$^+$ recipient mice. After 21 days, T_{REG} cells that preserved Foxp3 expression (GFP$^+$, stable T_{REG} [sT_{REG}]) and T_{REG} cells that lost Foxp3 expression (GFP$^-$, unstable T_{REG}, termed *ex*T_{REG} cells) were isolated based on GFP expression from donor Ly5.1$^+$ CD4$^+$ splenocytes and subjected to transcriptional microarray analysis. Donor Ly5.1$^+$CD4$^+$GFP$^-$ T_{EFF} cells were also FACS-isolated at day 21 from mice that received only T_{EFF} (Ly5.1$^+$CD4$^+$GFP$^-$) cells, thus devoid of any Foxp3-expressing cells at day 0 (Figure 1A). Freshly-sorted CD4$^+$ GFP$^-$ and GFP$^+$ T cells from Foxp3^{GFPki} reporter mice were also included in the microarray analysis as control. Hierarchical cluster analysis of the top 654 genes ($p<0.05$) identified between T_{REG}, *ex*T_{REG} and activated T_{EFF} cells reveals a closer relationship between *ex*T_{REG} cells and activated T_{EFF} cells compared to stable T_{REG} cells (Figure 1B). We observed that the majority of genes that are significantly modulated upon transfer (>1 log2 fold change) are not shared between sT_{REG} and *ex*T_{REG} cells (Figure 1C). We observed that *ex*T_{REG} cells down-regulated some of the major T_{REG} signature genes (Ctl4, IL-10, Nrp1) and up-regulated genes associated with T_H1 and T_H17 cells (Tbx21, Cxcr6) (Figure 1D). Interestingly, the mRNA levels of IL-33 receptor (*Il1rl1,* IL-33R, ST2) were significantly higher in T_{REG} cells that maintained Foxp3 (sT_{REG}) compared to *ex*T_{REG} and T_{EFF} cells. In contrast, mRNA expression of IL-1 receptor (*Il1r1*) was significantly reduced in stable T_{REG} cells when comparing with *ex*T_{REG} (Figures 1E). Analysis of cell surface-bound ST2 expression by flow cytometry confirmed findings of increased mRNA levels, demonstrating that a subset of sT_{REG} cells readily expressed ST2 after transfer (Figure 1F). In order to further confirm these results, we transferred total CD4$^+$ T cells from the

fate-mapping Foxp3$^{GFP\text{-}Cre}$ X Rosa26-lox-stop-lox tdTomato mice (4) into TCRβ Ly5.1^{+} mice (Figure 1G-H; Figure S1). Again, we observed a difference in the surface expression of ST2 between stable (tdTomato^{+}GFP^{+}) vs. *ex*T$_{REG}$ cells (tdTomato^{+} GFP^{-}) in the spleen (Figure 1G-H; Figure S1). We also observed a tendency towards higher expression of the IL-1R1 in the *ex*T$_{REG}$ when compared to sT$_{REG}$ cells (Figure S1). These results illustrate that differential expression of ST2 and IL1R1 segregates distinct subsets T$_{REG}$ cells with divergent effector outcomes.

2. ST2 expression delineates stable T$_{REG}$ cells from IFNγ- and IL-17A-producing T$_{REG}$ cells during viral infection.

We hypothesized that ST2^{+} T$_{REG}$ cells increase in an inflammatory microenvironment that harbours elevated levels of IL-33, an alarmin induced by infectious pathogens particularly at mucosal surfaces (5). To test this hypothesis, we examined the functional dynamics of ST2^{+} T$_{REG}$ cells in the context of a type-1 immune-driven respiratory viral infection *in vivo*. Infection with Influenza A H1N1/PR8 induces pulmonary expression and release of IL-33 early during the course of disease (6, 7). Infected mice demonstrated an increase in pulmonary CD4^{+} T cells that coincided with the peak of weight loss (Figure 2A). ST2^{+} T$_{REG}$ cell numbers in the lungs were found to increase starting by day 4 and to peak at day 10 post-infection (Figures 2B-D). Interestingly, the increase in ST2^{+} T$_{REG}$ cells during the course of infection was seen only in lungs of infected mice (Figure 2E), suggesting that IL-33 acts locally in the lung mucosal environment. Since Foxp3^{+} T$_{REG}$ cells homing to inflammatory sites have the potential to adopt an inflammatory phenotype by co-expressing RORγT as well as IL-17A or IFNγ (3, 8), we determined whether ST2 expression by T$_{REG}$ cells correlated with expression of these inflammatory phenotypes. We observed that at peak infection the production of IFNγ and IL-17A originated exclusively from pulmonary ST2^{-} T$_{REG}$ and ST2^{-} T$_{EFF}$ cells (Figures 2F-H). Similarly, the proportions and absolute cell numbers of IFNγ and IL-17A secreting Foxp3^{+} T$_{REG}$ cells increased during peak infection and were restricted to the ST2^{-} cell subset (Figures 2G-H). These data show that IL-33-responsive (ST2^{+}) T$_{REG}$ cells

do not secrete inflammatory cytokines and represent a stable population of T_{REG} cells during influenza virus infection.

3. IL-1 inhibits ST2 expression on Helios$^+$ T_{REG} cells.

To further detail the nature and origin of ST2$^+$ T_{REG} cells, we assessed their phenotype in secondary lymphoid organs (pLN) as well as mucosal tissues (9) of naïve C57BL/6 mice. We observed a significant frequency of ST2$^+$ T_{REG} cells in the colon lamina propria that is not observed in pLN (Figure 3A). At steady state, ST2$^+$ T_{REG} cells possess a memory profile (Figure S2), a result consistent with a study by Schiering et al. (10). Spleen-derived ST2$^+$ T_{REG} cells consistently expressed high levels of Nrp1, Helios and TIGIT, a phenotype reflecting thymic origin (Figure 3B). Furthermore, thymic-derived CD4^{SP} Foxp3-GFP$^+$ T cells upregulate the ST2 receptor (11), and also maintain high levels of Helios expression (Figure S3A-B). We confirmed that IL-33 was sufficient to induce the expression of ST2 and the transcription factor GATA3 in T_{REG} cells (Figure S3C) (10, 11). Moreover, this induction in ST2 expression correlated with the accumulation of Helios$^+$ T_{REG} cells in IL-33 treated cultures (Figure 3C-D), and an increase in CD25 and Foxp3 expression in ST2$^+$ T_{REG} cells (Figure 3E-F). As IL-33 enhanced the proliferative capacity of T_{REG} cells(12), but did not affect the suppressive ability of T_{REG} cells *in vitro* (Figure S4), we then hypothesized that the specific accumulation of Helios$^+$ T_{REG} cells could lead to an overall increase in a suppressive environment at the mucosa, as Helios was recently associated with increased suppressive function in T_{REG} cells (13). Interestingly, upon transfer of GFP$^+$ (Foxp3$^+$) T cells in TCRβ$^{-/-}$ C57BL/6 recipient mice, stable Helios$^+$ T_{REG} cells expressed ST2 (Figure 3G, green panel) whereas Helios$^-$ T_{REG} cells showed increased IL-1R1 expression (Figure 3G, orange panel). Analysis of ST2 expression on *de novo* generated GFP$^+$ (pT_{REG}) cells from mice that received only GFP$^-$ T cells revealed that pT_{REG} cells, in contrast to T_{REG} cells, fail to upregulate the ST2 receptor (Figure 3H). This was further confirmed *in vitro*, as TGF-β generated iT_{REG} cells did not upregulate ST2 in the presence of IL-33 (S5).

Since IL-1R1 was preferentially expressed in unstable T_{REG} cells with reprogramming potential, we further assessed ST2 expression in T cells from mice

lacking IL-1R1 (IL-1R1$^{-/-}$; Figure 3J-L). Importantly, we observed an increase in the frequency of ST2^{+} Helios^{+} T_{REG} cells at the steady-state in the lungs of IL-1R1$^{-/-}$ mice when compared to WT mice (Figure 3J-K). Moreover, we show that ST2^{+} T_{REG} cells from IL-1R1$^{-/-}$ mice expressed higher levels (MFI) of ST2 than their counterparts from WT mice (Figure 3L) suggesting that IL-1 inhibits the expression of ST2 in T_{REG} cells. Finally, we show that ST2 and IL-1R1 are differentially expressed among T_{REG} cells at the resting state in the lung of BALB/c mice (Figure 3M-N). Overall, these results illustrate that subsets of T_{REG} cells possess the ability to respond to either IL-1 or IL-33 at mucosal sites, and that IL-1 signaling regulates that ST2 expression in in T_{REG} cells.

4. Absence of IL-33-, but not IL-1-, signaling converts Helios^{+} T_{REG} cells to a pro-inflammatory phenotype that fuels intestinal inflammation.

To determine whether IL-1 or IL-33 signaling affected the stability and suppressive function of T_{REG} cells *in vivo*, we exploited the CD4^{+} T_{EFF} cell-induced model of intestinal inflammation (colitis). Specifically, we transferred T_{EFF} cells alone (Thy1.1^{+}, CD90.1^{+}) or in the presence of WT, ST2$^{-/-}$ or IL-1R1$^{-/-}$ T_{REG} cells (Thy1.2^{+}, CD90.2^{+}) into SCID/beige mice and monitored the dynamics of T_{REG}/T_{EFF} responses relative to colitis development (Figure 4A). In contrast to mice receiving T_{EFF} cells alone (Thy1.1^{+}), all mice receiving T_{REG} cells did not display significant weight loss by day 28 post-transfer (Figure 4B). SCID/beige mice that received ST2$^{-/-}$ T_{REG} cells displayed a higher colitis score than the ones with WT T_{REG}, which confirmed the results from Schiering and colleagues (10). Strikingly, we show that mice that received IL-1R1$^{-/-}$ T_{REG} effectively prevented colitis onset by day 28 (Figure 4C). Concomitantly, mice co-transferred with ST2$^{-/-}$ T_{REG} cells showed significantly increased numbers of CD11b^{+}Ly6G^{+} neutrophils in the colon compared to mice receiving WT or IL-1R1$^{-/-}$ T_{REG} cells (Figure 4D). In addition, IL-1R1$^{-/-}$, but not ST2$^{-/-}$, T_{REG} fully inhibited the accumulation of IL17A-producing T_{EFF} cells in the colon highlighting the lack of suppressive ability of T_{REG} cells in the colon when IL-33, but not IL-1, signaling is abrogated in T_{REG} cells (Figure 4E). Moreover, mice co-transferred with IL-1R1$^{-/-}$ T_{REG} cells show enhanced frequencies of Foxp3^{+} T_{REG} cells in the colon and

mesLN, but not in the spleen, compared to mice that received WT or ST2$^{-/-}$ Foxp3^{+} T_{REG} cells (Figures 4F; Figure S6). IL1R1$^{-/-}$ T_{REG} cells expressed higher levels of ST2, and this coincided with increased Helios expression in the colon (Figure 4G-H). Consistently, mice that received IL-1R1$^{-/-}$ T_{REG} cells also showed increased numbers of ST2^{+} T_{REG} cells and reduced numbers of RORγT^{+}IL-17A^{+}T_{REG} cells, compared to mice that received WT or ST2$^{-/-}$ T_{REG} cells (Figure 4I-J). Strikingly, a greater number of IL-17A producing T_{REG} cells that lost Foxp3 expression (exT_{REG}) were found in mice that received ST2$^{-/-}$ T_{REG} cells in contrast of recipients of WT or IL-1R1$^{-/-}$ T_{REG} cells (Figure 4K). Importantly, the increased numbers of colonic neutrophils positively correlated with the increased frequencies of RORγT^{+}IL-17A^{+} T_{REG} cells in all groups (Figure 4L). Finally, we assessed if we could prevent T_{REG} cell reprogramming *in vivo*, as determined by the production of IL-17A and expression of RORγT in T_{REG} cells by IL-33 supplementation. To this end, as described in Figure 1, we adoptively transferred 2x10^{5} Ly5.1^{+}CD4^{+}GFP^{+} (Foxp3^{+}) T_{REG} cells i.v. into TCRβ$^{-/-}$ mice, and injected rIL-33 i.p. (100ng/mouse) every 48 hours. 14 days after the T_{REG} transfer, we observed a significant ($p<0.05$) reduction in the frequency of IL-17A^{+} T cells and an increase in the accumulation of IL-17A^{+} exT_{REG} cells (Figure S7A-B). Concomitantly, we observed a trend towards reduced expression of the transcription factor RORγT in Foxp3^{+} T cells and a significant increase in ST2 expression (Figure S7C-D). These results highlight that IL-1 and IL-33 exert opposite functions in controlling adaptation and functional specialization of T_{REG} cells during intestinal inflammation.

5. T_H17 polarizing conditions induce IL-1R1 expression on T_{REG} cells and promote their proliferation.

Our results show that lack of IL-33 signaling in T_{REG} cells compromises their function, while IL-1R1 deficiency favours T_{REG} cell responses highlighting the inhibitory role of IL-1 in the stability and function of T_{REG} cells. We hypothesized that IL-1R1 expression could promote RORγT expression in T_{REG} cells. We then examined the effects of IL-1β on the phenotype and function of T_{REG} cells *in vitro* by establishing a mixed culture of Ly5.2^{+} T_{REG} and Ly5.1^{+} T_{EFF} cells under neutral or T_H17 differentiated conditions (Figure

5A). Indeed, under T_H17 polarizing conditions, Foxp3$^+$ T_{REG} cells readily express RORγT, which is undetectable when cultured in control medium (Figures 5B). Moreover, RORγT$^+$Foxp3$^+$ T cells expressed higher levels of IL1R1 than medium-treated T_{REG} and RORγT$^+$ T_{EFF} cells (Figure 5C-D). Inclusion of IL-1β to the T_H17 polarizing conditions was critical for enhanced proliferation and survival of T_{REG} cells *in vitro* (Figures 5E-F). Consistently, Anakinra, a potent IL-1R antagonist (Kineret®), significantly blocked the proliferation of T_{REG} cells in T_H17 polarizing conditions, suggesting a specific role for IL-1β in the expansion and/or survival of RORγT$^+$ T_{REG} cells (Figures 5E-F; Figure S8). This feature was not due to an indirect effect of IL-1β on T_{EFF} cells, as replacing WT with IL-1R1$^{-/-}$ T_{REG} in this assay impaired the proliferation of these cells upon IL-1β stimulation (S8C-D). Furthermore, we observed that RORγT expression was highest in Helios$^-$ T_{REG} cells, which coincided with the expression of IL-1R1 (Figure S8B). Finally, IL-1R1$^+$ T_{REG} cells displayed a diminished suppressive ability on *in vitro* differentiated T_H17 cells in the presence of IL-1β (Figure 5G), an observation not due to a reduction in the frequency of T_{REG} in this culture (Figure S9). Thus, T_H17 polarizing conditions skew the functional adaptation of T_{REG} cells and modulate their responsiveness to IL-1.

6. IL-33 favours the stability of Helios$^+$ST2$^+$ T_{REG} cells in T_H17 polarizing conditions.

We showed that ST2$^+$ T_{REG} cells are robust suppressors and refractory to plasticity. We then determined whether ST2$^+$ T_{REG} cells maintain this resistance and their phenotype even in T_H17 polarizing conditions. To this end, we TCR-activated ST2$^+$ and ST2$^-$ T_{REG} cell subsets in the presence or absence of Th17 differentiation conditions (IL-6, TGF-β and IL-1β), and assessed their ability to lose Foxp3 and Helios expression and acquire RORγT and IL-1R1 expression. We confirmed that IL-33 contributed to the maintenance of Helios and ST2 expression by ST2$^+$ T_{REG} cells (Figure 6A). Interestingly, even when deprived of IL-33, only a small portion of ST2$^+$ T_{REG} cells downregulates ST2 and Helios expression in the span of 72 hours and up-regulated RORγT and IL-1R1 (Figure 6B). This portion of T_{REG} cells was significantly lower than what we observed in ST2$^-$ T_{REG} cells. Since ST2$^+$ T_{REG} could resist the inflammatory signals that lead to T_{REG} instability, we

wanted to assess the effect of IL-33 in the total pool of T_{REG} cells in T_H17 polarizing conditions. When we added increasing amounts of IL-33 to these conditions, we observed an increase in RORγT$^-$ IL-1R1$^-$ ST2$^+$ T_{REG} cells in culture (Figure 6C). In fact, the IL-33-induced expression of ST2 was not affected by T_H17 conditions (Figure 6D). We observed that the ST2$^+$ T_{REG} cells in the culture resisted IL-1R1 and RORγT expression (Figures 6E-F). Finally, in order to understand the effect of IL-2 on the differentiation of IL-1R1$^+$ T_{REG} cells, we added IL-2 to T_H17 polarizing conditions in the presence of saturating amounts of IL-1β and IL-33 (Figure S10). We observed that IL-2 was sufficient to skew the differentiation of Foxp3$^+$ T cells towards stable ST2$^+$ T_{REG} cells even in increasing amounts of IL-6 (Figure S10 A-B). Interestingly, although ST2$^+$ T_{REG} cells expand in the presence of IL-2 and IL-33, IL-1R1$^+$ T_{REG} show an increased competitive expansion as they accumulate faster in the presence of IL-6 (Figure S10C). In fact, the expression of IL1R1 on T_{REG} cells increased in the presence of IL-6 in a dose-dependent manner (Figure S10D). These conditions reveal the dichotomy between ST2$^+$ and IL-1R1$^+$ T_{REG} cells and illustrate how distinct polarizing signals *in situ* promote the accumulation of stable vs. unstable T_{REG} cells.

7. Lack of IL-1 signaling alters the dynamics of ST2$^+$ and RORγT$^+$ Foxp3$^+$ T_{REG} subsets in fungal infection.

It is well established that IL-1 and IL-33 mediate distinct cellular responses of various immune cell types, including T cells (14). Notably, IL-1 signalling plays a fundamental role in host defence against fungal diseases (15), whereas IL-33 expression is exploited by the pathogen to evade clearance (16). After observing that IL-1R1$^{-/-}$ mice exhibited increased ST2-expressing T_{REG} cells at the steady state (Figure 3J-L), we hypothesized that absence of IL1R1 signaling would further favour IL-33 activity *in vivo* to enhance ST2$^+$ T_{REG} cell responses during infection. To understand the roles of ST2 and IL1R1 on the dynamics of the T_{REG} cell response in disease, we infected mice intratracheally with the fungal pathogen *Cryptococcus neoformans* which induces the production of IL-33 (16) and IL-1α/β (17, 18). When compared to WT mice, IL-1R1$^{-/-}$ mice exhibited reduced fungal clearance in lung and brain (Figure 7A). Further characterization

of $CD4^+$ T cell populations revealed that lack of IL-1 signalling leads to significantly reduced T_H17 responses during the course of infection, as evidenced by the expression of the T_H17 associated transcription factor RORγT in both $Foxp3^+$ and $Foxp3^-$ T cells (Figure S11A). Interestingly, the lack of IL-1 signalling significantly increased the proportion and production of ST2 in $Foxp3^+$ T_{REG} cells in the lung at day 14 of infection (Figure 7B-C). These differences correlated with a significant increase in the frequency of $CD4^+Foxp3^+$ T cells in the lungs of IL-$1R1^{-/-}$ mice (Figure 7D) and an increased ratio of T_{REG} (# $CD4^+Foxp3^+$) to T_{EFF} (# $CD4^+Foxp3^-$) in the lungs (Figure S11B-C-D) underlining how the lack of IL-1 signalling affected the T_{REG}/T_{EFF} cell balance in the lungs. Importantly, the frequencies of pulmonary $ST2^+$ T_{REG} cells initially increased in both WT and IL-$1R1^{-/-}$ mice but remained high throughout the course of infection in the lungs of IL-$1R1^{-/-}$ mice when compared to WT mice (Figure 7E). Moreover, IL-$1R1^{-/-}$ mice showed a significantly decreased frequency of RORγT^+ T_{REG} cells in the lungs in the course of disease (Figure 7F). Concomitantly, we observed a decrease in IL-17A-producing T_{REG} cells at day 14 (Figure 7G). As we previously observed *in vitro*, RORγT^+ T_{REG} cells were largely $ST2^-$ and $Helios^-$ (Figure 7H; Figure S11E). In fact, $ST2^+$ T_{REG} cells expressed the transcription factor GATA3 (Figure 7I). Moreover, $ST2^+$ contrary to RORγT^+ T_{REG} cells maintained high levels of Helios expression (Figure S11F). As such, $GATA3^+$ and RORγT^+ expressing $Foxp3^+$ T cells represent two distinct subpopulations of pulmonary T_{REG} cells during infection (Figure 7J), and the absence of IL-1R1 significantly shift the populations in the lungs at day 14 of infection in favour of $GATA3^+Foxp3^+$ T_{REG} cells (Figure 7K; Figure S11G). These results reveal the antagonistic roles of IL-33 and IL-1 in modulating the functional specialization of $Foxp3^+$ T_{REG} cell during lung infection.

2.4 Discussion

$CD4^+Foxp3^+$ T_{REG} cells, key regulators of innate and adaptive immunity, accumulate locally during the course of various acute and chronic infections, inhibit immune responses and limit pathology (19). In viral infections, T_{REG} cells reduce the efficacy of $CD4^+$ and $CD8^+$ T cell responses in the early stages of viral infections (20). However, $Foxp3^+$ T_{REG} cells also have a protective role after viral clearance by controlling immune-mediated and microbe-associated pathology (21, 22). These divergent roles suggest a delicate control in the functional dynamics of $Foxp3^+$ T_{REG} cells for the regulation of immune responses to infections.

An increasing body of evidence reveals that $Foxp3^+$ T_{REG} cells do not have a fixed lineage identity but rather display considerable functional adaptability by altering their epigenetic and transcriptional landscapes to adapt to inflammatory conditions, in turn acquiring specialized roles for efficient modulation of immune responses (23). This functional specialization was characterized by the loss of Foxp3 expression in T_{REG}, the consequential loss of their suppressive phenotype, and reprogramming into inflammatory T_H1/T_H17-like *ex*Foxp3 T_{EFF} cells in lymphopenia, infections, organ-specific autoimmunity, and in tumour microenvironments (1, 3, 4, 24). Moreover, $Foxp3^+$ T_{REG} cells can also undergo a more subtle form of functional adaptability by transiently co-expressing lineage-specifying transcription factors for a more efficient control of T_{EFF} cells expressing the same transcription factors in inflammatory sites (24-26). These cellular outcomes suggest a delicate control in the stability and fate of $Foxp3^+$ T_{REG} cells, allowing them to adjust their suppressive potential, and concurrently differentiate into inflammatory T_{EFF} cells.

By applying an unbiased screen to delineate the mechanistic events leading to the loss of Foxp3 in T_{REG}, we identified the differential expression of the cell surface receptor for IL-33 (IL-33R, ST2) or IL-1 (IL-1R1) between stable ($Foxp3^{hi}$) and *ex*T_{REG} cells, respectively. We make the novel finding that IL-33 and IL-1 play opposing roles in dictating the dynamics and functional specialization of T_{REG} cells at inflamed sites during viral and fungal infection as well as chronic inflammation.

Distinct types of T_{EFF} cells express the receptors for IL-1 (IL-1R1), IL-18 (IL-18RA) and/or IL-33 (T1/ST2, IL-33 receptor) in order to enhance their proliferation and cytokine production (27). Recent studies reveal the presence of an $ST2^+$ T_{REG} cell subset in mucosal and lymphoid tissues (10, 11, 28). IL-33 and IL-1α/β are released locally by many non-hematopoietic and hematopoietic cells at external and internal barrier surfaces upon cell stress at where they function as endogenous alarmins (5). Interestingly, IL-33 was recently shown to favour the expansion of T_{REG} cells in the gut as $ST2^{-/-}$ T_{REG} cells show diminished functions in the same tissue environment (10). However, the role of IL-1 for T_{REG} cell function was largely unknown. This was probably due to the requirement for $IL\text{-}1R1^+$ T_{REG} cells to differentiate into $ROR\gamma T^+Foxp3^+$ dual expressers prior to IL-1 signalling.

We confirm here that IL-33-mediated effects on T_{REG} cells occur in the presence of IL-2 (10) whereas expression of IL-1R1 is induced in the presence of IL-6 and TGF-β, essential polarizing cytokines for T_H17 differentiation (29). Interestingly, IL-33-stimulated dendritic cells (30) and mast cells (31) were shown to produce IL-2 upon IL-33 stimulation, highlighting a positive regulatory loop to support $ST2^+$ T_{REG} cell stimulation. On the other hand, production of IL-1β by dendritic cells is enhanced by IL-6, IL-21 and IL-23, and promotes T_H17 differentiation (32). Interestingly, both IL1β and IL-33 enhanced survival and proliferation of polarized T_{REG} cells within their respective inflammatory milieu *in vitro*. Previous studies have shown that thymic-derived T_{REG} cells were able to respond to IL-33 and IL-2 in order to upregulate the ST2 receptor (11, 33). We also confirm the strong link between ST2 and the expression of the transcription factor Helios and the surface receptors Neuropilin 1 and TIGIT, whose collective expression reflects T_{REG} cells of thymic origin and enhanced T_{REG} stability (13, 34-36). In contrast, $ROR\gamma T^+$ T_{REG} cells show reduced Helios expression both *in vivo* and *in vitro*. We did not observe ST2 expression in *in vitro* or peripherally-induced T_{REG} cell subsets (pT_{REG} and iT_{REG}, respectively), in exT_{REG} cells, nor was ST2 expression prominently seen in $Helios^-$ T_{REG} cells, in contrast to a recent report (10), although further characterization of $ST2^+$ T_{REG} cells in the gut environment, relative to the lung, is required. Interestingly, Helios expression by tT_{REG} cells was necessary to maintain immune homeostasis in later stages of life (13). Future studies are required to confirm whether Helios plays an important role

in stabilizing tT_{REG} cell function, and if inflammatory conditions can modulate Helios expression or function.

To characterize the dynamics of $ST2^+$ T_{REG} cells in the context of an immune challenge, we studied their fate in two mouse models of lung infections: an acute model of murine-adapted Influenza A virus H1N1/PR8, and a chronic *Cryptococcus neoformans* fungal infection. In both models, we observed that $ST2^+$ T_{REG} cells peak early in the course of infection and at timepoints that precede the onset of peak anti-pathogen immune responses. Recently, it was suggested that $ST2^+$ T_{REG} cells play a particularly important role in tissue protection (28). As such, we observed that $ST2^+$ T_{REG} cells resisted production of pro-inflammatory cytokines throughout the course of disease, in contrast to their $ST2^-$ counterparts, which display significant plasticity by co-expressing RORγT and develop the potential to secrete inflammatory cytokines like IL-17. The early accumulation of $ST2^+$ T_{REG} cells during lung injury may reflect an attempt by local mucosal T_{REG} cells to maintain homeostasis in the early events. However, as inflammation progresses, the adaptive immune response switches to an effective inflammatory response, where newly adapted $ST2^-$ T_{REG} cells appear. This mechanism is particularly evident during chronic infection with *Cryptococcus neoformans*, where the pathogen favours IL-33 (16), IL-1α and IL-1β (17, 18) release in the early phase of disease to enhance pathogenicity. In fact, we observed that marked elevation of $ST2^+$ T_{REG} cells lead to an overall increase of the T_{REG} to T_{EFF} ratio and increased virulence and pathogenicity. Thus, IL-33 release observed with pathogenic *Cryptococcus neoformans* strains may promote the stability of immunosuppressive $ST2^+$ T_{REG} cell pool, thereby counteracting anti-microbial immune responses. On the other hand, recent evidence reveals that excessive IL-33 levels, as evidenced by lung treatment with IL-33, could alter the suppressive function of $ST2^+$ T_{REG} cells (37), a phenomenon we did not observe in our T_H1 and T_H17-driven infectious models. Thus, other signalling pathways are probably involved in the loss of function of $ST2^+$ T_{REG} cells and will be the focus of further investigation.

In this study, we assessed the effect of IL-1β on the suppressive response of T_{REG} on T_H17 cells. We show that IL-1β stimulation almost completely abrogated the suppressive ability of T_{REG} cells. The inability of $IL\text{-}1R1^+$ T_{REG} cells to suppress T_H17 cells is further substantiated by their increased survival and proliferation in the presence of IL-

1, consistent with a study by Ben-Sasson et al. showing that IL-1 enhances T_H17 cell proliferation (38). In accordance with these results, during infection with *Cryptococcus neoformans*, no elevation of RORγT and IL-17 expression in $ST2^-$ T_{REG} cells was observed in IL-1R1-deficient mice, unlike their wild-type counterparts. In contrast, we found that IL-1R1-deficiency led to marked elevation of $GATA3^+$ $ST2^+$ T_{REG} cells with an overall increase of the T_{REG} to T_{EFF} ratio and increased virulence and pathogenicity. Moreover, we observed that lack of IL-1R1 on T_{REG} cells caused enhanced stability and suppressive ability of the $Foxp3^+$ T_{REG} cell population during the course of T cell-induced colitis. In agreement with a previous report (10), we observed that $ST2^{-/-}$ T_{REG} cells were inefficient in suppressing T cell-induced colitis. Strikingly, we show that the lack of IL-33 signaling in T_{REG} cells leads to the acquisition of pro-inflammatory characteristics, and promotion of the colitogenic process. Conversely, we also show that IL-$1R1^{-/-}$ T_{REG} cells have increased maintenance of Foxp3 expression in donor T_{REG} cells, and an up-regulation of the IL-33R in this inflammatory setting. In addition, we observed that IL-1R1 is required for the proliferation of co-expressing $ROR\gamma T^+Foxp3^+$ cells, further highlighting the role of IL-1R1 for the functional adaptability of T_{REG} cell responses. In their 2010 report, Li *et al.* showed that IL-1β enhanced IL-17A production by *ex vivo* T_{REG} cells in undifferentiated co-cultures with T_{EFF} cells *in vitro (29)*, an observation not aligned with our conclusions. The reasons for this are unclear but may relate to the reduced T_{REG} cell-mediated regulation of APC function in the absence of IL-1R signaling, and consequential induction of IL-17 secretion by T_{EFF} cells in culture supernatants. Nonetheless, these results illustrate that distinct polarizing signals on T_{REG} cells are required for them to acquire the ability to respond to the distinct members of the IL-1 family induced during immune challenge such as IL-1 and IL-33.

Overall, we show that two distinct members of the IL-1 family of cytokines, IL-33 and IL-1, have differential effects on the functional adaptation of $Foxp3^+$ T_{REG} cells at mucosal surfaces during immune challenge. We show that IL-1, but not IL-33, impedes the suppression of T_{EFF} cells by T_{REG} cells in highly inflammatory conditions. In contrast, IL-33 stabilizes the T_{REG} cell phenotype and function, all-the-while restricting the potential of $Foxp3^+$ T_{REG} cells to adopt inflammatory features in challenged mucosal sites. These results highlight a mechanism by T_{REG} cells to adapt to the inflammatory conditions

throughout the evolving nature of an infection to fine-tune anti-pathogen adaptive immunity and protect the host from pathology. They provide an additional dimension to the role and fate of T_{REG} cells in disease. Further characterization of the processes that lead to the adaptation of T_{REG} cells will pave the way towards the development of therapies that aim to modulate their response in the course of disease.

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Author Contributions

F.A., J.H.F. and C.P. designed the conceptual framework of the study, designed experiments and wrote the paper. F.A. designed and performed experiments and analyzed data. I.R. contributed to the *in vivo* Influenza experiments. M.S. and S.Q. contributed on the design and experiment involving *Cryptococcus neoformans*. N.P. and T. A. contributed to the *Influenza* and Colitis experiments. S.S. provided support in the analysis of the microarray. All authors provided valuable input throughout the study and the writing of the manuscript.

Competing financial interests

All authors declare that they have no competing financial interests.

2.6 Materials and Methods

Mice

WT (Ly5.2$^+$) and congenic (Ly5.1$^+$)TCR-β$^{-/-}$ C57BL/6 mice were obtained from Taconic Laboratories while C57BL/6.Foxp3^{GFP} reporter knock-in (Foxp3^{GFP}) mice (3) were provided by Alexander Rudensky and bred into the congenic background (Ly5.1$^+$) for more than 10 generations. Inbred BALB/c were purchased from Charles River. Thy1.1+ BALB/c (JAX® CBy.PL(B6)-Thy1^a/ScrJ #005443), T1/ST2$^{-/-}$ BALB/c mice (39) and IL1R1$^{-/-}$ BALB/c mice (10-generation backcross from JAX #003018 B6 IL1R1^{Tm1Rom1} strain on the BALB/c background) were bred on site. IL1R1$^{-/-}$ mice were kept as homozygous for breeding purposes. Foxp3$^{GFP\text{-}CRE}$ X Rosa26 lox-stop-lox tdTomato fate-tracking mice on C57Bl/6 background were obtained from Jeffrey Bluestone (University of California) (4). All mice were used at 10-12 weeks of age. All mice were housed and bred under specific pathogen-free conditions and used according to institutional guidelines at McGill University.

Lymphocyte isolation

Isolation of T cells was performed, as previously described (7). Briefly, after CO_2 euthanasia, the lungs were perfused with cold PBS through the right ventricle. The collected lungs were then digested in RPMI 1640 with 5% FBS (Wisent) containing collagenase A (0,5mg/ml) and collagenase D (0,5mg/ml) in the presence of DNAse I (0.005uM) (Sigma-Aldrich) 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 lamina propria T cells (9) were obtained through the processing of the colon as previously described (3). The cells were then filtered through a cell strainer and kept in complete RPMI medium.

Purification of T cell subsets

For *in vivo* adoptive transfers, T_{REG} cells from splenocytes of Ly5.1$^+$ Foxp3^{GFPki} C57BL/6 were isolated the following; first CD4$^+$ T cells were enriched using an autoMACS (Miltenyi Biotec), and then CD4$^+$GFP$^+$ T cells (purity >99%) were sorted using a

FACSAria™ (BD Biosciences). ST2$^+$ and ST2$^-$ Foxp3$^+$ T_{REG} cells were isolated by FACSAria™ as CD4$^+$GFP$^+$ ST2$^+$ or ST2$^-$ T cells. When working on the BALB/c background, CD4$^+$CD25^{hi} (top 50% of CD25$^+$ cells) and CD4$^+$CD45RB^{hi}CD25$^-$ cells were sorted using a FACSAria™ the day of the transfer.

***In vitro* assays**

FACS-sorted CD4$^+$Foxp3$^+$ T_{REG} cells (GFP$^+$, 50x10^3) expressing ST2 (ST2$^+$) or not (ST2$^-$) were activated in 96-well flat-bottomed (0.2ml) plates previously coated with α-CD3 (5µg/ml) and α-CD28 (2µg/ml), and in the presence of IL-2 (100U/ml- or otherwise indicated) in RPMI (Wisent) supplemented with 10% FBS at 37°C for 72 hours. For *in vitro* stimulation assays, IL-33 (10ng/ml – or otherwise indicated), IL-6 (10 ng/ml- or otherwise indicated), mouse recombinant TGFβ1 (1ng/ml) and/or IL-1β (25 ng/ml) (R&D biosystems) were added at the start of the culture unless otherwise stated. In some studies, T cell cultures were performed in the presence of Anakinra (Kineret™; Swedish Orphan Biovitrum, Stockholm, Sweden). For suppression and polarization assays, 5.0x10^4 FACS-sorted CD45.2$^+$CD4$^+$GFP$^-$ were plated in 96-well flat-bottomed plates together with 2.0x10^5 irradiated feeder cells (CD4^{neg} fraction) and activated with soluble α-CD3 (1µg/mL) in the absence of IL-2. CD45.1$^+$CD4$^+$GFP$^+$ T_{REG} cells were added at a 1:2 or 1:4 ratio (2.5x10^4 or 1.25x10^4). The cells were labeled with either Violet proliferation dye V450 (BD Bioscience) or CellTrace™ Violet (Thermofisher), depending on the experiment.

Adoptive T cell transfer

For the microarray, FACSAria™ sorted CD4$^+$GFP$^+$ or GFP$^-$ cells from Ly5.1$^+$ Foxp3^{GFPki} C57BL/6 mice were suspended in PBS and transferred intravenously into Ly5.2$^+$ TCRβ$^{-/-}$ animals (1.0x10^5 cells/mouse). Mice were monitored for dehydration and weight loss. Necropsy was performed at day 21 post-injection.

For the recombinant IL-33 injections, the Ly5.2$^+$ TCRβ$^{-/-}$ mice received 2x10^5/mouse of FACSAria™ sorted CD4$^+$GFP$^+$ T_{REG} cells (Ly5.1$^+$ Foxp3^{GFPki} C57BL/6) intravenously and were injected with 100 ng/mice of recombinant IL-33 (R&D biosystems)

intra-peritoneum (I.P.) starting from day of injection (Day 0) every 48 hours until the day of necropsy (Day 14).

In the colitis experiments, FACSAria^{TM} sorted Thy1.1^{+} CD4^{+}CD45RB^{hi}CD25^{-} T cells (naïve T_{EFF}) were kept separate from CD4^{+}CD25^{hi} cells (T_{REG}) originating from WT, T1/ST2$^{-/-}$ or IL-1R1$^{-/-}$ BALB/c mice. At the time of injection, 4.0x10^{5} naïve T_{EFF} and 2.0x10^{5} T_{REG} cells were mixed and injected in the lower right quadrant of the peritoneum (I.P.). Weight of mice was monitored daily. CD4^{+} T cells from Foxp3$^{GFP\text{-}CRE}$ X Rosa26 lox-stop-lox tdTomato fate-tracking mice were sorted and adoptively transferred (I.P) into a TCR-β$^{-/-}$ Ly5.1^{+} mouse and left for homeostatic proliferation for 10 days.

Microarray analysis

Freshly isolated (Day 0) CD4^{+} GFP^{+} (fresh T_{REG}) and GFP^{-} (fresh T_{EFF}) were isolated from Foxp3^{GFPki} Ly5.1^{+} mice and transferred into TCRβ$^{-/-}$ Ly5.1^{+} (see Figure 1A). At day 21 post-transfer, Ly5.2^{+}CD4^{+} T cells were isolated and further separated based on GFP signal: 1) GFP^{hi} (T_{REG}), 2) GFP^{neg} (*ex*T_{REG}) or 3) GFP^{neg} (T_{EFF} from GFP^{-} transferred mice). Two additional groups of cells were analyzed: 4) freshly-sorted T_{REG} (fresh T_{REG}) and 5) freshly-sorted T_{EFF} cells (fresh T_{EFF}) from day 0. RNA was isolated using the RNeasy Mini Kit from Qiagen as per the manufacturer's instructions. Samples were run on a Illumina® MouseWG-6 v2.0 chip which contains 45281 mouse probes and 974 control probes (Illumina® – Genome Québec), and the resulting raw expression data were extracted, annotated, robust spline normalized and background-adjusted using illuminaMousev2.db and bead array packages in R. The top 654 genes that varied ($p<0.05$ cut-off) were selected and the expression patterns were analyzed between the groups. A modified ANOVA for microarray analysis (eBayes function from the Limma package) was used to compare across all conditions for each gene.

Intranasal infection with Influenza A

The mouse-adapted Influenza A virus (40) H1N1 strain A/Puerto Rico/8/34 was propagated and titrated by plaque assay on Madin-Darby canine kidney cells, as described (41). Mice were anesthetized by intramuscular injection with a mixture of 10

mg/kg ketamine (Ayerst Veterinary Laboratories) and 125 mg/kg xylazine (Bayer). For a sublethal infection, 20 plaque-forming units (PFU) of IAV per 20 g body weight were administered intranasally. The mice were monitored daily for clinical score and weight loss.

Intratracheal infection with C*. neoformans*

C.neoformans 52D (ATCC no. 24067) was grown and prepared as previously described (16). Briefly, a single colony of *C. neoformans* from a Sabouraud dextrose agar (Becton Dickinson) was resuspended in Sabouraud dextrose broth (Becton Dickson) and then grown further in a rotating culture until stationary phase (48 h) at room temperature for *in vivo* infections. Subsequently, the culture was spun, washed twice with PBS, and resuspended to a desired concentration in PBS. Concentrations of *C. neoformans* were verified by plating on Sabouraud dextrose agar at 37°C for 72 h followed by determination of CFU. The mice were anesthetized with 10 mg/kg ketamine (Ayerst Veterinary Laboratories) and 125 mg/kg xylazine (Bayer) intra-peritoneally. To access the trachea, a vertical 1-cm incision of the skin was made below the jaw. A 22-gauge catheter (Becton Dickson) was inserted into the trachea. In a volume of 50 µl sterile PBS, $2X10^5$ CFU/ml *C. neoformans* was instilled, followed quickly by 50 µl volume of air. The incision was closed using a 9 mm EZ Clip Wound Closing Kit (Stoelting). Mice were monitored daily following surgery.

Colitis score

Mean colitis score of the colon of each mouse was assessed by 5 distinct double-blinded observers following the guidelines from Erben et al. 2014 (42). (4-5 mice per group; 3 distinct experiments).

Flow cytometry

After lymphocyte isolation, single-cell suspensions were stained with the following fluorescence-conjugated mAbs, purchased from Thermofisher (eBioscience) unless otherwise stated: α-CD4–Alexa700 (GK1.5), α-CD8-V500 (53-6.7) (BD Biosciences), α-ST2-PerCP710 (RMST2-2), α-CD25-PECy7 (PC61) (BD Biosciences), α-Foxp3-FITC or

PE (FJK-16s), α-IL17A-APC (eBio17B7), α-IFNg-PECy7 (XMG1.2), α- RORγT-PE (AFKJS-9) or α-RORγT BV786 (Q31-378) (BD Bioscience), α-GATA3-Alexa647 (BD Biosciences), α-CD45.1 PE (A20) (PharMingen), α-Helios-Pacific Blue or PE (22F6) (Biolegend), α-CD121a/IL1R1 (35F5) (BD Biosciences) and CD90.2 Alexa 780 (53-2.1). Non-viable cells were excluded using fixable viability dye eFluor 780 or 506 reagent (Thermofisher). Data were acquired using a FACS Fortessa X-20 flow cytometer (BD Biosciences) and analyzed using FlowJo version 9 software (TreeStar).

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, N unpaired student T-test was used with the p-value expressed in the figure legend. All statistical analysis was performed with GraphPad Prism version 5 software (GraphPad Software).

2.7 Figures

Figure 1. Identification of ST2 as a marker of functionally stable T_{REG} cells.

(A) $CD4^+GFP^+Ly5.1^+$ T cells from $Foxp3^{GFPki}$ C57BL/6 mice were FACS-sorted and adoptively transferred into $TCR\beta^{-/-}$ mice. The cells were harvested 21 days post-transfer based on GFP expression and microarray analysis (see Methods) was performed (splenocytes of $TCR\beta^{-/-}$ mice were isolated and pooled before sorting the distinct populations; Two separate T cell transfers were performed, the cells isolated and processed individually for microarray analysis). The top 654 genes that varied ($p<0.05$ cut-off) between the groups are shown.

(B) Transcriptional signatures of stable T_{REG} (sT_{REG}), exT_{REG} and T_{EFF} cells isolated at day 21 post transfer.

(C) Venn diagram showing the distribution of differentially expressed genes (cut-off of >1 log2 fold change) between exT_{REG} and sT_{REG} relative to T_{REG} ($CD4^+GFP^+$) from day 0.

(D) Highest ranking (>2 fold change) genes in exT_{REG} and sT_{REG} at day 21 relative to T_{REG} cells from day 0.

(E) Pearson correlation of log2 fold change gene expression directly comparing sT_{REG} cells and exT_{REG} cells identifying the differential expression of *Il1r1* (blue) and *Il1rl1* (red). Dotted lines show ±1 log2 fold change.

(F) Representative flow cytometry profile of surface expression of IL1RL1 (IL-33R, ST2) and IL-1R1 (bottom) in $CD4^+CD45.1^+$ T cells in the spleen at day 21 after transfer (N=4); Controls are fluorescence minus one (FMO).

(G-H) $CD4^+$ T cells from $Foxp3^{GFP\text{-}CRE}$ $Rosa26^{RFP}$ fate-tracking mice were sorted and adoptively transferred (i.p.) into a $TCR\beta^{-/-}$ $Ly5.1^+$ mouse and examined 10 days post-transfer (N=4). **(G)** (Left) Representative plot showing frequencies of GFP^+RFP^+ (Foxp3+; sT_{REG}) and GFP^-RFP^+ (exT_{REG}) cells in $CD4^+CD45.2^+$ (right panel). Cut-off population of GFP^{hi} and GFP^{lo} T cells. **(H)** Representative histogram of expression levels of ST2 (left panel) and IL-1R1 (right panel) between GFP^{hi} (red) and GFP^{lo} (blue) $CD4^+CD45.2^+$ RFP^+ T cells.

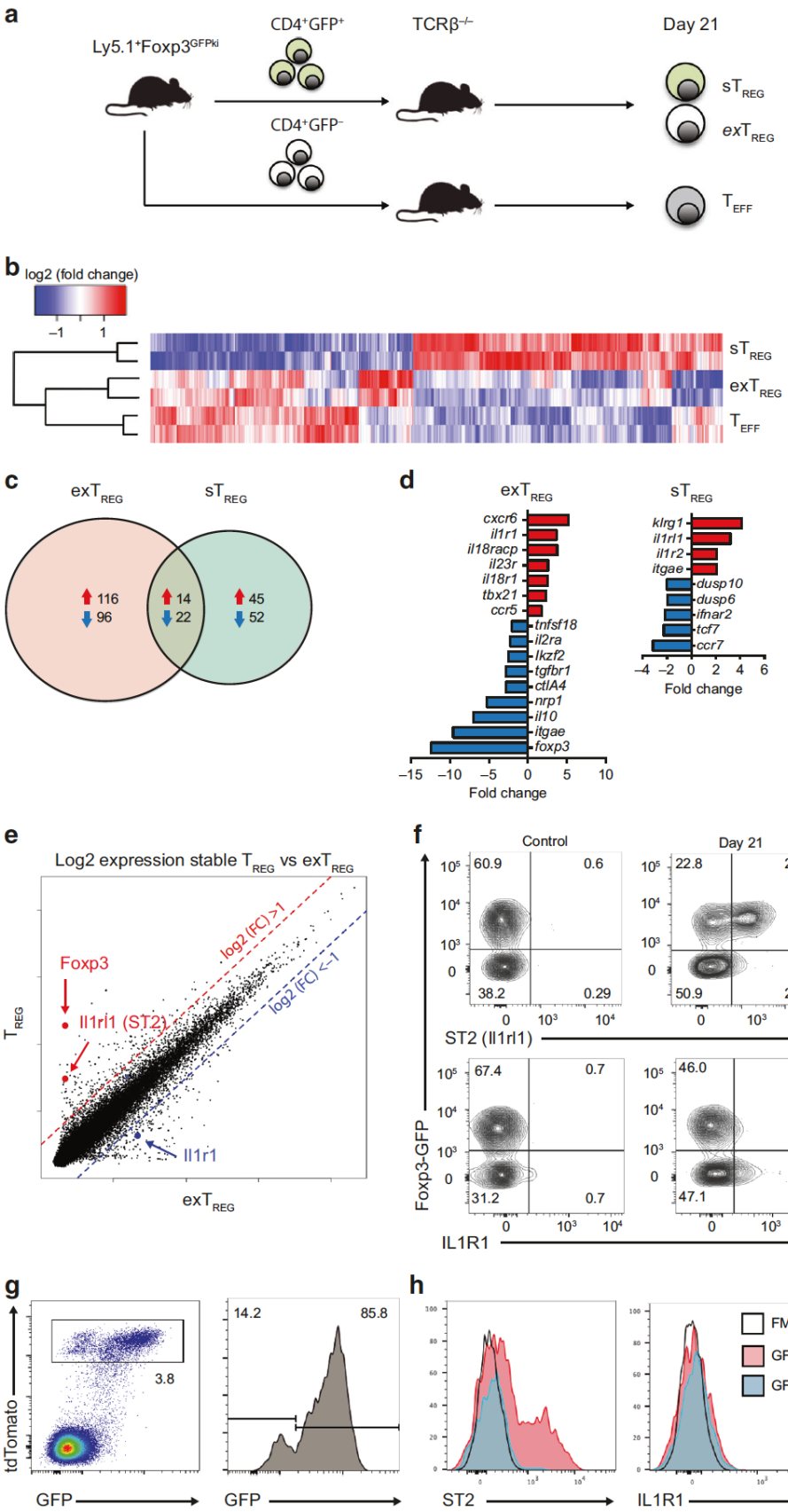

a
Ly5.1+Foxp3GFPki
CD4+GFP+
CD4+GFP−
TCRβ−/−
Day 21
sTREG
exTREG
TEFF
b
log2 (fold change)
−1
1
sTREG
exTREG
TEFF
c
exTREG
sTREG
116
96
14
22
45
52
d
exTREG
cxcr6
il1r1
il18racp
il23r
il18r1
tbx21
ccr5
tnfsf18
il2ra
lkzf2
tgfbr1
ctlA4
nrp1
il10
itgae
foxp3
−15 −10 −5 0 5 10
Fold change
sTREG
klrg1
il1rl1
il1r2
itgae
dusp10
dusp6
ifnar2
tcf7
ccr7
−4 −2 0 2 4 6
Fold change
e
Log2 expression stable TREG vs exTREG
Foxp3
Il1rl1 (ST2)
Il1r1
log2 (FC) >1
log2 (FC) <-1
TREG
exTREG
f
Control
Day 21
60.9
0.6
38.2
0.29
22.8
24.3
50.9
2.0
ST2 (Il1rl1)
67.4
0.7
31.2
0.7
46.0
0.69
47.1
6.24
Foxp3-GFP
IL1R1
g
tdTomato
3.8
GFP
14.2
85.8
GFP
h
ST2
IL1R1
FMO
GFPhi
GFPlo

Figure 2. The ST2 receptor segregates stable T_{REG} cells from pro-inflammatory IFNγ and IL-17A-producing T_{REG} cells *in vivo*.

(A) $CD4^+$ T cells peak in the lung by day 4 post-infection with Influenza A PR8/H1N1. C57BL/6 mice were infected with 20 pfu of PR8/H1N1 intra-nasally and necropsy was performed at day 0, 4, 10 and 17 post-infection. Representative of 4 distinct experiments.

(B) ST2 expression (red quadrant) is selectively increased in $CD4^+Foxp3^+$ T cell in the course of an Influenza A infection. (N=5).

(C-D) T_{REG} cells and $ST2^+$ T_{REG} cells accumulate in the lung and peak at day 10 after infection. (One way ANOVA. ***p<0.001; **p<0.01.)

(E) The increase in $ST2^+$ T_{REG} cell at day 10 post-infection is conscribed to the lung.

(F) Representative flow cytometry plot between $Foxp3^+$ (left) and $Foxp3^-$ (43) CD4+ T cells in the lungs of infected mice at day 10 post-infection.

(G-H) $ST2^-$ (blue), but not $ST2^+$ (red), T_{REG} cells produce IFNγ or IL17A at day 10 post-infection. Student T-test. * p<0.05; ** p<0.01; ***p<0.001.

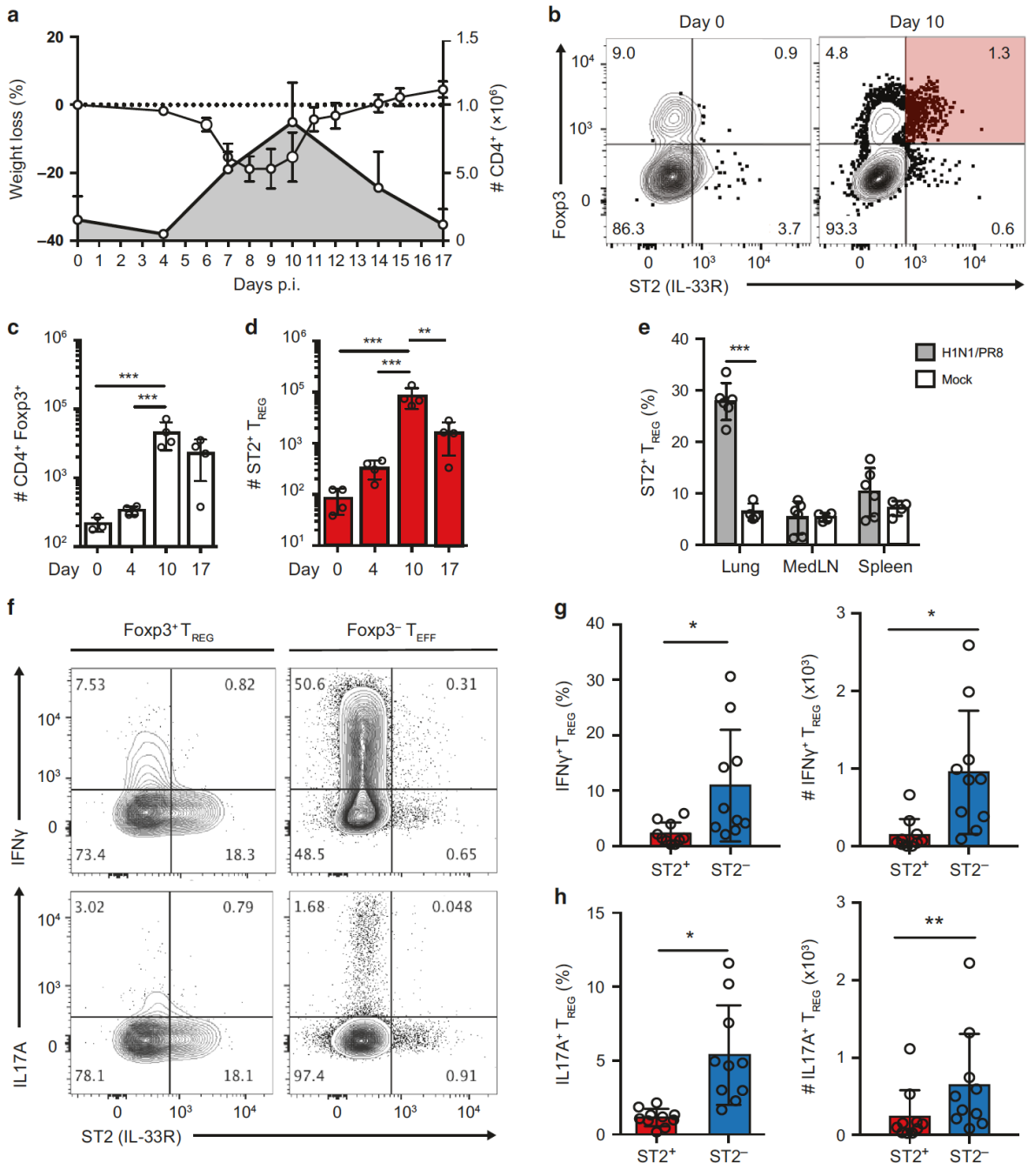

a
Weight loss (%)
CD4+ (×10^6)
Days p.i.
b
Day 0
Day 10
Foxp3
ST2 (IL-33R)
9.0
0.9
86.3
3.7
4.8
1.3
93.3
0.6
c
CD4+ Foxp3+
Day 0 4 10 17
d
ST2+ TREG
Day 0 4 10 17
e
ST2+ TREG (%)
H1N1/PR8
Mock
Lung
MedLN
Spleen
f
Foxp3+ TREG
Foxp3− TEFF
IFNγ
IL17A
ST2 (IL-33R)
7.53
0.82
73.4
18.3
50.6
0.31
48.5
0.65
3.02
0.79
78.1
18.1
1.68
0.048
97.4
0.91
g
IFNγ+ TREG (%)
IFNγ+ TREG (x10^3)
ST2+
ST2−
h
IL17A+ TREG (%)
IL17A+ TREG (x10^3)
ST2+
ST2−

Figure 3. IL-1 inhibits ST2 expression on Helios$^+$ T_{REG} cells.

(A) Representative flow cytometry profile of ST2 expression in T_{REG} cells from pLN and colon of naïve C57BL/6 mice (N=5 mice).

(B) Expression levels of Neuropilin 1, Helios and TIGIT by ST2$^+$ (red) and ST2$^-$ (blue) T_{REG} cells from the spleen of naïve C57BL/6 mice were analyzed by flow cytometry. Light grey histograms represent staining with isotype control. Representative of 3 mice, repeated in 3 separate experiments.

(C-D) CD4$^+$ T cells from spleens of WT and ST2$^{-/-}$ BALB/c mice were FACS-isolated and activated by plate-bound α-CD3 and α-CD28 in the presence of IL-2 (50U/ml) and/or IL-33 (10ng/ml) for 72 hours. Expression levels of Foxp3, ST2 and Helios were analyzed by flow cytometry, and (D) frequencies of Foxp3$^+$Helios$^+$ T_{REG} cells were determined. One-way ANOVA; *p<0.05. Triplicates. Representative of 3 distinct experiments.

(E-F) Mean fluorescence intensity of CD25 and Foxp3 expression of ST2$^+$ (red) and ST2- (blue) T_{REG} cells in (C). Student T-test. *** p<0.001. (triplicates; N=5)

(G) ST2$^+$ T_{REG} cells segregate with Helios expression in the T_{REG} adoptive transfer model. CD4$^+$GFP$^+$ T cells were transferred as described in Figure 1A and analyzed at day 21. Expression levels of ST2 and IL1R1 in Helios$^+$Foxp3$^+$ (green), Helios$^-$Foxp3$^+$ (blue) and Helios$^-$Foxp3$^-$ (red). (N=4 per group; Representative of 3 experiments).

(H) Representative flow cytometry profile of ST2 and GFP (Foxp3) expression on the transferred GFP^{neg} and GFP^{pos} (Ly5.1$^+$ T cells) in the spleen at day 21.

(I) Peripherally-induced T_{REG} cells (pT_{REG}) do not express ST2 expression after transfer. Student T-test. ***p<0.001. (N=4 per group; Representative of 3 experiments).

(J-K-L) IL1R1$^{-/-}$ mice show increased frequencies of ST2$^+$ T_{REG} cells in the lung at the steady state. **(J)** Representative flow cytometry of Helios vs. ST2 expression in the lung of naïve (WT), IL-1R1$^{-/-}$ or ST2$^{-/-}$ BALB/c mice. **(K)** Frequency of ST2$^+$ T_{REG} cells in the lung. **(L)** Mean fluorescence intensity (MFI) of ST2 among ST2$^+$ T_{REG} cells. One-way ANOVA; Tukey correction. ***p<0.001. (N=3-6 per group; Representative of 3 separate experiments).

(M) Representative flow cytometry plots of the expression of ST2 and IL-1R1 on $CD4^{+}Foxp3^{+}$ T_{REG} cells isolated from the lungs of BALB/c mice, and (N) Mean Fluorescence intensity (MFI) of IL1R1 on ST2+ (red) and ST2- (blue) T_{REG} cells. Paired Student T test; ***$p<0.01$. Representative of 3 distinct experiments (N=5 mice per experiment).

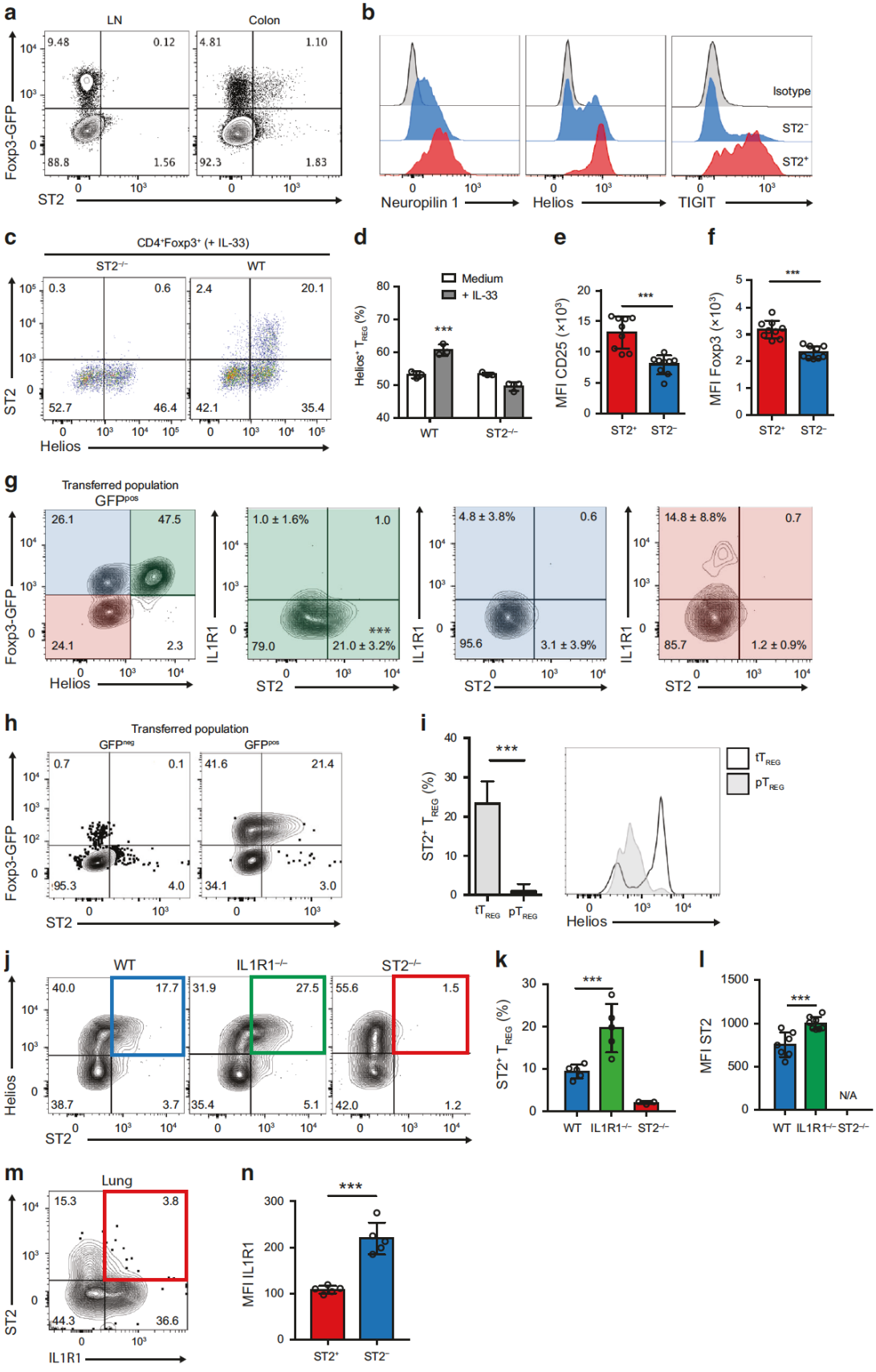

a
LN
Colon
9.48
0.12
88.8
1.56
4.81
1.10
92.3
1.83
Foxp3-GFP
ST2
b
Isotype
ST2⁻
ST2⁺
Neuropilin 1
Helios
TIGIT
c
CD4⁺Foxp3⁺ (+ IL-33)
ST2⁻/⁻
WT
0.3
0.6
52.7
46.4
2.4
20.1
42.1
35.4
ST2
Helios
d
Medium
+ IL-33
***
Helios⁺ TREG (%)
WT
ST2⁻/⁻
e
***
MFI CD25 (×10³)
ST2⁺
ST2⁻
f
***
MFI Foxp3 (×10³)
ST2⁺
ST2⁻
g
Transferred population
GFPpos
26.1
47.5
24.1
2.3
1.0 ± 1.6%
1.0
79.0
***
21.0 ± 3.2%
4.8 ± 3.8%
0.6
95.6
3.1 ± 3.9%
14.8 ± 8.8%
0.7
85.7
1.2 ± 0.9%
Foxp3-GFP
Helios
IL1R1
ST2
h
Transferred population
GFPneg
GFPpos
0.7
0.1
95.3
4.0
41.6
21.4
34.1
3.0
Foxp3-GFP
ST2
i
***
ST2⁺ TREG (%)
tTREG
pTREG
Helios
j
WT
IL1R1⁻/⁻
ST2⁻/⁻
40.0
17.7
38.7
3.7
31.9
27.5
35.4
5.1
55.6
1.5
42.0
1.2
Helios
ST2
k
***
ST2⁺ TREG (%)
WT
IL1R1⁻/⁻
ST2⁻/⁻
l
***
MFI ST2
N/A
WT
IL1R1⁻/⁻
ST2⁻/⁻
m
Lung
15.3
3.8
44.3
36.6
ST2
IL1R1
n
***
MFI IL1R1
ST2⁺
ST2⁻

Figure 4. Absence of IL-33-, but not IL-1-, signaling converts Helios$^+$ T_{REG} cells to a pro-inflammatory phenotype that fuels intestinal inflammation.

(A) SCID/beige mice received $CD4^+CD45RB^{hi}CD25^-Thy1.1^+$ effector T cells (T_{EFF}, $4x10^5$) either alone or with $CD4^+CD25^{hi}$ T_{REG} cells ($2x10^5$) from WT, $ST2^{-/-}$ or $IL\text{-}1R1^{-/-}$ BALB/C mice. Necropsy was done at day 28 post-transfer. Pooled results shown from 3 separate experiments. Where applicable, the results were compiled using a common denominator in each experiment (average of the WT).

(B) Mice that received T_{EFF} alone show weight loss by day 28 post-transfer.

(C) Mean colitis score of each mouse (see Methods). 5 blinded observers. One-way ANOVA.** $p<0.01$

(D) Relative number of cells (to the average of WT) of $CD11b^+Ly6G^+$ cells in the colon of mice, assessed by flow cytometry.

(E) Relative number of cells (to the average of WT) of Th17 cells ($CD4^+IL\text{-}17A^+$ amongst $Thy1.1^+$ cells) assessed by flow cytometry. One-way ANOVA. ***$p<0.001$; **$p<0.01$; *$p<0.05$

(F) $IL\text{-}1R1^{-/-}$ T_{REG} cell show less Foxp3 loss in the colon after transfer. Two-way ANOVA. (Tukey) **$p<0.01$.

(G) $IL\text{-}1R1^{-/-}$ T_{REG} cells co-express Helios and ST2.

(H) ST2-expressing $CD90.2^+$ T_{REG} cells are $Helios^+$.

(I) A higher frequency of $IL1R1^{-/-}$ T_{REG} cells express ST2 compared to WT T_{REG}. Two-way ANOVA. Tukey correction. ***$p<0.001$.

(J) $CD90.2^+$ ($Thy1.2^+$) $CD4^+Foxp3^+$ T_{REG} cells from $ST2^{-/-}$ donors accumulate as $ROR\gamma T^+$ in the colon of the mice (Relative to the average number of WT). One-way ANOVA. **$p<0.01$.

(K) exT_{REG} cells originating from $ST2^{-/-}$ T_{REG} cells accumulate as $IL\text{-}17A^+$ T cells in the colon of mice (Relative to the average number of WT).

(L) The numbers of T_H17-like exT_{REG} cells correlate with increased neutrophils accumulation in the colon of mice. Pearson correlation analysis. Representative of 3 separate experiments.

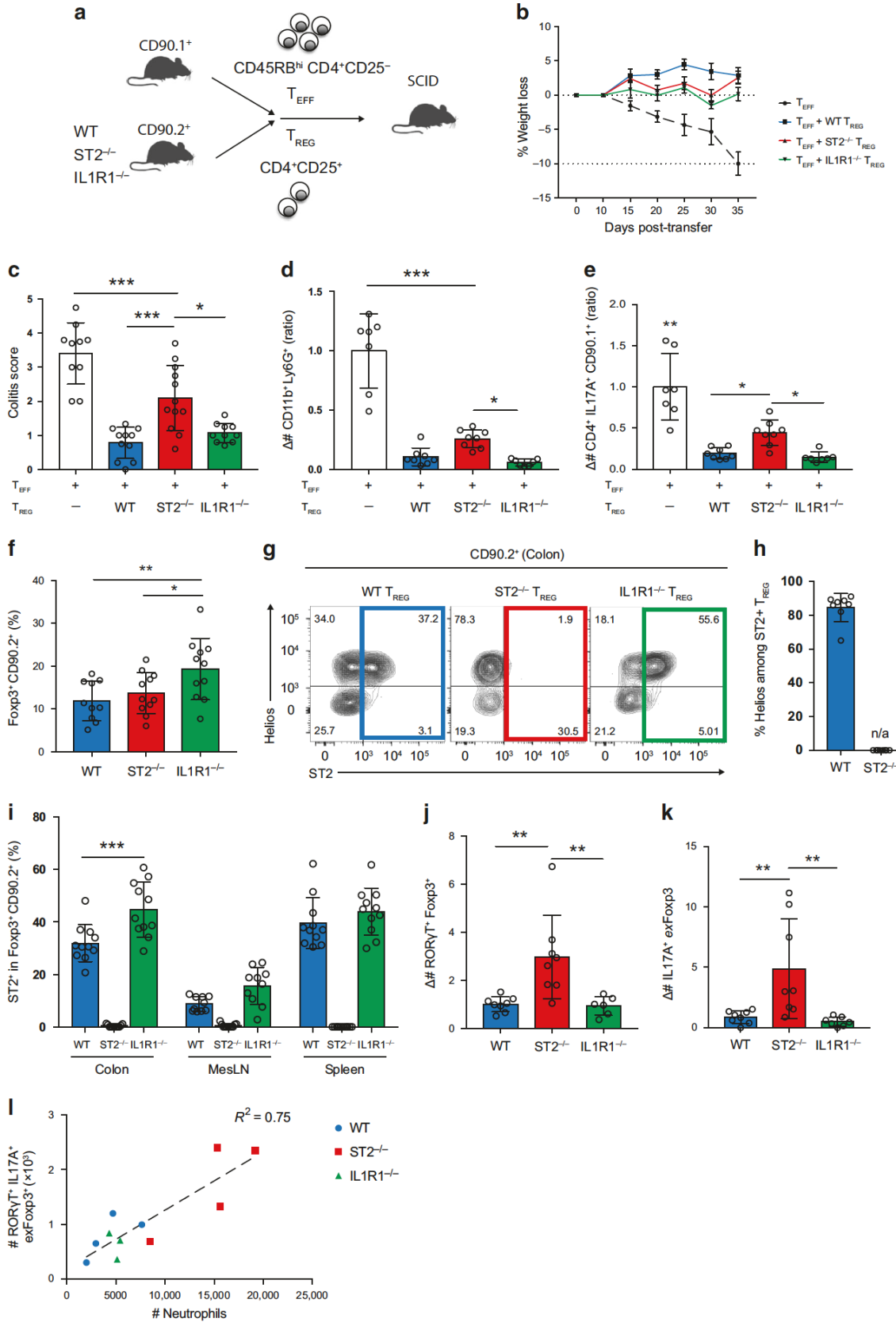

a
CD90.1+
CD45RBhi CD4+CD25−
SCID
TEFF
TREG
WT
ST2−/−
IL1R1−/−
CD90.2+
CD4+CD25+
b
% Weight loss
Days post-transfer
TEFF
TEFF + WT TREG
TEFF + ST2−/− TREG
TEFF + IL1R1−/− TREG
c
Colitis score
d
Δ# CD11b+Ly6G+ (ratio)
e
Δ# CD4+ IL17A+ CD90.1+ (ratio)
f
Foxp3+CD90.2+ (%)
g
CD90.2+ (Colon)
WT TREG
ST2−/− TREG
IL1R1−/− TREG
Helios
ST2
34.0
37.2
25.7
3.1
78.3
1.9
19.3
30.5
18.1
55.6
21.2
5.01
h
% Helios among ST2+ TREG
n/a
i
ST2+ in Foxp3+CD90.2+ (%)
Colon
MesLN
Spleen
j
Δ# RORγT+ Foxp3+
k
Δ# IL17A+ exFoxp3
l
R2 = 0.75
# RORγT+ IL17A+ exFoxp3+ (×10³)
# Neutrophils

Figure 5. T_H17 polarizing conditions induce IL1R1 expression on T_{REG} cells, favour their proliferation but hinder their suppressive ability.

$Ly5.2^+CD4^+GFP^+$ T_{REG} cells were FACS-sorted from C57BL/6 $Foxp3^{GFPki}$ mice and co-cultured (1:2 ratio) with $Ly5.1^+CD4^+GFP^-$ (T_{EFF}) sorted from similar mice, α-CD3 (1ug/ml) and irradiated APCs ($CD4^{neg}$ fraction from the $Ly5.2^+$) for 72 hours. Representative of 5 experiments in triplicate.

(A) Representative flow plot of the gating strategy.

(B) T_H17 polarizing conditions [IL-6 (10 ng/ml) + TGFβ (1 ng/ml)], not IL-1β (25 ng/ml), upregulate RORγT in CD4+ Foxp3+ T cells *in vitro*. One-way ANOVA. Tukey correction. ***p<0.001

(C) RORγT is co-expressed with IL-1R1 on T_{REG} cells in T_H17 polarizing conditions.

(D) $ROR\gamma T^+$ $Foxp3^+$ T cells express the IL-1R1 receptor upon T_H17 polarization to a greater extent than $ROR\gamma T^+$ T_{EFF} cells.

(E) IL-1β enhances the proliferation of T_{REG} cells in T_H17 polarizing conditions. $Ly5.2^+CD4^+GFP^+$ T cells were stained with V450 proliferation dye prior to culture. ANAKINRA (Kineret®). T_{REG} in medium (blue), in T_H17 pol. conditions (red), in T_H17 + IL-1β (green) or in T_H17 + IL-1β + ANAKINRA (100ug/ml) (grey).

(F) $CD4^+Foxp3^+Ly5.2^+$ T cell counts after 72 hours. One-way ANOVA, Tukey correction. *p<0.05; **p<0.01; ***p<0.001.

(G) IL-1β signaling enhanced proliferation of T_{EFF} ($Ly5.2^+$) cells even in the presence of T_{REG} cells at a 1:2 ratio. $CD4^+Foxp3^-Ly5.1^+$ T_{EFF} cells alone (white), in the presence of T_{REG} at 1:2 ratio (blue), in Th17 pol. conditions (red) or in the presence of IL-1β (green).

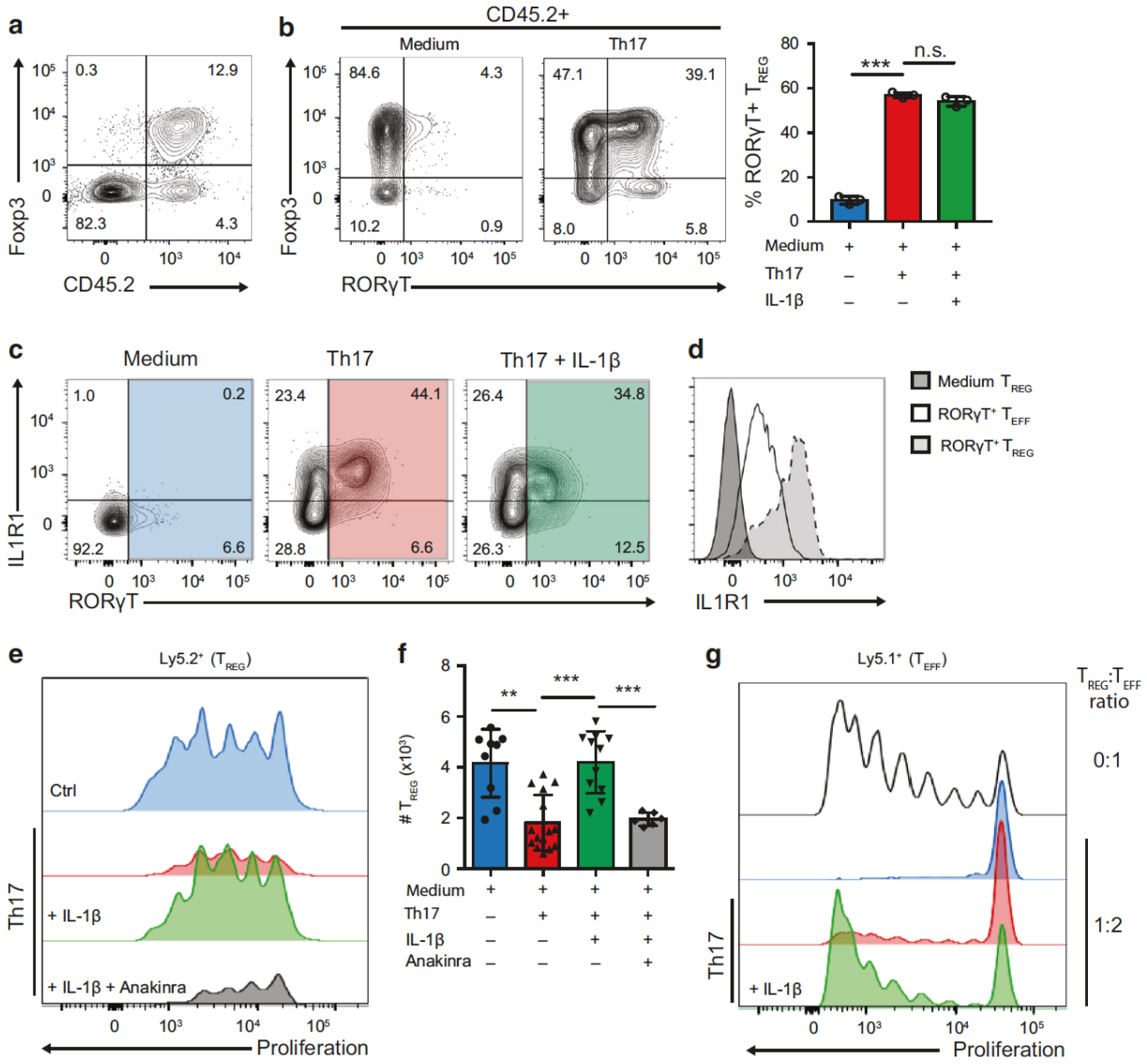

a
Foxp3
CD45.2
0.3
12.9
82.3
4.3
b
CD45.2+
Medium
Th17
Foxp3
RORγT
84.6
4.3
10.2
0.9
47.1
39.1
8.0
5.8
% RORγT+ TREG
***
n.s.
Medium
Th17
IL-1β
c
Medium
Th17
Th17 + IL-1β
IL1R1
RORγT
1.0
0.2
92.2
6.6
23.4
44.1
28.8
6.6
26.4
34.8
26.3
12.5
d
IL1R1
Medium TREG
RORγT+ TEFF
RORγT+ TREG
e
Ly5.2+ (TREG)
Th17
Ctrl
+ IL-1β
+ IL-1β + Anakinra
Proliferation
f
TREG (x10³)
**
***
***
Medium
Th17
IL-1β
Anakinra
g
Ly5.1+ (TEFF)
TREG:TEFF ratio
0:1
1:2
Th17
+ IL-1β
Proliferation

Figure 6. IL-33 favours the stability of Helios^{+}ST2^{+} T_{REG} cells in T_H17 polarizing conditions.

(A-B) ST2^{+} and ST2^{-} CD4^{+}GFP^{+} (T_{REG}) were isolated from the spleen of Foxp3^{GFPki} mice and plated in the presence of Ly5.1^{+}CD4^{+}GFP^{-} (T_{EFF}) sorted from Ly5.1^{+}Foxp3^{GFPki} mice, soluble α-CD3 (1ug/ml) and irradiated APCs (CD4^{neg} fraction from the Ly5.2^{+}) at a 1:2 ratio for 72 hours. Representative of 4 distinct experiments in triplicates.

(A) T_{REG} cells resist the loss of ST2 and Helios expression under pro-inflammatory conditions *in vitro.* ST2^{+} and ST2^{-} CD4^{+}GFP^{+} T cells from the spleen of Foxp3^{GFPki} were activated in the presence of IL-6 (10 ng/ml), TGF-β (1ng/ml) and IL-1β (25ng/ml) for 72 hours.

(B) Loss of Helios expression is associated with an increase in RORγT expression in Foxp3^{+} T_{REG} cells *in vitro*. One-way-ANOVA; *p<0.05; **p<0.01; ***p<0.001

(C-D-E-F) CD4^{+}GFP^{+} T_{REG} cells were isolated from the spleen of Foxp3^{GFPki} mice and co-cultured in the presence of CTV-labeled CD4^{+}GFP^{-} T_{EFF} cells (1:2 ratio), α-CD3 (1ug/ml) and irradiated APCs (CD4^{neg} fraction from the Ly5.2^{+}) for 72 hours.

(C-D) IL-33 facilitates the up-regulation of the ST2 receptor on T_{REG} cells in T_H17 polarizing conditions. One-way ANOVA. Tukey correction. **p<0.01

(E-F) ST2^{+} T_{REG} cells resist the expression of RORγT and IL-1R1. Paired Student T test; *p<0.05; **p<0.01; ***p<0.001

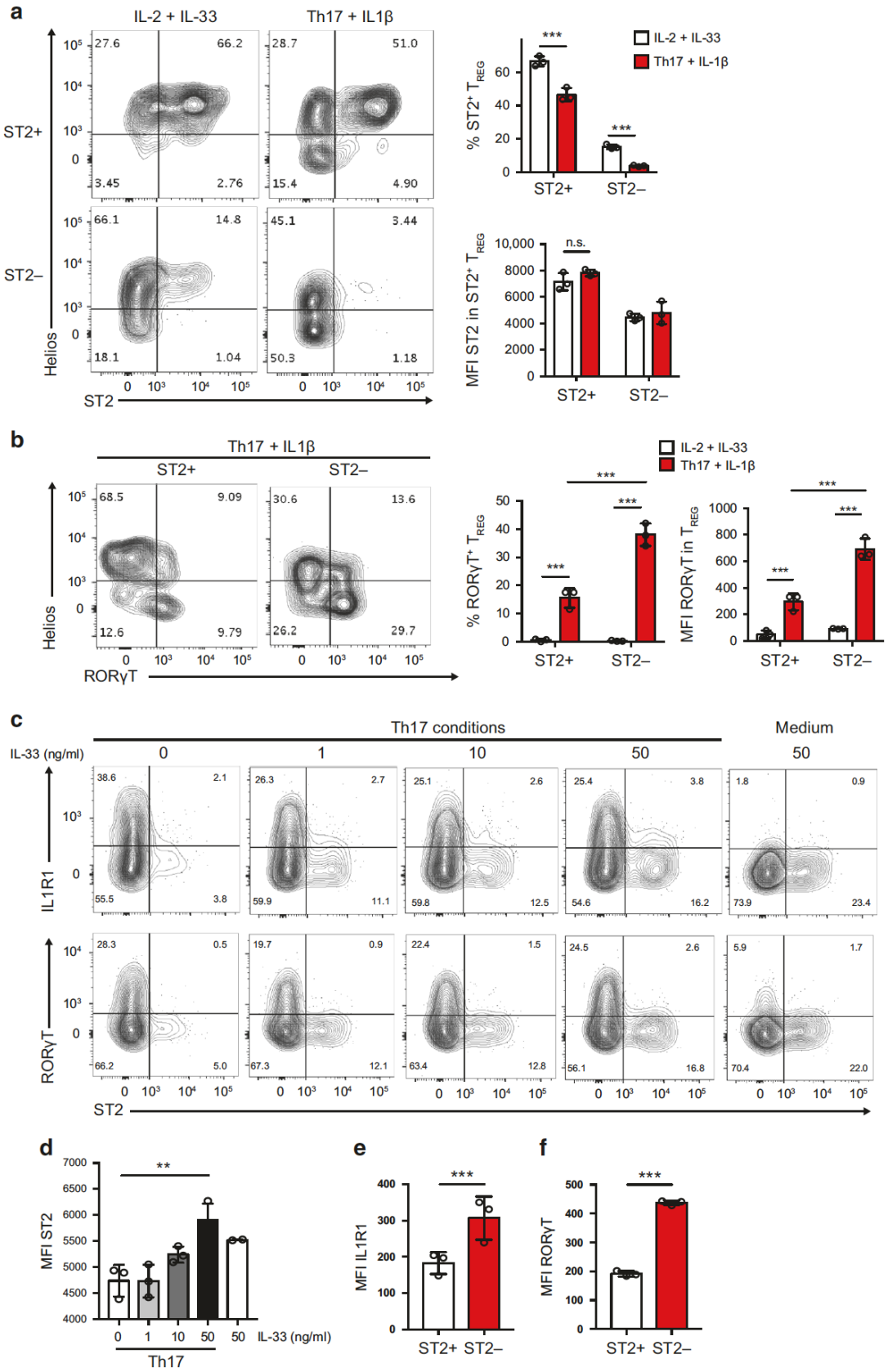

a
IL-2 + IL-33
Th17 + IL1β
ST2+
ST2–
Helios
ST2
27.6
66.2
3.45
2.76
28.7
51.0
15.4
4.90
66.1
14.8
18.1
1.04
45.1
3.44
50.3
1.18
IL-2 + IL-33
Th17 + IL-1β
% ST2+ TREG
***
ST2+
ST2–
MFI ST2 in ST2+ TREG
n.s.
b
Th17 + IL1β
ST2+
ST2–
Helios
RORγT
68.5
9.09
12.6
9.79
30.6
13.6
26.2
29.7
% RORγT+ TREG
MFI RORγT in TREG
c
Th17 conditions
Medium
IL-33 (ng/ml)
0
1
10
50
50
IL1R1
RORγT
ST2
38.6
2.1
55.5
3.8
26.3
2.7
59.9
11.1
25.1
2.6
59.8
12.5
25.4
3.8
54.6
16.2
1.8
0.9
73.9
23.4
28.3
0.5
66.2
5.0
19.7
0.9
67.3
12.1
22.4
1.5
63.4
12.8
24.5
2.6
56.1
16.8
5.9
1.7
70.4
22.0
d
MFI ST2
**
Th17
IL-33 (ng/ml)
e
MFI IL1R1
f
MFI RORγT

Figure 7. Absence of IL-1 signaling *in vivo* skews the T_{REG} cell response to favour $ST2^+$ T_{REG} cells in the course of disease.

(A) IL-$1R1^{-/-}$ mice show increased fungal burden in the lung (left) and brain (43) upon intra-tracheal exposure to 10^4 CFU/mice of *Cryptococcus neoformans* 52D. (N=4-5 per group; Representative of 3 distinct experiments).

(B) Representative flow cytometry profile of ST2 relative to Foxp3 expression in $CD4^+$ T cells at day 14 post-infection (lung).

(C) Higher frequency of $Foxp3^+$ T_{REG} cells in the IL-$1R1^{-/-}$ (red) compared to WT mice (white).

(D) ST2-expressing T_{REG} cells represent a major population of $Foxp3^+$ T cells in IL-$1R1^{-/-}$ mice (red) throughout the course of infection in opposition to WT mice (white).

(E) $ST2^+$ T_{REG} cells accumulate early in the lungs during infection but decrease in WT (white) compared to IL-$1R1^{-/-}$ mice (red).

(F) IL-$1R1^{-/-}$ mice (red) show a decreased frequency of $ROR\gamma T^+$ $CD4^+Foxp3^+$ T_{REG} cells in the lungs at the peak of adaptive immunity.

(G) IL-17A production by $Foxp3^+$ T cells originates from $ST2^-$ T_{REG} cells in the lung at day 14 post-infection.

(H) $ST2^-$ T_{REG} expressing RORγT represent a significant fraction of T_{REG} cells in the lung in the course of infection. Two-way ANOVA. Tukey correction. ***p<0.001. **p<0.01. *p<0.05.

(I) ST2 expression on T_{REG} cells correlates with increased GATA3 expression in the lung at day 14 in both WT (white) and $IL1R1^{-/-}$ (red).

(J-K) Absence of IL-1R1 influences the balance between $GATA3^+$ and $ROR\gamma T^+$ T_{REG} cells in the lung (day 14) in WT (white) when compared to IL-$1R1^{-/-}$ (red). One-way ANOVA. ***p<0.001. **p<0.01. *p<0.05.

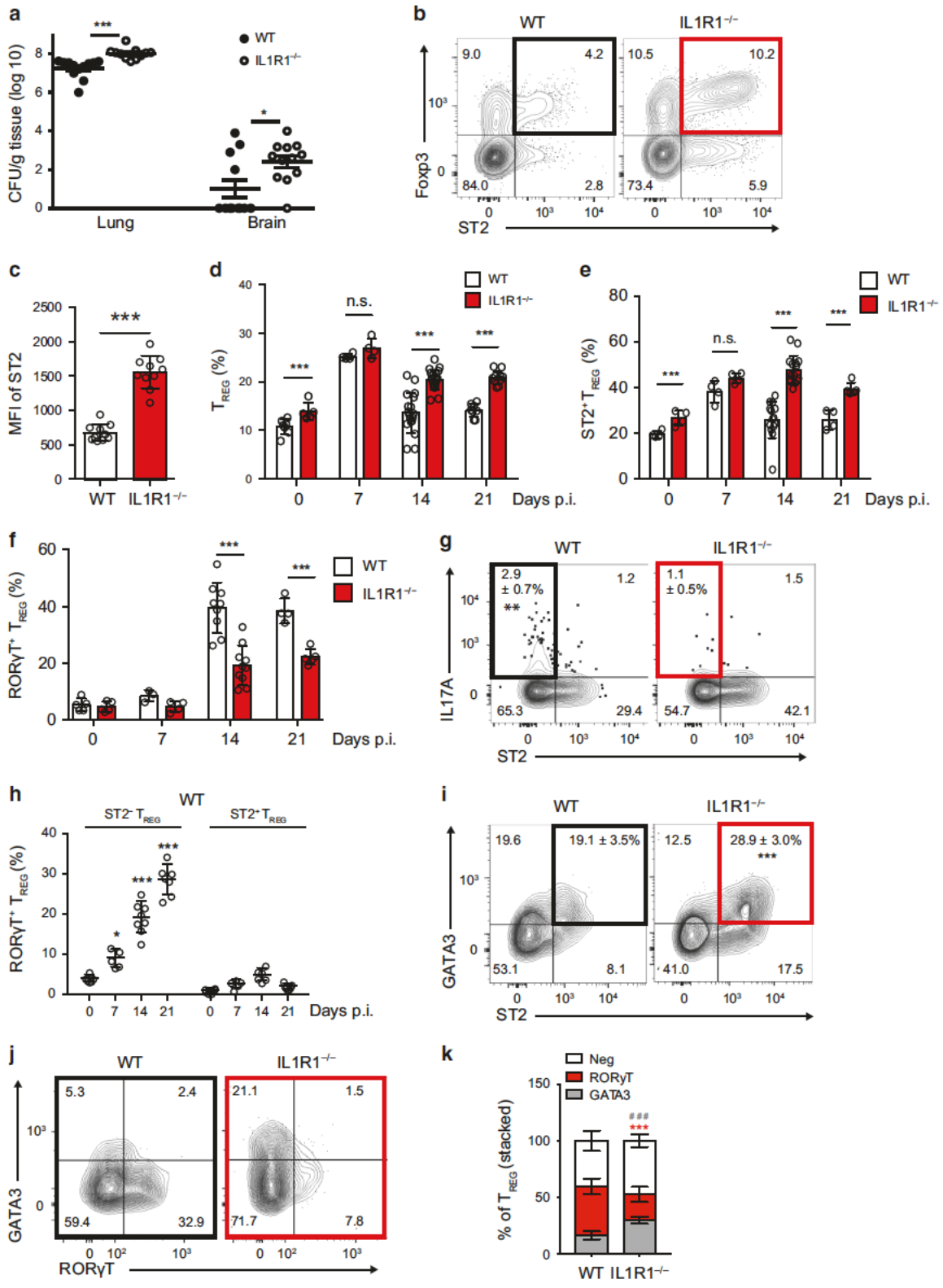

a
CFU/g tissue (log 10)
WT
IL1R1−/−
Lung
Brain
b
WT
IL1R1−/−
Foxp3
ST2
c
MFI of ST2
WT
IL1R1−/−
d
TREG (%)
n.s.
Days p.i.
e
ST2+ TREG (%)
n.s.
Days p.i.
f
RORγT+ TREG (%)
Days p.i.
g
IL17A
ST2
2.9 ± 0.7%
1.1 ± 0.5%
h
ST2- TREG
ST2+ TREG
RORγT+ TREG (%)
Days p.i.
i
GATA3
ST2
19.1 ± 3.5%
28.9 ± 3.0%
j
GATA3
RORγT
k
Neg
RORγT
GATA3
% of TREG (stacked)

Figure S1. Stable T_{REG} cells express ST2 upon transfer in lymphopenic hosts.

$CD4^+$ T cells from $Foxp3^{GFP\text{-}CRE}$ X Rosa26 lox-stop-lox tdTomato fate-mapping mice were FACS-sorted and adoptively transferred (i.p.) into $TCR\beta^{-/-}$ $Ly5.1^+$ recipient for 10 days (4 distinct mice).

(A) Percentage of $CD4^+CD45.2^+$ ($Ly5.2^+$) among total cells isolated from the recipient mice at day 10 post-transfer.

(B) Representative flow cytometry plots of GFP vs. ST2 and IL1R1 expression in $CD4^+CD45.2^+$ ($Ly5.2^+$) RFP^+ T cells at day 10.

(C) Mean fluorescence intensity (MFI) of IL-1R1 in GFP^{hi} and GFP^{lo} in the colon after transfer.

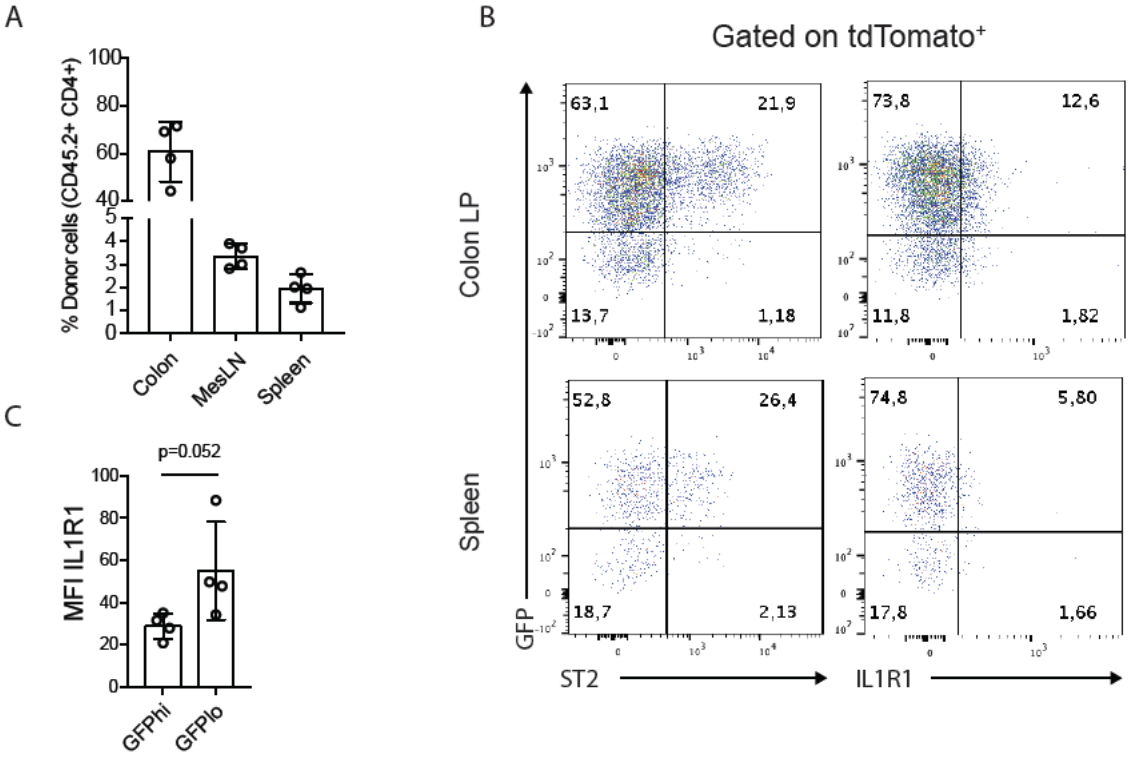


Figure S2. ST2^{+} T_{REG} cells express a memory phenotype.

(A) Expression levels of CD44 and CD62L by ST2^{+} (left) and ST2^{-} (43) T_{REG} cell subsets were analyzed by flow cytometry from spleen of Foxp3^{GFPki} mice. Representative of 5 distinct mice.

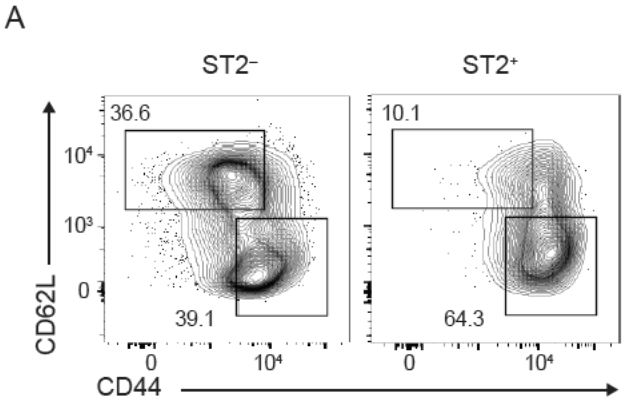


Figure S3. *In vitro* stimuli with IL-33 facilitates the up-regulation of ST2 and the expression of GATA3 in thymic T_{REG} cells.

(A) $CD4^{SP}$ (Single-positive) GFP^{+} thymocytes of naïve 8-week-old B6 $Foxp3^{GFPki}$ mice were FACS-sorted and activated with α-CD3 and α-CD28, and in the presence of IL-2 (100U/ml) and/or IL-33 (10ng/ml) for 72 hours. Representative flow plot.

(B) Thymic-isolated $ST2^{+}$ T_{REG} respond to IL-33 by up-regulating the ST2 receptor. Student T-test. *p<0.05 Representative of 3 distinct experiments is shown

(C) $CD4^{+}GFP^{+}$ T cells from spleens of $Foxp3^{GFPki}$ mice were FACS-sorted and activated by plate-bound α-CD3 and α-CD28 in the presence of IL-2 (50U/ml) and/or IL-33 (10ng/ml) for 72 hours. Expression levels of Foxp3 and GATA3 were analyzed by flow cytometry, and frequencies of Foxp3-GFP^{+} $GATA3^{+}$ T_{REG} cells (depicted in the gates) were determined (N=3). Representative of 3 distinct experiments is shown.

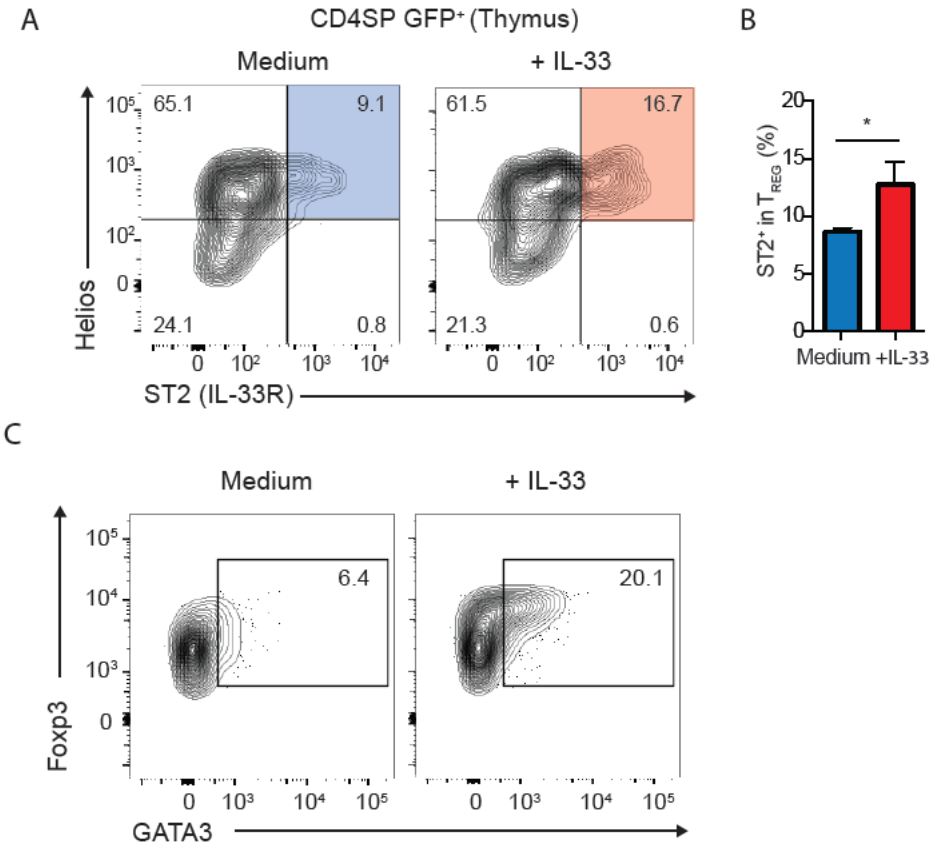


Figure S4. IL-33 promotes the proliferation of ST2^{+} Helios^{+} T_{REG} cells.

(A) CD4^{+}GFP^{+} T cells from spleens of Foxp3^{GFPki} mice were FACS-sorted, V450 labeled, and plated at a 1:2 ratio with responder T cells (as described in Figure 2M and N) for 72 hours. Left panel: representative histogram of proliferating T cells (V450 dilution). Right panel: Total number of T_{REG} cells at 72 hours. Student T test. ***p<0.001. (N=4).

(B) CD4^{+}CD25^{hi} T cells from WT or ST2$^{-/-}$ BALB/C mice were activated in plated α-CD3 (3μg/ml) and α-CD28 (2μg/ml) in the presence or absence of IL-33 (10ng/ml). Numbers of T_{REG} cells after 72 hours (N= 3). Student T test; ***p<0.001. **p<0.01

(C-D) IL-33 does not affect T_{REG} suppression *in vitro*. V450 proliferation dye labeled responder (CD4^{+}GFP^{-}) T cells were activated in the presence of irradiated APCs (CD4^{neg} fraction) and soluble αCD3 (1ug/ml), and with titrated numbers of CD4^{+}GFP^{+} or GFP^{-} T cells from Foxp3^{GFPki} mice. Suppressive function, as determined by V450 dye dilution, was assessed at 72 hours post-activation. Representative histogram of proliferating T cells (V450 dilution) is shown. One-way ANOVA. Tukey correction. (N=3)

(E) The generation of ST2^{+} T_{REG} cells is dependent on IL-33 and IL-2 *in vitro*. CD4^{+} GFP^{+} T cells were isolated form Foxp3^{GFPKi} mice and activated with αCD3 (3μg/ml) and αCD28 (2μg/ml) in the presence of IL-2 (100U/ml) with or without IL-33 (10ng/ml) for 72 hours. Student T-test. *** p<0.001. (triplicates; N=5 separate experiments)

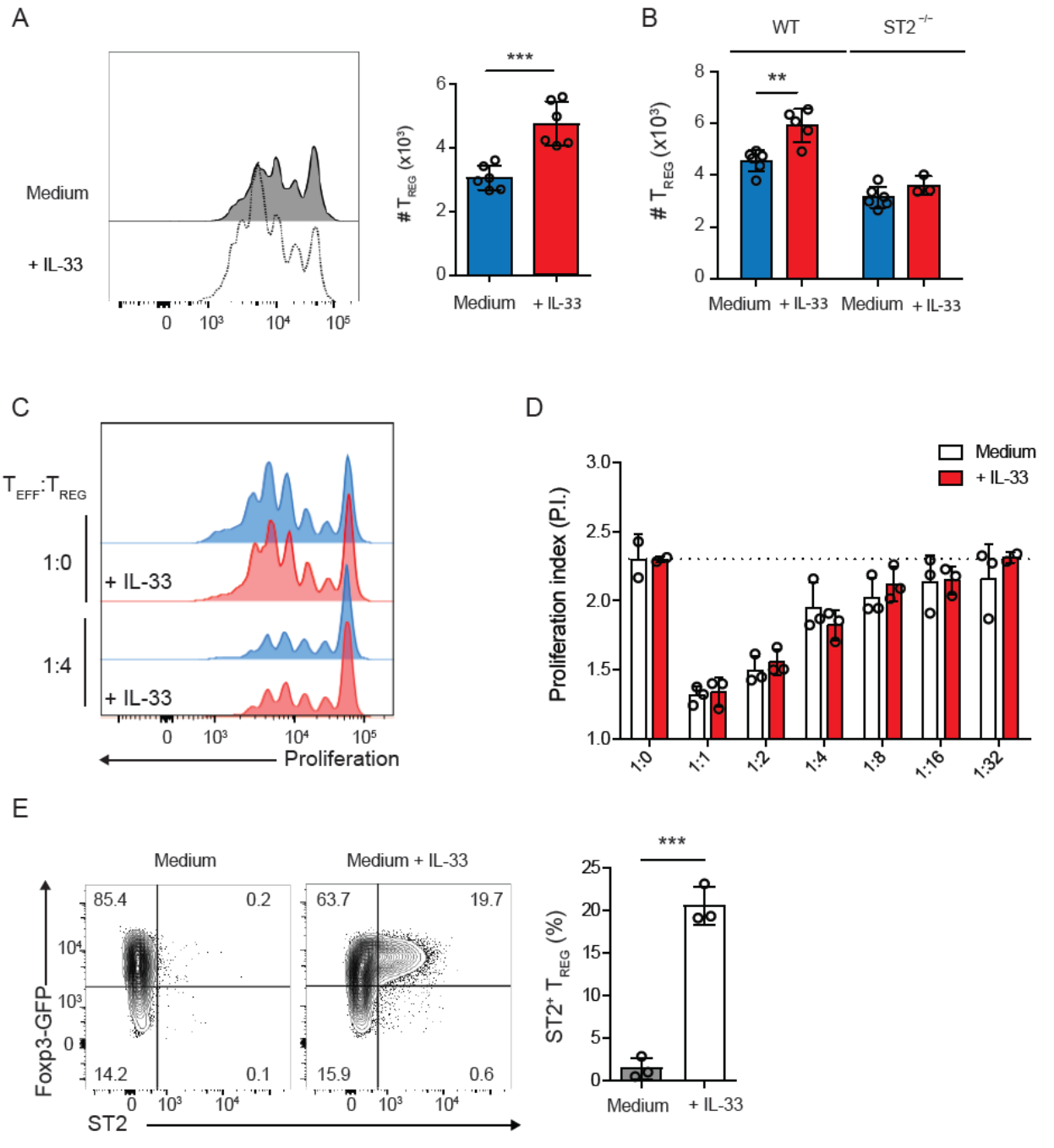

A
Medium
+ IL-33
0
10³
10⁴
10⁵
***
T_REG (x10³)
Medium
+ IL-33
B
WT
ST2 -/-
**
T_REG (x10³)
Medium
+ IL-33
Medium
+ IL-33
C
T_EFF:T_REG
1:0
+ IL-33
1:4
+ IL-33
Proliferation
D
Medium
+ IL-33
Proliferation index (P.I.)
1:0
1:1
1:2
1:4
1:8
1:16
1:32
E
Medium
Medium + IL-33
85.4
0.2
14.2
0.1
63.7
19.7
15.9
0.6
Foxp3-GFP
ST2
***
ST2⁺ T_REG (%)
Medium
+ IL-33

Figure S5. IL-33 does not induce ST2 expression in iT_{REG} cells in the presence of TGFβ and IL-2.

(A) $CD4^+GFP^+$ T cells from spleens of $Foxp3^{GFPki}$ mice were FACS-isolated and activated in plated anti-CD3 (3μg/ml) and anti-CD28 (2μg/ml) in the presence of IL-2 (100U/ml) with or without rhTGFβ1 (5ng/ml) and IL-33 (10ng/ml) for 72 hours. Representative flow cytometry plots of triplicate cultures. (N=3 separate experiments).

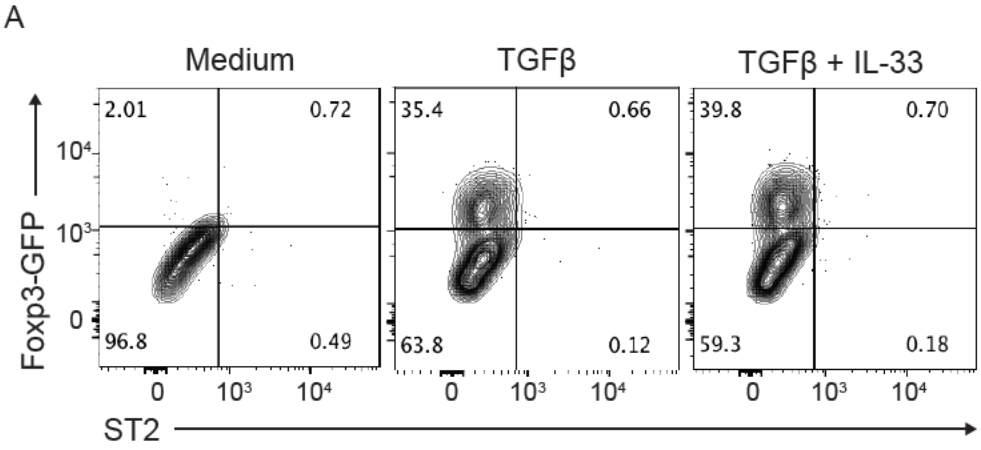


Figure S6. Lack of IL-1 signaling facilitates the accumulation of Foxp3^{+} T_{REG} cells in the colon.

(A) Representative flow cytometry plot of CD90.2 (Thy1.2) vs. Foxp3 among CD4+ T cells isolated from the colon of SCID/bei mice 28 days after transfer of $CD4^{+}CD45RB^{hi}CD25^{-}Thy1.1^{+}$ effector T cells (T_{EFF}, $4x10^{5}$) either alone or with $CD4^{+}CD25^{hi}$ T_{REG} cells ($2x10^{5}$) from WT, $ST2^{-/-}$ or $IL\text{-}1R1^{-/-}$ BALB/C mice. (see Methods).

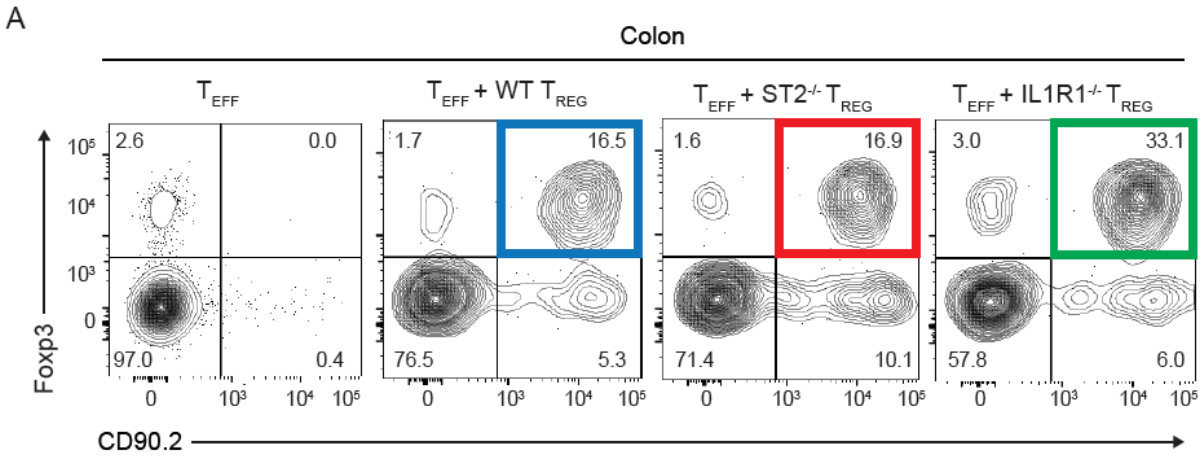


Figure S7. IL-33 supplementation prevents the development of Th17-like T_{REG} and exT_{REG} cells *in vivo*.

$CD4^+GFP^+$ T cells from the spleen of $Foxp3^{GFPki}$ $Ly5.1^+$ female mice were cell-sorted and transferred intravenously (i.v.) into $TCR\beta^{-/-}$ female mice. 8 mice were divided into two groups, one group received PBS (PBS) while the other received 100ng/mouse of rIL-33 (+IL-33) intra-peritoneally (i.p.) every two days starting at day 0 of transfer. Necropsy was performed at day 14 post-transfer. **(A)** IL-33 prevents the accumulation of $IL\text{-}17A^+$ T cells in both $Foxp3^+$ and $Foxp3^-$ ($CD4^+$ $Ly5.1^+$) compartments. **(B)** IL-33 prevents the accumulation of $IL\text{-}17A^+$ exT_{REG} cells in the colon. **(C)** IL-33 prevents RORγT expression in splenic T_{REG} cells ($CD4^+GFP^+$). **(D)** IL-33 supplementation favoured the accumulation of $ST2^+$ T_{REG} cells (Spleen shown). Student T test. $p<0.05$.

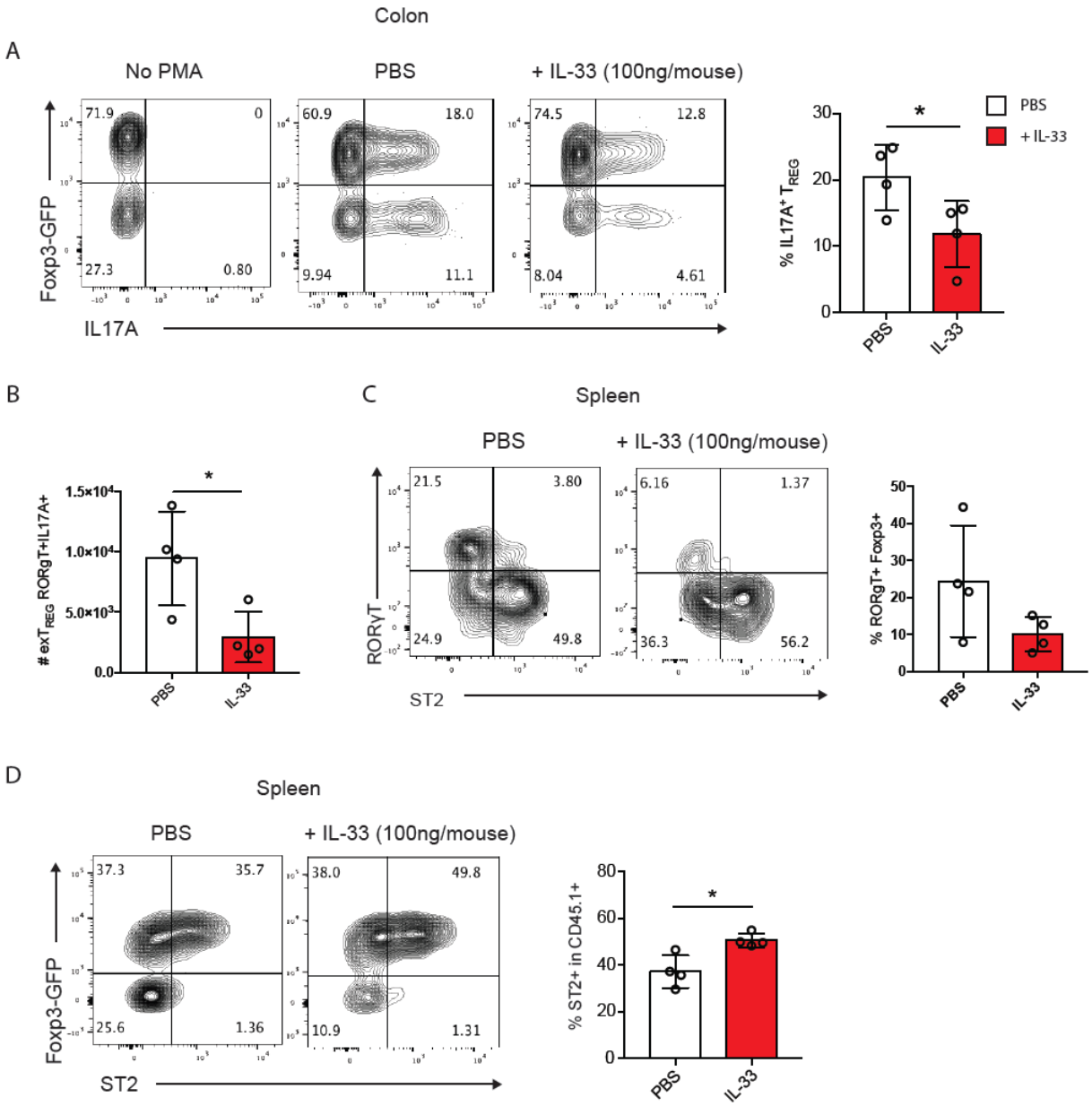

Colon
A
No PMA
PBS
+ IL-33 (100ng/mouse)
71.9
0
27.3
0.80
60.9
18.0
9.94
11.1
74.5
12.8
8.04
4.61
Foxp3-GFP
IL17A
% IL17A+ TREG
PBS
+ IL-33
B
exTREG RORgT+IL17A+
C
Spleen
RORγT
ST2
21.5
3.80
24.9
49.8
6.16
1.37
36.3
56.2
% RORgT+ Foxp3+
D
37.3
35.7
25.6
1.36
38.0
49.8
10.9
1.31
% ST2+ in CD45.1+

Figure S8. Blockade of IL-1 impairs T_{REG} cell expansion in T_H17 conditions.

$CD4^+CD25^{hi}$ T cells were FACS-sorted from the spleen of WT or IL-$1R1^{-/-}$ BALB/c mice, and co-cultured with WT $CD4^+CD25^-$ T cells (1:2 ratio) in the presence of irradiated APCs and soluble αCD3 (1μg/ml) for 72 hours, and in the absence (Ctrl) or presence of IL-6 (10 ng/ml), TGFβ (1ng/ml), or IL-1β (25 ng/ml). In some cases, Anakinra (100 ng/ml) was added. **(A)** Expression of RORγT and IL-1R1 in $CD4^+Foxp3^+$ T_{REG} cells. **(B)** Expression of IL-1R1 in $Helios^+$ and $Helios^-$ T_{REG} cell subsets (N=4). Student T-test *p<0.05. **(C-D)** Proliferation and absolute cell counts of $Foxp3^+$ T cells after culture. One-way ANOVA. **p<0.01.

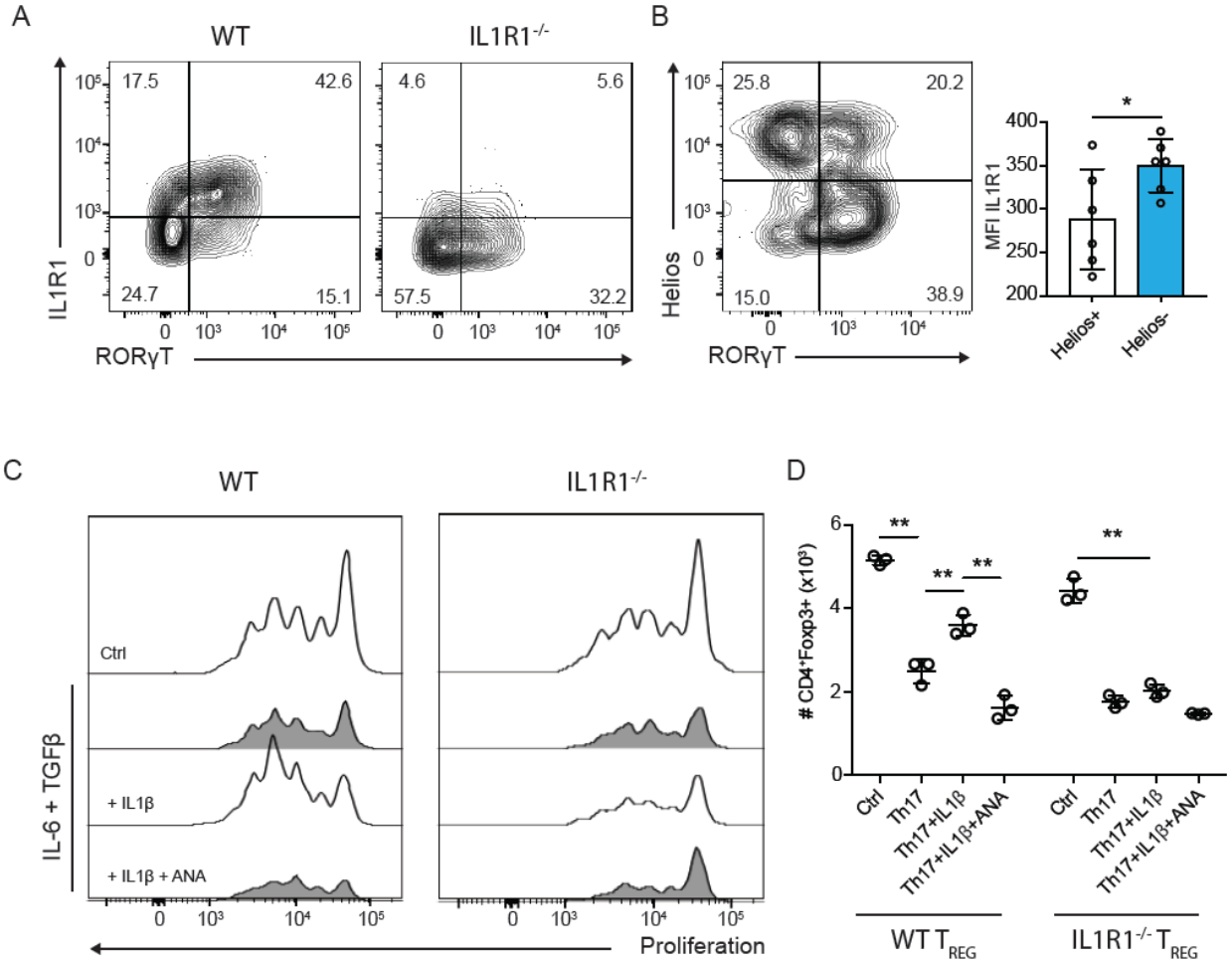


Figure S9. The presence of IL1β does not alter the T_{EFF}/T_{REG} balance.

T_{REG} cells (GFP^+) were FACS-sorted from $Ly5.2^+$ $Foxp3^{GFPki}$ reporter mice, and were activated in the presence of congenic $Ly5.1^+$ T_{EFF} cells (GFP^-) for 72 hours in the presence of irradiated APCs ($CD4^{neg}$ fraction) and soluble αCD3 (1μg/ml). **(A)** Representative gating strategy in flow cytometry analysis. **(B)** Frequency of $Foxp3^+$ $CD45.2^+$ ($Ly5.2^+$) T cells in the culture after 72 hours. One-way ANOVA. ***p<0.001. (N=5).

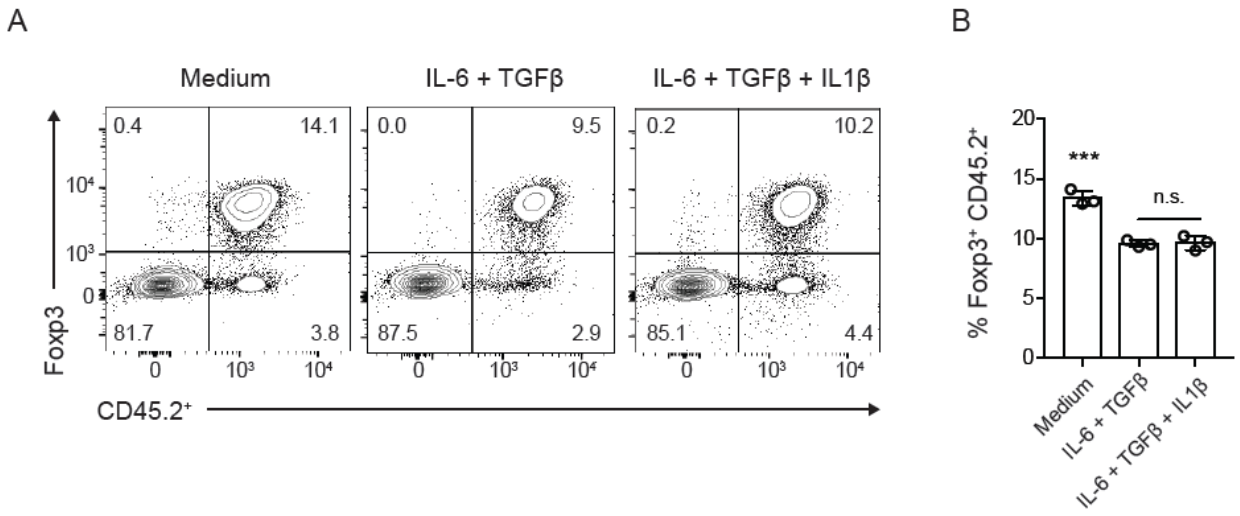


Figure S10. IL-2 facilitates the accumulation of ST2$^+$ T_{REG} cells.

FACS-sorted CD4$^+$GFP$^+$ T_{REG} cells from Ly5.2$^+$ Foxp3^{GFPki} mice were co-cultured for 72 hours with CD4$^+$GFP$^-$ T_{EFF} cells FACS-sorted from congenic Ly5.1$^+$ Foxp3^{GFPki} mice (1:2 ratio) in the presence of irradiated APCs and soluble αCD3 (1μg/ml). During the culture period, cells were exposed to TGFβ (1ng/ml), IL-33 (10ng/ml) and IL-1β (25 ng/ml) and increasing concentrations of IL-2 or IL-6. **(A)** IL-2, but not IL-6, facilitates the accumulation of ST2$^+$ T_{REG} cells in inflammatory conditions. Two-way ANOVA. Tukey correction. **(B)** The mean fluorescence of ST2 (MFI) on T_{REG} cells is dependent on IL-2 and is not influenced by IL-6. **(C)** IL-2 and IL-33 are not sufficient to skew the ratio of ST2$^+$ to IL-1R1$^+$ T_{REG} cells (# ST2$^+$/# IL-1R1$^+$) in the presence of IL-6 and IL1β. Two-way ANOVA. Tukey correction. *p<0.05; **p<0.01; ***p<0.001. **(D)** IL-6 induces IL-1R1 expression on T_{REG} cells in a dose-dependent manner. One-way ANOVA. *p<0.05; **p<0.01.

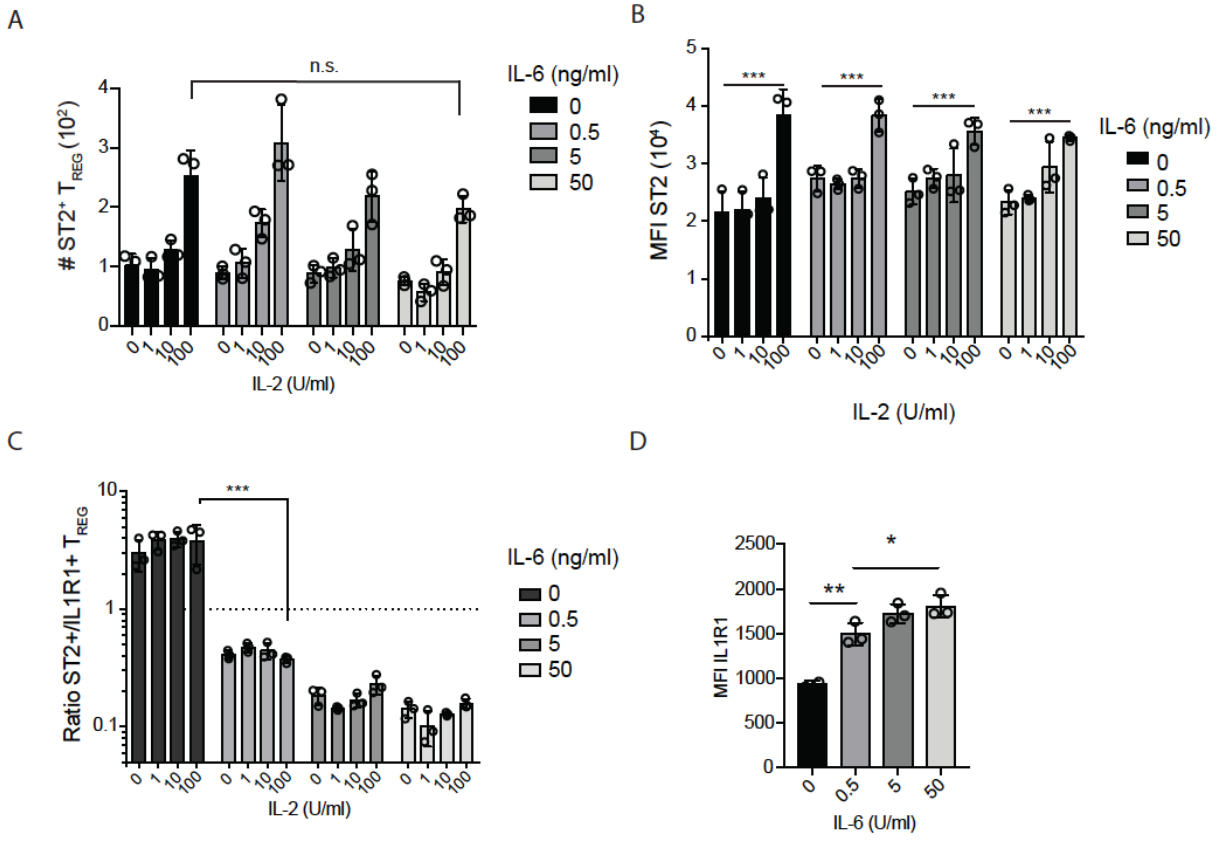
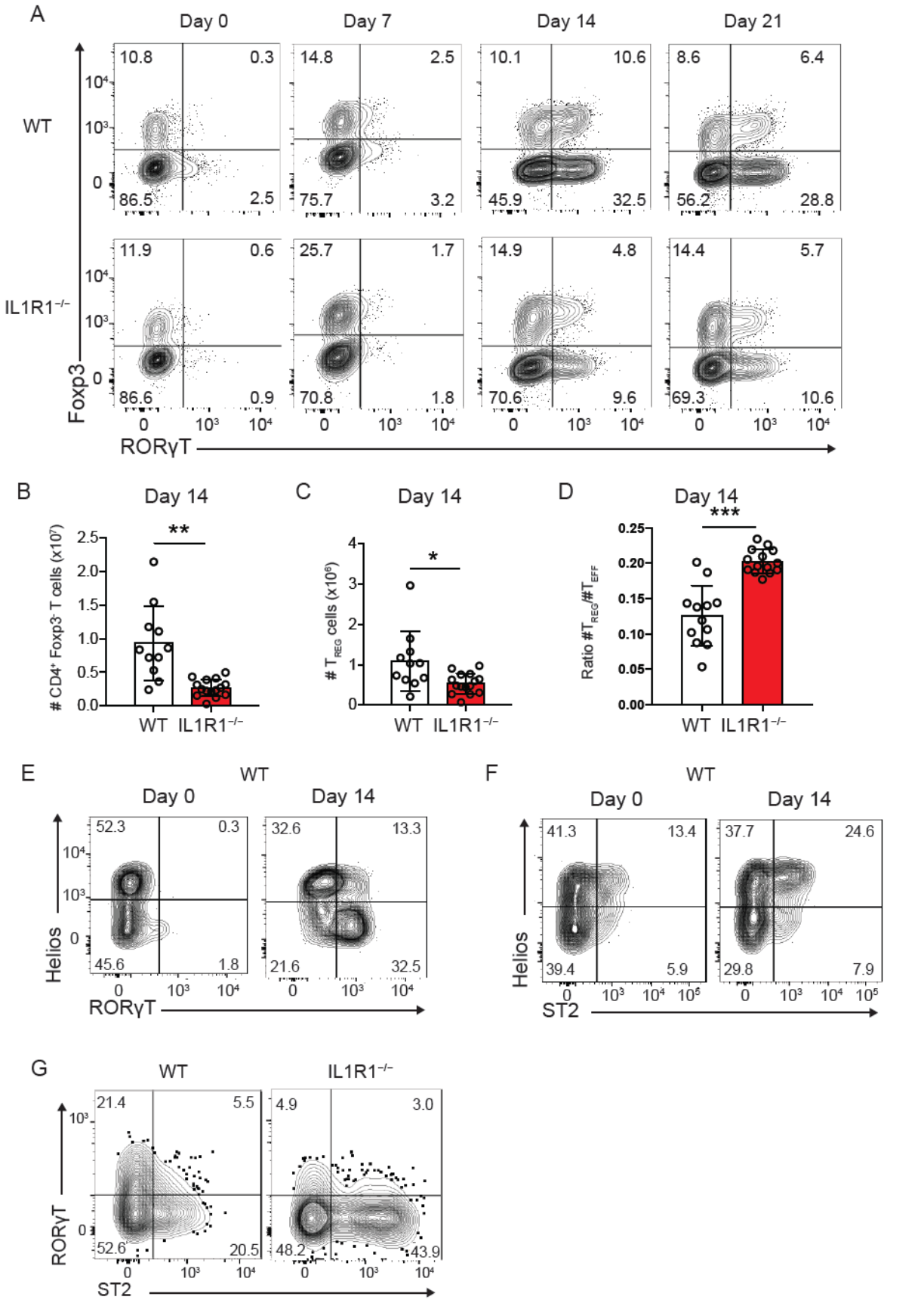


Figure S11. Accumulation of RORγT^{+} T_{REG} and T_{EFF} cells depend on IL-1R1 signaling in the course of infection.

WT and IL-1R1$^{-/-}$ BALB/c mice received 10^5 CFU/mice of *Cryptococcus neoformans* 52D intra-tracheally, and the groups (n=4-5) were necropsied at days 0, 7, 14 and 21 post-infection. Data compiled from 3 distinct experiments. **(A)** Representative flow cytometry profiles of CD4^{+} T cells in the lung of mice at days 0, 7, 14 and 21 post-infection. **(B)** Number (#) of T_{EFF} cells (CD4^{+}Foxp3^{-} T cells) in the lungs at day 14 of infection. **(C)** Number of T_{REG} (CD4^{+}Foxp3^{+} T cells) in the lungs at day 14 of infection. **(D)** Ratio (# of T_{REG}/# of T_{EFF}) of T_{REG} cells over T_{EFF} cells in the lungs of mice at day 14. Student T-test. *p<0.05; **p<0.01; ***p<0.001. **(E)** Expression of Helios and RORγT, and **(F)** ST2 in CD4^{+}Foxp3^{+} T cells in the lungs of WT mice at day 0 and 14 post-infection. **(G)** Representative flow cytometry plot of CD4^{+}GFP^{+} T cells in the lung of mice at day 14 post-infection between WT (left) and IL-1R1$^{-/-}$ (43) mice.


A
Day 0
Day 7
Day 14
Day 21
WT
IL1R1−/−
Foxp3
RORγT
10.8
0.3
86.5
2.5
14.8
2.5
75.7
3.2
10.1
10.6
45.9
32.5
8.6
6.4
56.2
28.8
11.9
0.6
86.6
0.9
25.7
1.7
70.8
1.8
14.9
4.8
70.6
9.6
14.4
5.7
69.3
10.6
B
Day 14
CD4+ Foxp3- T cells (x10^7)
**
WT
IL1R1−/−
C
Day 14
TREG cells (x10^6)
*
WT
IL1R1−/−
D
Day 14
Ratio #TREG/#TEFF
***
WT
IL1R1−/−
E
WT
Day 0
Day 14
Helios
RORγT
52.3
0.3
45.6
1.8
32.6
13.3
21.6
32.5
F
WT
Day 0
Day 14
Helios
ST2
41.3
13.4
39.4
5.9
37.7
24.6
29.8
7.9
G
WT
IL1R1−/−
RORγT
ST2
21.4
5.5
52.6
20.5
4.9
3.0
48.2
43.9

2.8 References

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CHAPTER 3 - Aberrant memory T_H2 responses against neonatal RSV infection impair the functional adaptation of $ST2^+$ T_{REG} cells through enhanced STAT6 signaling

Chapter 3- Aberrant memory T_H2 responses against neonatal RSV infection impair the functional adaptation of $ST2^+$ T_{REG} cells through enhanced STAT6 signaling

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3.1 Bridging statements for Chapter 3

In the previous chapter, we described how IL-33 and IL-1β alarmins determine the function and fate of T_{REG} cells at mucosal surfaces. Specifically, we showed that IL-33 signaling in T_{REG} cells promoted the stability of Foxp3 expression and the suppressive ability of T_{REG} cells, while IL-1β blocked the suppressive ability of T_{REG} cells, drove the expansion of pro-inflammatory T_H17-like exT_{REG} cells and contributed to exacerbated T_H17 responses during pulmonary viral and fungal infections. Collectively, our results positioned IL-33 as a self-preserving alarmin that contributes to immune homeostasis by stabilizing the T_{REG} phenotype and promoting T_{REG} cell suppressive functions. In **Chapter 2**, we focused our investigation of IL-33 on infectious disease models characterized by hallmark T_H1 and T_H17-driven responses. However, IL-33 was shown to play a pro-inflammatory role in T_H2-driven diseases (1), suggesting that the immune context may dictate the pro- and anti-inflammatory effect(s) of IL-33 on T_{REG} cells. As such, we investigated the effect of IL-33 on T_{REG} cells in the course of a respiratory syncytial virus (RSV) model characterized by T_H2-associated pathology. In line with our study of how infectious diseases drive T_{REG} cell adaptation to the lungs, we studied the effect of a neonatal infection with RSV. This unique murine model is driven in part by the pro-inflammatory action of IL-33 (2) and is thought to be a valid model to explain the link between infantile pulmonary viral disease and the onset of asthma (3). In **Chapter 3**, we investigated how IL-33 signaling modulates the suppressive function of pulmonary T_{REG} cells and enables an aberrant and pathogenic T_H2 response using a neonatal RSV infection model. In **Chapter 3**, we highlight that the immunopathological context is key in determining the effect of IL-33 on the suppressive function of T_{REG} cells.

3.2 Summary

Respiratory viral diseases, including infections caused by the Respiratory syncytial virus (RSV), are a major cause of morbidity in infants and are, in severe cases, associated to the development of asthma in children. RSV infections in the neonatal period, when the immune system is still immature, can have long-term consequences that alter immuno-regulatory mechanisms that serve to preserve lung homeostasis. As such, dysregulated regulatory T (T_{REG}) cell responses have been associated to RSV-induced asthma, although the mechanisms remain ill-defined. Since IL-33 is a key alarmin involved in the exacerbation of T_H2 responses, we hypothesized that a neonatal RSV infection impairs the long-term suppressive function of pulmonary IL-33-responding ($ST2^+$) T_{REG} cells and contributes to the RSV-induced pathology.

In this study, we investigated the role of IL-33 on T_{REG} cells in the course of a neonatal RSV A2 infection and followed the long-term effect of the infection on their functional adaptation in mucosal sites. Here, we uncovered that, contrary to conventional T_H2 cells, $ST2^+$ T_{REG} cells fail to accumulate in the lungs at the time of neonatal RSV infection but expand robustly upon RSV re-infection, at which time they fail to prevent an aberrant T_H2 response in the lungs. Importantly, we show that this effect is dependent on STAT6 signaling in $ST2^+$ T_{REG} cells. While IL-33 potentiated the expression of the IL4Rα, IL-4, in turn, impaired the suppressive function and drove the production of IL-5 and IL-13 by T_{REG} cells. Moreover, this mechanism was antigen-independent in the adult, since IL-33 alone was sufficient to generate the aberrant T_H2 response in mice infected with RSV as neonates.

We identify a novel feed-back mechanism by which IL-33 impairs the suppressive ability of T_{REG} cells by potentiating STAT6 signaling in these cells. These results highlight how the micro-environment dictates the functional adaptation of T_{REG} cells and provides a novel target for the development of therapies.

3.3 Introduction

Respiratory viral diseases are a major cause of morbidity and mortality in infants (4). Throughout the neonatal period and early childhood, the newborn is highly susceptible to disease as the immune system requires maturation and "education" in order to accelerate and optimize its response (5). Interestingly, immune immaturity in the neonatal period is not characterized by complete immunosuppression but rather by an immune skewing towards type 2 immunity mediated by innate and adaptive immune cells (5). Activation of dendritic cells (DCs), macrophages and innate lymphoid-like cells (ILCs) from the lung lead to a lower production of type 1-related cytokines like IL-12 and IFNγ and an increase in type 2 associated cytokines like IL-4, IL-5 and IL-13 compared to cells from an adult (6) while T cell responses are biased towards type 2 helper T cells (T_H2) (5). This is not without consequence, as it is suggested that many viral infections in infancy can lead to airway hyperreactivity in the adult (7). In mice, it is now well established that early (<7 day old) infection with a respiratory syncytial virus (RSV) leads to an exacerbated type 2 response upon re-challenge in adult mice, in a pathology reminiscent of asthma (3). Many aspects of this model have been characterized, notably the role of T_H2-associated alarmins like TSLP, IL-25 and IL-33, all prominent cytokines involved in the pathology of asthma (2, 8). However, the aspects of immune regulation that are altered during these exacerbated responses in adulthood remain ill-defined.

Although components of innate and adaptive immunity play a role in regulating the immune response, $CD4^+Foxp3^+$ regulatory T cells (T_{REG}) are major players in the maintenance of immune homeostasis in the lungs (9). Interestingly, in mice that were infected with RSV as neonates, the frequency of pulmonary T_{REG} cells in the adult are significantly reduced when exposed to a foreign antigen, suggesting a potential effect of RSV in facilitating immune evasion from T_{REG} cells and contributing to late-onset airway hyper-reactivity (10). Furthermore, it is suspected that neonatal RSV infection concomitantly drives a long term exacerbated type 2 response by impairing T_{REG} cell function (11, 12). T_{REG} cells are critical to prevent exacerbated disease in RSV-infected adult mice, revealing that the suppressive function of T_{REG} cells is required for a quick

return to homeostasis (13). The mechanisms by which T_{REG} cells fail to fully prevent RSV-induced airway hyperreactivity upon neonatal challenge remains elusive.

In recent years, T_{REG} cells were found to adapt to local inflammatory conditions in order to migrate, modulate their suppressive function, facilitate repair mechanisms and/or contribute to pathogen clearance (14-17). Among the factors that can influence T_{REG} adaptation, IL-33, an IL-1 family alarmin associated with increased T_H2 response in RSV (2), was shown to directly influence T_{REG} cells differentiation at mucosal surfaces (14, 15, 18, 19). IL-33 was shown to enhance T_{REG} cells proliferation, promote low levels of IL-5 and IL-13 production (20) and restrain IFNγ and IL-17A production by T_{REG} cells in inflammatory sites (14). Moreover, $ST2^+$ T_{REG} cells can suppress T_H2 cells directly (21) and produce amphiregulin in order to facilitate lung repair and control inflammation (15). However, in a murine lung allergy model, IL-33 signalling was shown to dysregulate the suppressive function of local T_{REG} cells (22). Thus, it remains to be understood how IL-33 could paradoxically drive both suppressive and pro-inflammatory functions in T_{REG} cells.

In this report, we investigated the role of locally adapted $ST2^+$ T_{REG} cells in the development of a dysregulated type 2 immune response upon secondary infection with RSV. We show that neonatal infection with RSV skews both the innate and adaptive immune response toward type-2 immunity and observed the development of strong IL-4 and IL-13-producing T_H2 cells in the lungs of infected mice. The exacerbated T_H2 response was associated with a significant increase in proliferating $ST2^+$ T_{REG} cells. Importantly, IL-33 signaling enhanced the expression of the IL4Rα on T_{REG} cells, and promoted IL-4 signaling. Indeed, exogeneous IL-4 synergized with IL-33 to promote a T_H2-like phenotype in $ST2^+$ T_{REG} cells *in vitro*, by enhancing GATA3 expression and promoting IL-5 and IL-13 production in T_{REG} cells. Importantly, IL-33 and IL-4 acted in concert to dysregulate the suppressive ability of T_{REG} cells. Collectively, RSV-generated memory T_H2 cells in the lungs impair the suppressive ability of pulmonary $ST2^+$ T_{REG} cells by releasing high amounts of IL-4 in an IL-33 dependent manner.

3.4 Results

1. Neonatal RSV A2 infection leads to the establishment of a type 2 immune response in the lungs.

Since neonatal mice are susceptible to the development of a type 2 response (6), we first investigated the immunological landscape in the lungs upon a neonatal infection with RSV. We inoculated neonatal BALB/c (5-7 day-old) mice with RSV A2 ($1x10^6$ $TCID_{50}$/g) I.N. and sacrificed the mice at day 4 post-infection to observe short-term immune changes in their lungs (Figure 1A). We observed a significant increase in the number of cells collected from the lungs at day 4, revealing that these mice generated a response to the infection (Figure 1B).

In recent years, the role of type 2 innate lymphoid cells (ILC2) has been described in airway hyper-reactivity (26). These cells are important contributors to the local release of IL-5, IL-9 and IL-13 in the lung during type 2 immunopathology and drive the dysregulated response to RSV since these signals contribute to the polarization of T_H2 responses and the recruitment of mast cells and eosinophils (27). In order to identify ILC2s, we gated on Lin^- (CD3, GR-1, B220, CD11b, CD11c, CD49b, TCRγδ), $Thy1.2^+$, $CD127^+$, $CD25^+$, $ST2^+$ (Figure 1C) and did not observe a difference in the frequency of these cells among pulmonary cells between mock and RSV-infected neonates (Figure 1D). On the other hand, we observed an increase in the absolute numbers of ILC2s (Figure 1E), in line with a previous report (2), suggesting that, while the proportion of these cells among all the inflammatory cells present in the lungs did not change, they too readily expanded upon infection. Similarly, pulmonary alveolar macrophages (PAMs) have also been shown to play an important role in inducing strong NK and T cells response upon neonatal RSV (28). We gated on live $CD45^+$ $Ly6G^{int}$ $F4/80^+$ cells differentiating $CD11c^+$ as PAMs (29). We observed an increase in the number of alveolar macrophages in the lungs of neonate mice (Figure 1F). Moreover, these PAMs displayed increased MHC-II expression on their surface (Figure 1G), illustrating a heightened level of activation. Finally, it was suggested that macrophages can also specialize through classic or alternative activation in a manner analogous to T_H1 and T_H2 cells. This is notably illustrated by the competing effects of

iNOS (M1) or Arginase-1 (M2) production and may reflect their ability to direct the differentiation of the adaptive immune response between T_H1 and T_H2 cells (30). Indeed, in the lungs of RSV-infected neonates, we observed a significant increase in Arg-1 in PAMs in the lungs of infected mice (Figure 1H). Collectively, these results highlight that RSV A2 favors a type 2 innate immune response in the neonate.

While RSV-specific T cells play a central role in enhancing the airway hyperactivity induced by RSV infections in the adult (31), it remains to be confirmed if an infection in neonatal mice lead to the generation of antigen-specific T_H2 cells that persist in the lungs in the adult. In the infected neonate mice, we observed an increase in the number of $CD44^{high}CD62^{low}$ $CD4^+$, but not $CD8^+$ T cells (Figure 1I-J), revealing $CD4^+$ T cells are rapidly activated in the lungs as early as 4 days post-infection. In order to confirm that the T cell response was skewed towards type 2 immunity, we isolated T cells from the lungs of RSV infected neonate mice and exposed them to heat-inactivated RSV in order to assess the generation of antigen-specific T cells. Interestingly, we observed a significant increase in RSV-specific $CD4^+$ T cells that produce IFNγ, as well as IL-4 and IL-13 (Figure 1K-L-M) very early in infection (day 4), suggesting that neonate mice are not prevented from generating T_H1 cells, but promote T_H2 cell accumulation during RSV A2 infection, in line with a prior report (32). These observations indicate that the neonatal immune response does not specifically block the development of RSV-specific $IFN\gamma^+$ T cells, but rather that the infection skews the immune response towards a more potent Type 2 response.

2. Neonatal RSV A2 infection impairs the accumulation of IL-33 responding T_{REG} cells and facilitates the establishment of memory T_H2 cells in the lungs.

$Foxp3^+$ T_{REG} cells have been shown to play a key role in the control of T_H2 responses in adults infected with RSV (13). To address how T_H2 cells generated during neonatal RSV A2 infection evade immune regulation by T_{REG} cells upon re-challenge, we characterized the pulmonary T_{REG} cells at day 4 of infection to observe the memory response induced in the lungs upon re-challenge and limit the number of migrating T_{REG} cells (13, 33). We observed a small increase in the total number of T_{REG} cells in the lungs

at day 4 (Figure 2A), suggesting that T_{REG} cells accumulate in situ in response with a mounting T_{EFF} cell response in the lungs. Indeed, we observed on average 3.0 (± 1.2) times more activated T_{EFF} cells relative to activated T_{REG} cells in the lungs of the infected mice (Figure 2B), revealing the expansion of T_{EFF} cells was not being suppressed. In order to understand the characteristics of these T_{REG}, we then characterized known phenotypic properties that distinguish T_{REG} subsets. The transcription factor Helios is commonly referred to as a marker delineating thymic-derived T_{REG} cells (34). Not surprisingly, most of the neonatal T_{REG} cells found in the lung by day 4 of infection expressed Helios, regardless of RSV infection (Figure 2C). This is in line with a prior report that peripherally-induced T_{REG} cells (pT_{REG}) are generated through exposure to environmental antigens later in life (34) and contribute to allergen tolerance (35). Thus, although more information is required on the dynamics of Helios expression, the majority of T_{REG} cells accumulating in the lungs of RSV-infected neonate mice were likely of thymic origin.

IL-33 is abundantly released in the lungs of both adult and neonatal mice in the course of RSV infection (2, 36). Coincidentally, T_{REG} cells require IL-33 and IL-2 in order to upregulate the ST2 (IL33R) receptor, proliferate at mucosal surfaces (14, 19) and suppress local T cell expansion (20). Interestingly, we observed a significant decrease in the frequency of $ST2^+$ T_{REG} at day 4 of infection relative to mock infected mice (Figure 2D-E), but not in the total number of $ST2^+$ T_{REG} cells (Figure 2F), suggesting that other T_{REG} cell populations were preferentially accumulating in the lungs. Importantly, $ST2^+$ T_{REG} cells also displayed a lower expression level of the receptor, as shown by the mean fluorescence intensity (MFI) of ST2 on $Foxp3^+$ T_{REG} cells (Figure 2G). These data reveal that the accumulation of $ST2^+$ T_{REG} cells in the lungs is impaired in the early phase of a neonatal RSV infection. Importantly, since this effect is likely not due to a lack of IL-33 in these mice (2), it may be due to a lack of IL-2 production by neonatal T cells (37), although more investigation is required. Nonetheless, we observed that the frequency of $ST2^+$ T_{REG} cells among $CD4^+$ T cells was comparable to that of mock infected mice 14 post-infection (Figure 2H-I-J) and even increased in number in the lungs (Figure 2K). Incidentally, this increase coincided with an elevated frequency (Figure 2L) and number of IL-4^+ T_H2 cells (Figure 2M) and IFNγ expressing T_H1 cells (Figure 2N). Collectively, these results suggest

that, while an RSV infection in the neonate facilitates the establishment of a potent T_H2 response, it does not induce $ST2^+$ T_{REG} cell accumulation in the lungs.

3. Neonatal infection leads to enhanced IL-4 and IL-13 production by memory T_H2 cells upon re-infection in the adult.

In order to understand the long-term effect of neonatal RSV on the lung adaptive immune system, we re-challenged mice 6 weeks after neonatal infection with the same strain of RSV A2 (Figure 3A). We isolated the lungs 4 days after RSV infection in order to study the memory T cell response at the peak of RSV infection, limit the presence of *de novo* infiltrating $CD4^+$ and $CD8^+$ T cells (38) and observe the expanding memory response that was acquired during the neonatal infection. We confirmed a prior report (31) that showed RSV infection induced a significant weight loss in re-challenged mice (secondary) at day 4 post-infection (Figure 3B). In the lungs, we observed an increase in the number of total cells in these mice compared to uninfected neonatal mice (primary infection) (Figure 3C). There was a significant increase in the numbers of ILC2s, eosinophils and neutrophils in the lungs of re-infected mice (Figure 3D-E-F), key cell types involved in asthma pathology (39, 40). These mice also had increased numbers of $CD4^+$ T cells in the lungs when compared to primary infected mice (Figure 3G). Moreover, we observed increased numbers of $CD4^+$ T cells and decreased numbers of $CD8^+$ T cells in the lungs of re-challenged mice (Figure 3H), although some memory IFNγ-producing $CD8^+$ T cells were observed (Figure 3I). A significant number of the lung-residing $CD4^+$ T cells in re-challenged mice expressed IL-4 and IL-13 (Figure 3J-K-L) while less IFNγ producing T cells were isolated from the lungs (Figure 3M). Indeed, a re-challenge with RSV A2 led to an overall skewing of the pulmonary $CD4^+$ T cells response towards type 2 immunity (Figure 3N). Overall, these results reveal that a neonatal RSV A2 infection induces a rapid and strong T_H2-skewed immune response in the lungs 6 weeks after the primary infection.

4. Re-infection with RSV promotes IL-13$^+$ ST2$^+$ T_{REG} cell accumulation in the lungs.

To further detail the effect of an RSV infection on T_{REG} cells, we assessed their phenotype in the lungs of infected (primary) and re-challenged (secondary) mice. When mice are re-challenged with RSV A2 as adults, we did not observe a significant change in the frequency of Foxp3$^+$ T_{REG} cells among CD4$^+$ T cells in the lungs by day 4 (Figure 4A). However, using the mitotic marker Ki67, we observed that re-challenged mice (secondary) showed a lower frequency of cycling T_{REG} cells (Figure 4B-C) compared to T_{EFF} cells. Concurrently, ST2 expression was restricted to Ki67$^+$ T_{REG} cells in both primary and re-challenged mice (Figure 4D) and confirmed an increase in the overall frequency of ST2$^+$ among pulmonary T_{REG} cells (Figure 4E). Interestingly, the level of ST2 expression did not change between ST2$^+$ T_{REG} cells in both groups, suggesting that the expansion observed was not due to a difference in IL-33 responsiveness. Importantly, ST2$^+$ T_{REG} cells accumulated more readily in the lungs of mice that were re-challenged by RSV (Figure 4G), suggesting that ST2$^+$ T_{REG} had a proliferative advantage over other potential T_{REG} subsets. During the infection, the proliferating ST2$^+$ T_{REG} cells expressed high levels of the transcription factor GATA3 (Figure 4H) and co-expressed Helios (Figure 4I). Finally, we assessed if a re-challenge with RSV induced the production of pro-inflammatory cytokines by T_{REG} cells in the lungs. At day 4 of RSV infection, we observed a higher frequency of IFNγ-producing T_{REG} cells in the lungs of primary infected mice compared to re-challenged mice (Figure 4J-K). On the other hand, T_{REG} cells in the lungs of re-challenged mice preferentially produced IL-13 at day 4 (Figure 4J-L), suggesting that the functional specialization of the pulmonary T_{REG} cells differed between the two groups. Collectively, these results suggest that the increased accumulation of pulmonary ST2$^+$ T_{REG} cells did not prevent the proliferation of T_H2 cells.

5. STAT6 signaling is not required for ST2 expression by T_{REG} cells but contributes to ST2$^+$ T_{REG} proliferation and GATA3 expression.

Since ST2$^+$ T_{REG} cells were shown to suppress T_H1 and T_H17-driven inflammation (14), we investigated if an additional signal provided during T_H2-driven inflammation favor immune evasion from ST2$^+$ T_{REG} cells. In particular, IL-4 release by T_H2 cells during an RSV re-challenge was shown to play a key role in driving a dysregulated response by T_{REG} cells (41, 42). IL-4, which signals through the transcription factor STAT6, was notably shown to promote the evasion of T_H2 cells from T_{REG} cell suppression *in vitro* (43). Thus, we next hypothesized that lung generated ST2$^+$ T_{REG} cells fail to control memory T_H2 immune responses in the adult through enhanced signaling by IL-4. Since the role of STAT6 in driving the IL-33 response of T_{REG} cells has never been directly addressed, we first investigated the effect of IL-4 on ST2 expression by splenic T_{REG} cells. Here, we confirmed that splenic T_{REG} cells upregulated the ST2 receptor upon TCR activation with IL-2 and IL-33 and did not require IL-4 (Figure 5A). On the other hand, IL-4 significantly potentiated both the frequency of ST2$^+$ T_{REG} cells and the expression of ST2 when in the presence of IL-33 (Figure 5A-B-C). Moreover, IL-4 and IL-33 synergistically enhanced T_{REG} cells numbers *in vitro* (Figure 5D), suggesting that these combined signals promote T_{REG} cell proliferation. Interestingly, while IL-33 induced a moderate expression of GATA3 in T_{REG} cells, IL-4 alone was sufficient to augment GATA3 expression in T_{REG} cells (Figure 5E). Finally, we observed that IL-33 signaling drove the expression of IL4Rα on T_{REG} cells (Figure 5F-G), suggesting a mechanism by which ST2$^+$ T_{REG} cells become more sensitive to IL-4 signaling.

To confirm these findings *in vivo*, we compared sorted CD4$^+$CD25^{hi} T_{REG} cells from WT or STAT6$^{-/-}$ mice. Comparable to WT mice, STAT6$^{-/-}$ T_{REG} cells (stained CD4$^+$Foxp3$^+$) responded to exogeneous IL-33 and IL-2 by acquiring ST2 expression (Figure 5I-J), confirming that IL-4 signaling was not required for the upregulation of the receptor on T_{REG} cells. We observed that GATA3 levels remained low in IL-33 stimulated T_{REG} cells from STAT6$^{-/-}$ mice *in vitro* (Figure 5K), suggesting that high levels of GATA3 expression may not be necessary for ST2 upregulation. Collectively, these results suggest that IL-33

signaling on T_{REG} cells facilitates IL-4 signaling and a GATA3-driven transcriptional program in these cells.

6. IL-4 potentiates Type-2 associated functions and disrupts the suppressive ability of ST2$^+$ T_{REG} cells *in vitro.*

Since IL-33 potentiated IL-4 signaling in T_{REG} cells, we studied the functional consequence of IL-4 signaling on these cells. To this end, isolated splenic CD4$^+$ Foxp3$^+$ T_{REG} cells were TCR-activated in the presence of IL-2 and IL-4 with or without IL-33. Since ST2$^+$ T_{REG} cells were shown to produce low amount of IL-13 upon IL-33 signalling *in vitro* (20) and we observed they produced IL-13 during RSV re-challenge, we assessed the effect of IL-4 on the production of cytokines by ST2$^+$ T_{REG} cells. After 72 hours, we observed that a fraction of T_{REG} cells stimulated to both IL-33 and IL-4 expressed low levels of IL-13 (Figure 6A-B). In fact, T_{REG} cells stimulated by both IL-4 and IL-33 produced higher quantities of IL-5 and IL-13 but not IFNγ compared to cells stimulated with only IL-33 or IL-4 when we analyzed the culture supernatants by multiplex ELISA (Figure 6C-D-E). Interestingly, we also observed a significant increase in the production of IL-10 by IL-4 and IL-33 stimulated T_{REG} cells (Figure 6F). This effect depended on STAT6 signaling, as we observed that IL-5, IL-13 and IL-10 production by T_{REG} cells exposed to both IL-4 and IL-33 depended on the presence of STAT6 whereas IFNγ production did not (Figure S1A-D).

We then explored the impact of IL-4/IL-33 on the suppressive function of T_{REG} cells *in vitro*. T_{REG} cells exposed to both IL-33 and IL-4 showed a significant reduction in their ability to suppress CD4$^+$ T cell expansion *in vitro* (Figure 6G). Although IL-4 enhances the proliferation of responder CD4$^+$ T cells (Figure S2A), T_{REG} cells, not responder T cells, expressed ST2 in these conditions (Figure S2B), suggesting that the T_{REG} cells were responding to IL-33 in these cultures. In order to confirm that the effect of IL-4 and IL-33 was directly affecting the suppressive ability of T_{REG} cells, we sorted CD4$^+$CD25^{HI} T cells from WT or STAT6$^{-/-}$ BALB/c mice and activated them in the presence of CTV-labelled STAT6$^{-/-}$ CD4$^+$CD25$^-$ T_{RESP} cells. We confirmed that joint signaling by IL-33 and IL-4 directly compromised the suppressive ability of T_{REG} cells *in vitro* as we observed an

increased division index (D.I.) of the T_{RESP} cells in these cultures (Figure 6H), whereas STAT6$^{-/-}$ T_{REG} cells fail maintained their suppressive functions. In all, these results indicate that IL-33 potentiates IL-4 signaling on T_{REG} cells in order to modulate their suppressive ability.

7. Neonatal RSV infection potentiates IL-33-driven ST2$^+$ T_{REG} cells expansion in the lung in an antigen-independent manner.

One of the important features of the exacerbated T_H2 response seen with neonatal RSV infection in humans is the long-term effect observed in the lungs that renders them susceptible to other Type 2 immune disorders like asthma (44). Importantly, the virus is cleared by the mice by day 14 of infection in the infected pups (45), suggesting that only the memory immune response persists in the lungs. To understand if this was observed in our model, we infected neonate mice with RSV A2 and, rather than re-infecting with RSV, instilled IL-33 I.N. with the goal of identifying antigen-independent effects of IL-33 on ST2$^+$ T_{REG} cell and T_H2 cells in the lungs (Figure 7A). 48 hours after instillation, we observed an increase in the number of CD4$^+$ T cells in the lungs in both groups that received IL-33, regardless if they had been challenged or not with RSV A2 as neonates (Figure 7B). On the other hand, CD4$^+$ T cells from mice that had experienced neonatal RSV displayed increased accumulation of IL-4 and IL-13 producing CD4$^+$ T cells in the lungs (Figure 7C-D) but not IFNγ producing CD4$^+$ T cells (Figure 7E). Moreover, RSV-infected mice, but not PBS infected mice, displayed a significant increase in rapidly proliferating CD4$^+$Foxp3$^+$ T_{REG} cells (Figure 7F). In the lungs, the frequency and number of ST2$^+$ T_{REG} cells was increased by IL-33 only in mice infected as neonates (Figure 7G-H) suggesting that the RSV-generated memory T_H2 cells favored the expansion of ST2$^+$ T_{REG} cells. These results suggest that the consequences of neonatal RSV on ST2$^+$ T_{REG} cells are maintained in adulthood and occur in the absence of a RSV re-challenge, revealing a long-term mechanism by which neonatal RSV potentiates T_H2 responses and compromise T_{REG} cell function in the lungs.

3.5 Discussion

The neonatal immune environment is characterized by a biased immune response that establishes an inadequate type 2 driven response that can persist through adulthood. Indeed, while a mature immune response will favor type 1 responses against pathogens such as viruses, neonatal immune response leads to a type 2 response (46). One such consequence of neonatal infections is the onset of asthma in older children where acute viral infections early in infancy lead to the establishment of T_H2 memory pool in the lungs. It is estimated that 15-53% of pre-school children and 15-40% of school-age children that have suffered from a sever viral infection in infancy develop asthma later in their life (44, 47, 48). As such, it is critical to understand how immunoregulatory mechanisms that normally prevent unwanted inflammatory responses in the lung fail to control the onset of an aberrant T_H2 response. To understand these processes, we investigated the effect of RSV A2 infection in neonate mice, a model described to cause asthma-like symptoms in adult mice upon re-challenge (31), on the functional adpatation $Foxp3^+$ T_{REG} cells.

$Foxp3^+$ T_{REG} cells play a critical role in the maintenance of lung homeostasis (9). To achieve this, tissue localized T_{REG} cells display considerable functional adaptability by altering their epigenetic and transcriptional programs to adapt to local conditions (49). In particular, mucosal T_{REG} cells gain the ability to respond to IL-33 in order to maintain their transcriptional program and suppress T cell responses (14, 18, 19). However, conflicting evidence has revealed that the suppressive ability of $ST2^+$ T_{REG} cells can also be hindered by IL-33 in the lungs (22), revealing gaps in our understanding of the effect of IL-33 on pulmonary T_{REG} cells. Interestingly, while the reports that suggest IL-33 to promote the suppressive function of T_{REG} cells have studied models of T_H1 and T_H17-driven inflammation (14, 18, 19), the report revealing that IL-33 impairs the suppressive functions of T_{REG} cells was investigating a T_H2-driven allergy model (22). As such, we hypothesized that the inflammatory context dictates the effect of IL-33 on pulmonary T_{REG} cells.

T_{REG} cell control exacerbated RSV responses in adults (11, 13, 50), revealing that the absence of control in re-challenged mice may be directly linked to T_{REG} cell function. To understand the factors that lead to the establishment of an aberrant response in the adult, we first investigated the effect of an RSV A2 infection in the lungs of neonate mice.

Using multiparametric flow cytometry, we characterized the early skewing of the type 2 response that occurs in 5 to 7-day-old infected pups. Here, we confirmed that ILC2s, whose function is associated to enhanced type 2 responses in the lungs (27), are increased during an RSV A2 infection (2). Moreover, we observed that the alveolar macrophage population was biased towards Arg-1 production. Collectively, these observations suggested that these cells, found to be important in determining the early neonatal immune response during RSV (51), were contributing to the type 2 skewing of the response. Indeed, while the infection did not block the generation of IFNγ$^+$ CD4$^+$ T cells, we observed the accumulation of IL-4 producing CD4$^+$ T cells in the lungs and confirmed an overall bias of T_H2 cell accumulation over T_H1 cell (31).

T_{REG} cells found in the lungs of adult mice can arise from two distinct populations, one of thymic origin and another that is locally induced from naïve CD4$^+$ T cells (52). In neonates, we observed that all the local pulmonary T_{REG} cells expressed Helios, suggesting an absence of peripherally induced T_{REG} cells during the initial RSV infection (34). We and others have shown that pulmonary Helios$^+$ T_{REG} cells can acquire the IL-33 receptor (ST2) through IL-2 and IL-33 stimuli (14, 22). Indeed, during the neonate infection with RSV, IL-33 was shown to play a key role in the establishment of a dysregulated memory T_H2 response (2), suggesting active IL-33 signaling during the infection. Surprisingly, we observed a decrease in IL-33 responding T_{REG} cells in the lungs of neonate mice, suggesting that T_{REG} cells failed to respond to IL-33 in these mice. Since IL-2 is required for the upregulation of the ST2 receptor (19), it is possible that the low production of IL-2 by neonatal T cells (53) negated the expression of ST2 by neonatal tT_{REG} cells. Although more investigation is required, the absence of neonatal ST2$^+$ T_{REG} cells may provide T_H2 cells with the opportunity to expand (36). Nonetheless, as the pups recuperate from the infection, we observed increased numbers of ST2$^+$ T_{REG} cells and IL-4-producing T_H2 cells, suggesting the accumulation of pulmonary ST2$^+$ T_{REG} cells were only temporarily delayed.

A skewed type 2 memory response persist in the lungs and contribute to aberrant response upon RSV A2 re-infection in the adult mice (2). While our model recapitulated the increased accumulation of eosinophils, neutrophils and ILC2s that characterizes the model (2, 31), we identify the potent IL-4, IL-5 and IL-13 producing T_H2 memory response

that occurs in these mice. In the re-challenged mice, $ST2^+$ T_{REG} cells proliferated readily and even produced low amounts of IL-13, revealing a T_H2-like differentiation (22). Since IL-4 signaling on T_{REG} cells was shown to cause a dysregulation of their suppressive response during RSV A2 infection (12), we investigated how IL-33 impacted IL-4 signaling in T_{REG} cells. We observed that IL-33 enhanced the expression of IL4Rα on T_{REG} cells. On the other hand, IL-4, in a STAT6-dependent manner, potentiated the expression of IL-5 and IL-13 on T_{REG} cells. Moreover, while IL-33 alone did not dampen the suppressive function of T_{REG} cells but potentiated the dysregulating effect of IL-4. Finally, we observed that the skewed T_H2 memory response generated during the neonatal RSV infection did not require their cognate antigen to release IL-4 and potentiate the expansion of $ST2^+$ T_{REG} cells. Collectively, we show that IL-33 signaling is sufficient to lead to the functional dysregulation of T_{REG} cells during aberrant T_H2 responses.

While these results provide a novel demonstration of how RSV-generated T_H2 cells evade T_{REG} cell suppression, it is important to consider that, in otherwise healthy infants, RSV is not the sole cause of asthma development in children (54). Nonetheless, some of the better identified contributing genetic factors include gain-of-function SNPs in *il4, il13 and IL-33* (55), revealing the strong association between these cytokines and the development of asthma. As such, understanding the role of neonatal infections in the dysregulation of the immune system is key towards establishing therapeutic and prevention strategies. This is the first description that links the effect of IL-33 and IL-4 in the functional adaptation of T_{REG} cells, suggesting a novel mechanism by which T_H2 cells evade immune suppression during allergy. These results provide the grounds for the development of novel therapies aimed at targeting the STAT6 signaling pathway during T_H2-exacerbated responses (24).

3.6 Acknowledgments

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Author Contributions

F.A., J.H.F. and C.P. established the conceptual framework of the study, designed experiments and wrote the paper. F.A. performed all the experiments and analyzed the data. L.L and K. R. prepared the RSV titers, performed the neonate infection with RSV and the adult-re-infection, as well as prepared the cell isolation form the lungs in this model. R.I. contributed to the *in vitro* experiments. All the authors provided valuable input throughout the study and the writing of the manuscript.

Competing financial interests

All authors declare that they have no competing financial interests.

3.7 Materials and Methods

Mice

Wild-type (WT) BALB/c and BALB/c.STAT6-KO ($STAT6^{-/-}$) mice were inbred in the RI-MUHC originally came from Jackson Laboratories (Jackson Laboratory, Bar Harbor, ME) obtained from. C57BL/6.$Foxp3^{GFP}$ reporter knock-in ($Foxp3^{GFPki}$) mice were provided by Alexander Rudensky and bred into the congenic background ($Ly5.1^{+}$) for more than 10 generations. BALB/c mice breeding cages were setup up and monitored daily. Neonate mice were infected I.N. between 5-7 day old. Adult mice re-infected at 6 weeks of age. All mice were housed and bred under specific pathogen-free conditions and used according to institutional guidelines at McGill University and the Canadian Council on Animal Care.

Lymphocyte isolation

Isolation of T cells was performed using a previously described method (23). Briefly, immediately after CO_2 euthanasia, the lungs are perfused with cold PBS through the right ventricle. The collected lungs are then 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 (0.005µM) (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 lamina propria T cells (colon) were obtained through the processing of the colon as previously described. 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 (FACSAria™, 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 $STAT6^{-/-}$ mice were used. To assess T cell cytokine production, isolated cells were then activated with phorbol myristate acetate (PMA), ionomycin and BD GolgiStop™ (BD Biosciences) for 3 hours.

In vitro assays

FACS-sorted $CD4^+GFP^+$ T_{REG} cells ($Foxp3^+$, $5x10^4$) 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 $CD4^+GFP^-$ ($5,0x10^4$) were co-cultured with titrated numbers of FACS-sorted $CD4^+GFP^+$ (Foxp3) T_{REG} cells [$2.5x10^4$ (1:2), $1.25x10^4$ (1:4), $6.25x10^3$ (1:8) and $3.75x10^3$ (1:16)] in the presence of soluble αCD3 (1 µg/ml) and $3.0x10^5$ 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 µl (100 µl 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 form BALB/c and $STAT6^{-/-}$ mice. The samples from 3 distinct experiments were sent were sent for multiplex ELISA to Eve Technologies using the Mouse High-Sensitivity Multiplex Discovery Assay-18 testing for GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, Lix, IL-17A, KC, MCP-1, MCP-2, TFNα (Eve Technologies, Calgary, AB, Canada).

Intranasal infection with Respiratory Syncytial Virus

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 the extensive cytopathic effect was assessed 4-5 d post-infection, cells were scraped and the supernatant was collected, as previously

described (24). 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 (24), and stored a -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 ($TCID_{50}$) were calculated by the Reed-Muench method (25).

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.) (10^6 $TCID_{50}$/g body weight, 2–3 g body weight), as described previously (24) and sacked 4 days later. For the adult re-infection model, mice were infected as neonates, and were left untreated until reaching 6 weeks of age, at which point were re-infected with RSV A2 (10^6 $TCID_{50}$/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 10^6 $TCID_{50}$/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) of IL-33 i.n. at were sacrificed two days later. Here, we the both 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℃) for 72 hours in the presence of BD GolgiStop™(BD Biosciences).

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-CD8-V500 (53-6.7) (BD Biosciences), 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-IFNg-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-TCRγδ-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-IFNγ-BUV737 (XMG1.2, BD biosciences). Non-viable cells were excluded using fixable viability dye eFluor 780 or 506 reagent (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).

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 5 software (GraphPad Software).

3.8 Figures

Figure 1. Neonatal RSV infection leads to the accumulation of IL-4$^+$ CD4 T cells in the lungs

(A) Methodology of the neonate infection. 10^6 $TCID_{50}$/g of RSV A2 was administered I.N. in 5 to 7-day-old BALB/c mice. The pups were then sacrificed 4 days p.i. and the lungs were collected for cell analysis. Compiled data of 3 distinct experiments (n=3-4 per experiment).

(B) Total cell numbers in the lungs at day 4 post-infection. Student T test. **p<0.01.

(C) Representative gating strategy of ILC2s in the lungs at day 4 p.i. Lin$^-$ (CD49b, B220, CD3, F4/80, CD11c, CD11b, Ly6G, TCRγδ) lymphoid sized cells were sub-gated based on the expression of Thy1.2$^+$ and CD127^{hi}, followed by CD25$^+$ and ST2$^+$.

(D) Frequency of ILC2s among all live cells in the lungs. Student T test.

(E) Total cell count ILC2s collected in the lungs of neonate mice at day 4 p.i. Student T test. *p<0.05

(F) Total cell count CD45$^+$Ly6G^{int}CD11c$^+$F4/80$^+$ alveolar macrophages (AMs). Student T test. *p<0.05. Representative of 3 distinct experiments.

(G) Mean Fluorescence intensity of MHC-II on AMs at day 4 p.i. Student T test. *p<0.05

(H) Representative histogram of the expression of Arginine 1 (Arg1) by flow cytometry on lung isolated AMs at day 4 p.i.

(I) Representative flow cytometry of the expression of CD44 and CD62L on lung isolated live CD4$^+$ T cells between mock and RSV infected neonate pups

(J) Total cell count of CD44^{high}CD62L^{low} CD4$^+$ T cells. Student T test. *p<0.05

(K) Total cell count of CD44^{high}CD62L^{low} CD8$^+$ T cells.

(L-M-N) Total isolated cells from the lungs of neonate pups were exposed with 2 µg protein/ml of heat-inactivated RSV A2 for 72 hours in the presence of BD GolgiStop™ and stained for flow cytometry. Representative of one experiment. **(L)** Total number of RSV specific IFNγ$^+$ CD4$^+$ T cells; **(M)** Total number of IL-4$^+$ CD4$^+$ T cells; **(N)** Total number of IL-13$^+$ CD4+ T cells. Student T test. *p<0.05

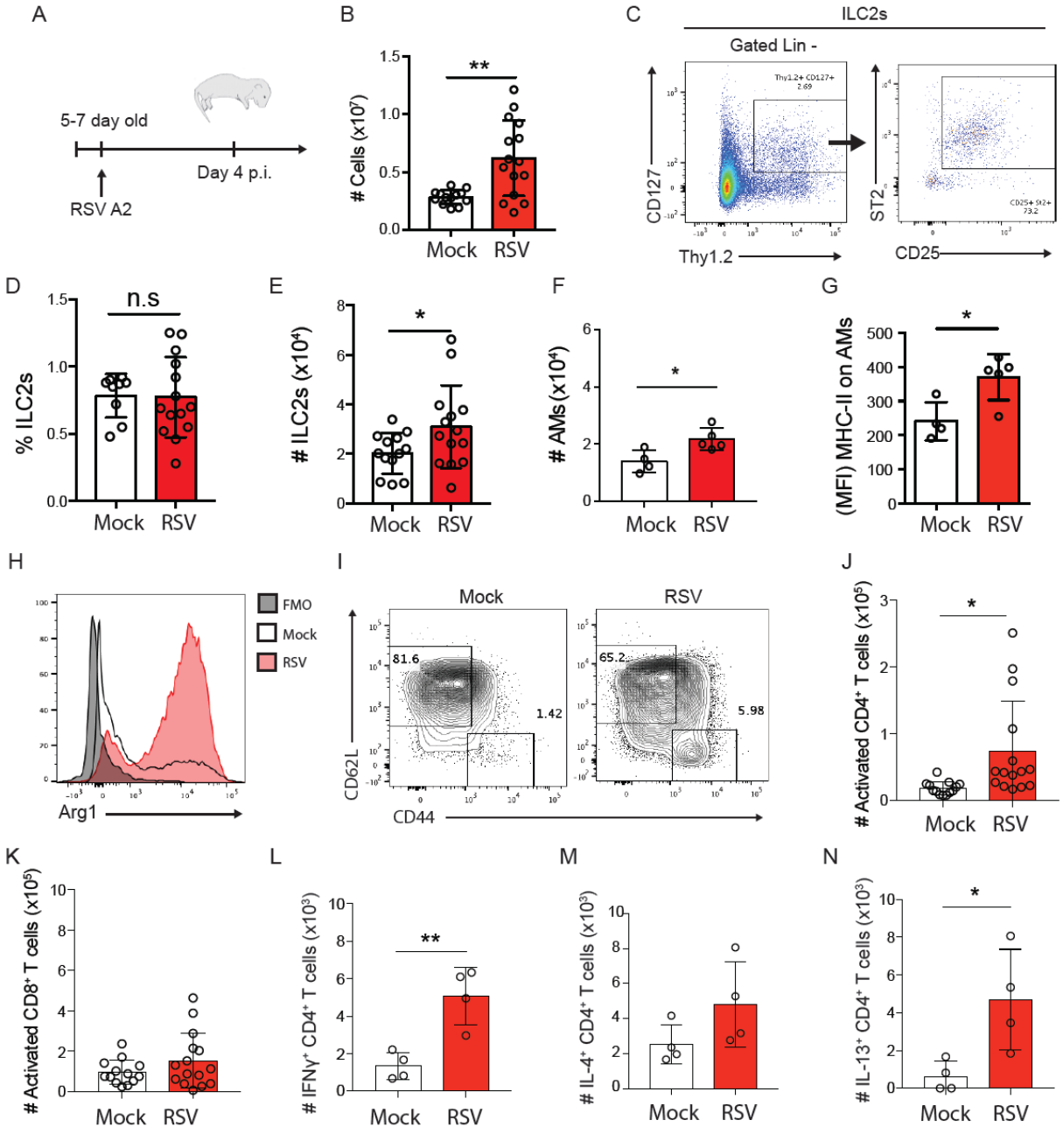

A
5-7 day old
Day 4 p.i.
RSV A2
B
Cells (x10⁷)
Mock
RSV
C
ILC2s
Gated Lin -
CD127
Thy1.2
ST2
CD25
D
n.s
% ILC2s
E
ILC2s (x10⁴)
F
AMs (x10⁴)
G
(MFI) MHC-II on AMs
H
FMO
Mock
RSV
Arg1
I
Mock
RSV
81.6
1.42
65.2
5.98
CD62L
CD44
J
Activated CD4⁺ T cells (x10⁵)
K
Activated CD8⁺ T cells (x10⁵)
L
IFNγ⁺ CD4⁺ T cells (x10³)
M
IL-4⁺ CD4⁺ T cells (x10³)
N
IL-13⁺ CD4⁺ T cells (x10³)

Figure 1

Figure 2. RSV infection in neonates leads to an initial decrease in IL-33 responsive T_{REG} cells and the establishment of a T_H2 pool in the lungs.

(A) Number of T_{REG} cells ($CD4^+Foxp3^+$) at day 4 post-infection. Student T-test. **p<0.01

(B) Ratio of activated ($CD44^{hi}CD62^{lo}$) $CD4^+$ $Foxp3^-$ (T_{EFF}) over activated $Foxp3^+$ (T_{REG}) in the lungs. Student T-test. *p<0.05

(C) Representative flow cytometry of Helios expression among $Foxp3^+$ CD4+ T cells in the lungs. Representative flow cytometry of n=8.

(D) Representative flow cytometry plot of ST2 and Helios expression among $Foxp3^+$ CD4+ T cells.

(E) Frequency of $ST2^+$ cells among $CD4^+Foxp3^+$ T cells in the lungs.

(F) Number of $ST2^+$ T_{REG} cells in the lungs. Student T test.

(G) Mean fluorescence intensity (MFI) of ST2 among $ST2^+$ T_{REG} cells. Representative of 3 distinct experiments.

(H) Methodology used. Briefly, 10-day-old neonate mice were infected with 10^6 $TCID_{50}$/g of RSV A2 and left to recuperate until day 24 of life. Control BALB/c mice received PBS as neonate.

(I) Expression of ST2 and Foxp3 among $CD4^+$ T cells in the lungs of mice at day 14 post-infection.

(J) Frequency of $ST2^+$ T_{REG} cells among total CD4+ T cells in the lungs of mice at day 14 post-infection. Representative of 3 distinct experiments.

(K) Number of $ST2^+$ T_{REG} cells in the lungs of 24-day-old mice. Student T test. ***p<0.001. Representative of 3 distinct experiments.

(L) Frequency of IL-4^+ T cells in the lungs of mice among all CD4+ T cells. Student T test. *p<0.05. Representative of 3 distinct experiments.

(M) Number of IL-4^+ T cells in the lungs of 24-day-old mice. Student T test. ***p<0.001. Representative of 3 distinct experiments.

(N) Number of IFNγ^+ T cells in the lungs of 24-day-old mice. Student T test. ***p<0.001. Representative of 3 distinct experiments.

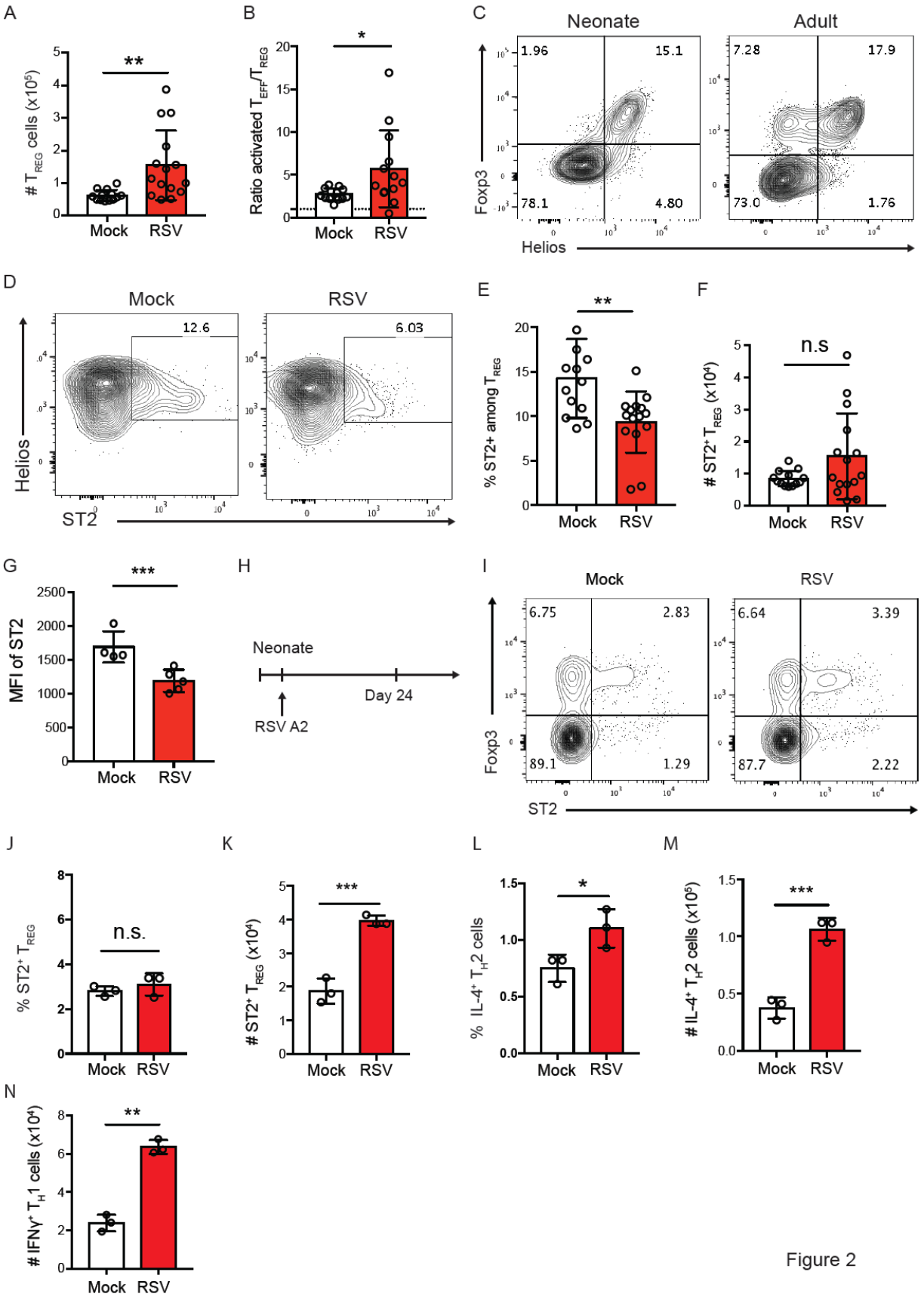

A
T_{REG} cells (x10^5)
**
Mock
RSV
B
Ratio activated T_{EFF}/T_{REG}
*
Mock
RSV
C
Neonate
Adult
1.96
15.1
78.1
4.80
7.28
17.9
73.0
1.76
Foxp3
Helios
D
Mock
RSV
12.6
6.03
Helios
ST2
E
% ST2+ among T_{REG}
**
Mock
RSV
F
n.s
ST2$^+$ T_{REG} (x10^4)
Mock
RSV
G
MFI of ST2
***
Mock
RSV
H
Neonate
Day 24
RSV A2
I
Mock
RSV
6.75
2.83
89.1
1.29
6.64
3.39
87.7
2.22
Foxp3
ST2
J
% ST2$^+$ T_{REG}
n.s.
Mock
RSV
K
ST2$^+$ T_{REG} (x10^4)
***
Mock
RSV
L
% IL-4$^+$ T_H2 cells
*
Mock
RSV
M
IL-4$^+$ T_H2 cells (x10^5)
***
Mock
RSV
N
IFNγ$^+$ T_H1 cells (x10^4)
**
Mock
RSV

Figure 2

Figure 3. Neonatal infection with RSV leads to an enhanced IL-4 and IL-13 production in memory T_H2 cells upon re-infection in the adult.

(A) Neonatal mice infected with 10^6 $TCID_{50}$/g of RSV A2 were left to recuperate and were re-infected at 6 weeks of age with 10^6 $TCID_{50}$/g of RSV A2 (Secondary). Control BALB/c mice received PBS as neonate and were infected with the same dose of RSV A2 (Primary). Compilation or representatives of 3 distinct experiment. The mice were sacrificed 4 days post-infection.

(B) Weight loss of adult mice after infection. Representative of 3 distinct experiments.

(C) Number of live cells in the lungs at day 4 p.i.. Student T test. ***p<0.001

(D) Number of ILC2s in the lungs. Student T test.

(E) Number of eosinophils (Siglec F^+, $Ly6C^{int}$, $CD11b^+$) in the lungs. Student T test. ***p<0.001

(F) Number of Neutrophils ($Gr1^+$, $CD11b^+$, $Ly6C^{lo}$) in the lungs. Student T test. **p<0.01

(G) Number of CD4+ T cells in the lungs of infected mice at day 4. Student T test. **p<0.01

(H) Stacked frequency of $CD4^+$ and $CD8^+$ T cells in the lungs. Student T test. ### ($CD4^+$ T cells) p<0.001, *** ($CD8^+$ T cells) p<0.00

(I) Number of IFNγ$^+$ CD8+ T cells in the lungs. Student T test. *p<0.05.

(J) Representative flow cytometry of IL-4 and IFNγ in $CD4^+$ T cells in the lungs.

(K) Number of IL-4^+ $CD4^+Foxp3^-$ (T_{EFF}) T cells in the lungs. Student T test. ***p<0.001.

(L) Number of IL-13^+ $CD4^+Foxp3^-$ (T_{EFF}) T cells in the lungs. Student T test. ***p<0.001.

(M) Number of IFNγ$^+$ $CD4^+Foxp3^-$ (T_{EFF}) T cells in the lungs. Student T test. ***p<0.001.

(N) Ratio of IL-4^+ over IFNγ$^+$ T_{EFF} cells in the lungs at day 4 post-infection. Student T test. ***p<0.001.

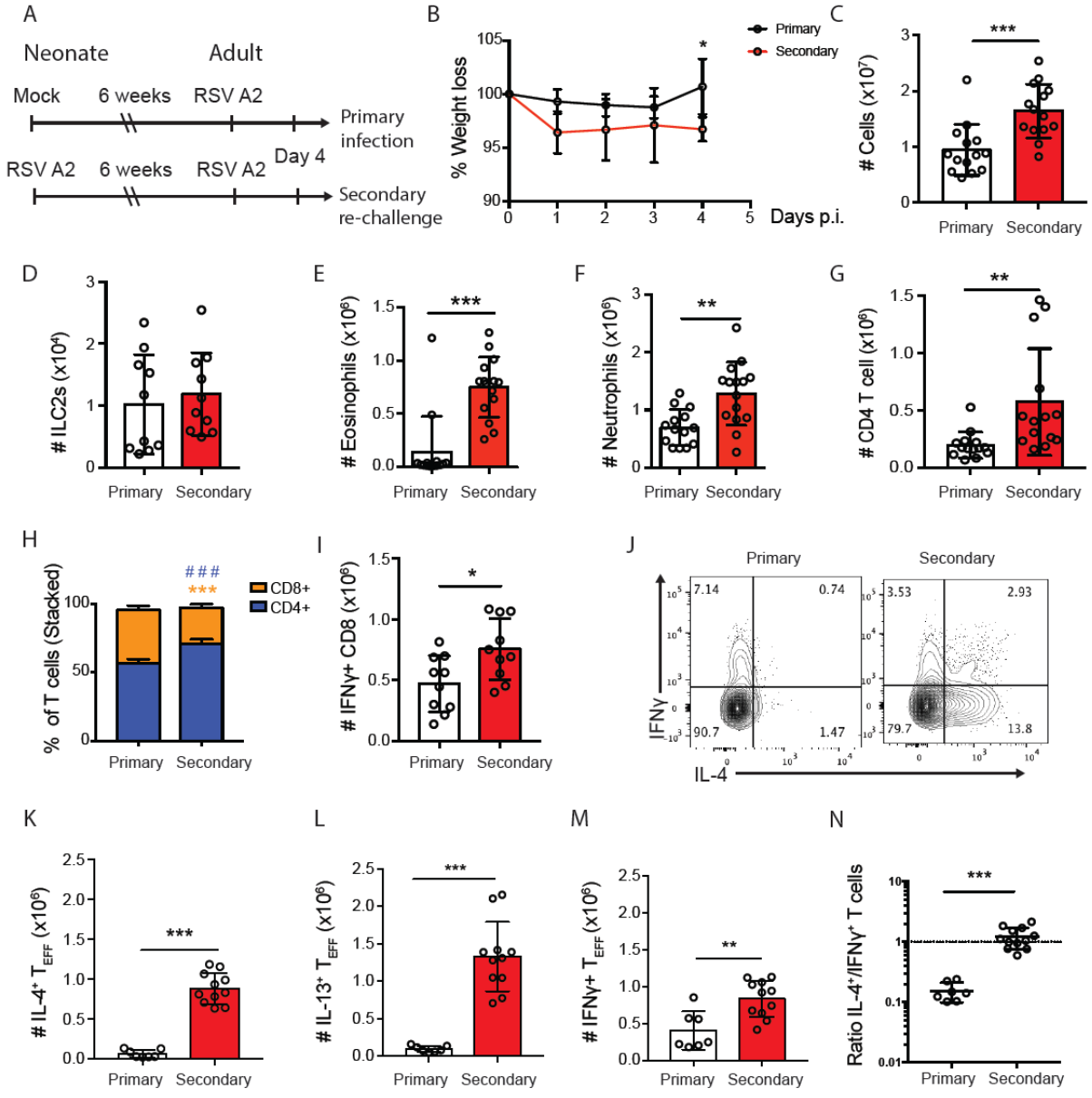

A
Neonate
Adult
Mock
6 weeks
RSV A2
Primary infection
RSV A2
6 weeks
RSV A2
Day 4
Secondary re-challenge
B
% Weight loss
Days p.i.
Primary
Secondary
C
Cells (x10^7)
D
ILC2s (x10^4)
E
Eosinophils (x10^6)
F
Neutrophils (x10^6)
G
CD4 T cell (x10^6)
H
% of T cells (Stacked)
CD8+
CD4+
I
IFNγ+ CD8 (x10^6)
J
IFNγ
IL-4
7.14
0.74
90.7
1.47
3.53
2.93
79.7
13.8
K
IL-4+ T_EFF (x10^6)
L
IL-13+ T_EFF (x10^6)
M
IFNγ+ T_EFF (x10^6)
N
Ratio IL-4+/IFNγ+ T cells

Figure 3

Figure 4. Re-infection with RSV promotes IL-13$^+$ ST2$^+$ T_{REG} cell accumulation in the lungs.

(A) Frequency of T_{REG} cells among CD4$^+$ T cells in the lungs of infected (primary) and re-infected (secondary) at day 4 post-infection.

(B) Ki67 and Foxp3 expression among live CD4$^+$ T cells in the lungs at day 4 post-infection.

(C) Frequency of CD4$^+$Foxp3$^+$ T_{REG} cells among Ki67$^+$ CD4$^+$ T cells in the lungs. Student T test. ***p<0.001. Representative of 3 distinct experiments.

(D) Representative flow cytometry of Ki67 and ST2among live T_{REG} cells in the lungs at day 4 post-infection.

(E) Frequency of ST2$^+$ cells among CD4+Foxp3+ T_{REG} cells in the lungs. Student T test. **p<0.01.

(F) Mean fluorescence intensity (MFI) of ST2 among ST2$^+$ T_{REG} cells.

(G) Number of ST2$^+$ T_{REG} cells in the lungs of primarily or secondarily infected mice. Student T test. ***p<0.001.

(H) 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. Representative of n=15.

(I) Representative flow cytometry of ST2 and Helios among live T_{REG} cells in the lungs at day 4 post-infection.

(J) Representative flow cytometry of IFNγ and IL-13 among CD4$^+$ Foxp3$^+$ T_{REG} cells in the lungs at day 4 post-infection.

(K) Frequency of IFNγ$^+$ among CD4$^+$ Foxp3$^+$ T cells. Student T test. ***p<0.001

(L) Frequency of IL-13$^+$ among CD4$^+$ Foxp3$^+$ T cells. Student T test. *p<0.05

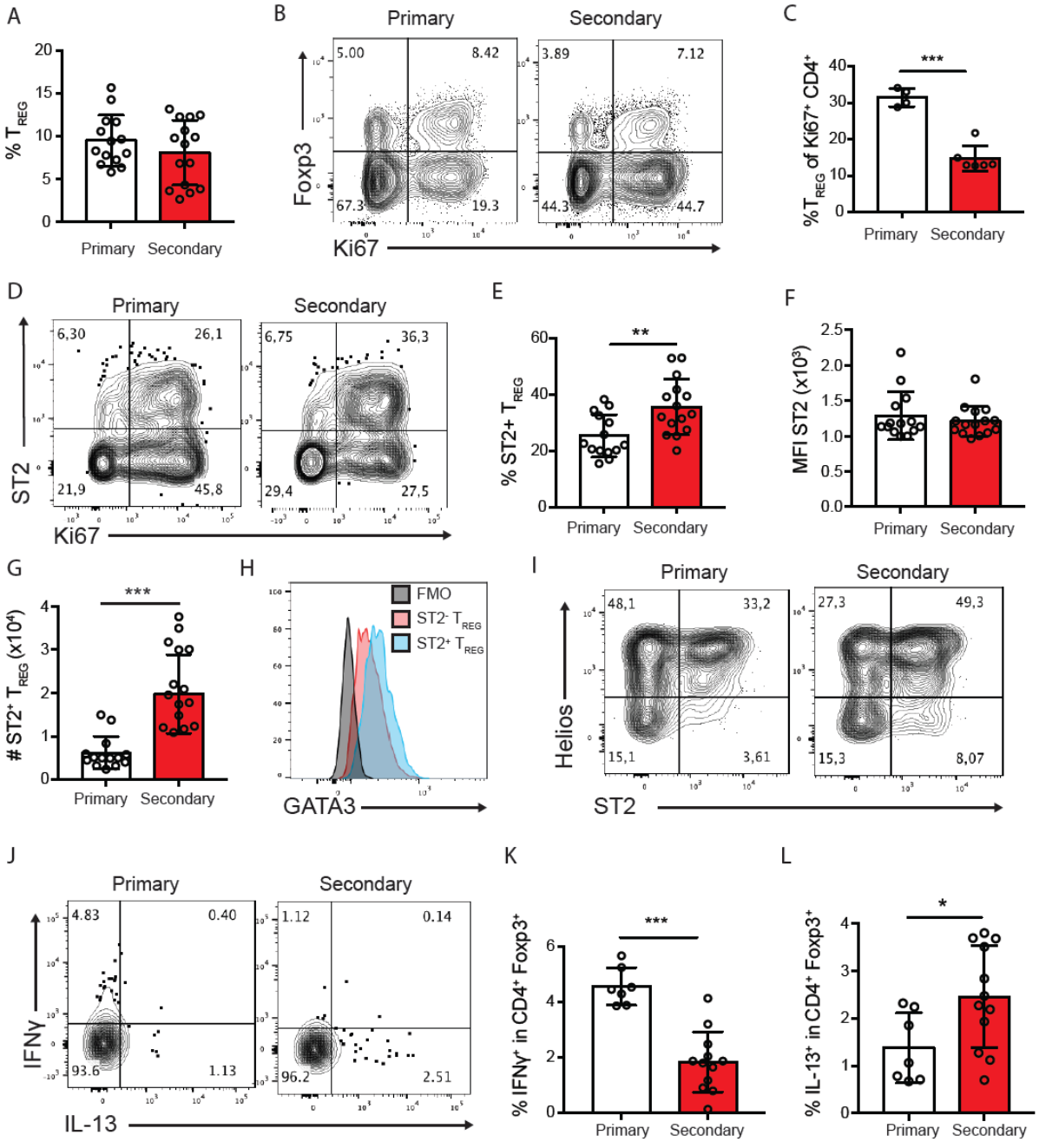

A
% T_REG
Primary
Secondary
B
Primary
Secondary
Foxp3
Ki67
5.00
8.42
67.3
19.3
3.89
7.12
44.3
44.7
C
% T_REG of Ki67+ CD4+
***
D
ST2
Ki67
6,30
26,1
21,9
45,8
6,75
36,3
29,4
27,5
E
% ST2+ T_REG
**
F
MFI ST2 (x10³)
G
ST2+ T_REG (x10⁴)
***
H
FMO
ST2- T_REG
ST2+ T_REG
GATA3
I
Helios
ST2
48,1
33,2
15,1
3,61
27,3
49,3
15,3
8,07
J
IFNγ
IL-13
4.83
0.40
93.6
1.13
1.12
0.14
96.2
2.51
K
% IFNγ+ in CD4+ Foxp3+
***
L
% IL-13+ in CD4+ Foxp3+
*

Figure 4

Figure 5. STAT6 signaling is not required for ST2 expression by T_{REG} cells but contributes to ST2$^+$ T_{REG} proliferation and GATA3 expression.

(A-H) CD4$^+$ T_{REG} cells were isolated from the spleen of Foxp3^{GFPki} mice and sorted as CD4$^+$GFP$^+$. The cells were then activated by plated αCD3 (5ug/ml) and αCD28 in the presence of IL-2 (200U/ml) (Medium) with added IL-33 (10ng/ml), IL-4 (50ng/ml) or a combination of both for 72 hours. Representative of 3 experiments

(A) Foxp3 and ST2 expression among T_{REG} cells in culture.

(B) Frequency of ST2$^+$ among live (CD4$^+$Foxp3$^+$) T_{REG} cells. One-way ANOVA. Tukey correction. ***p<0.001.

(C) Mean fluorescence intensity (MFI) of ST2 on live (CD4$^+$Foxp3$^+$) T_{REG} cells. One-way ANOVA. Tukey correction. ***p<0.001. Representative of 3 distinct experiments.

(D) Number of on live (CD4$^+$Foxp3$^+$) T_{REG} cells per 100µl of culture media. One-way ANOVA. Tukey correction. ***p<0.001.

(E) Frequency of GATA3$^+$ among live CD4$^+$Foxp3$^+$ T_{REG} cells in culture after 72 hrs. One-way ANOVA. Tukey correction. ***p<0.001.

(F) Mean fluorescence intensity (MFI) of ST2 in ST2$^+$ T_{REG} cells. One-way ANOVA. Tukey correction. ***p<0.001.

(G) Representative histogram of IL4Rα expression on CD4$^+$Foxp3$^+$ T cell in culture.

(H) Mean fluorescence intensity (MFI) of IL4Rα on T_{REG} cells. One-way ANOVA. Tukey correction.

(I-K) CD4$^+$CD25^{hi} (T_{REG}) cells from the spleen of BALB/C (WT) and STAT6$^{-/-}$ mice were FACs sorted and cultures in the same conditions as above. Compiled from 2 experiments.

(I) Frequency of ST2$^+$ among live CD4$^+$Foxp3$^+$ T_{REG} cells in culture after 72 hrs. One-way ANOVA. Tukey correction. ***p<0.001.

(J) Number of live ST2$^+$ CD4$^+$Foxp3$^+$ T_{REG} cells per 100µl in culture after 72 hrs. One-way ANOVA. Tukey correction. ***p<0.001.

(K) Representative histogram of the expression of GATA3 in CD4+Foxp3+ T_{REG} cells from STAT6$^{-/-}$ (red) or WT (grey).

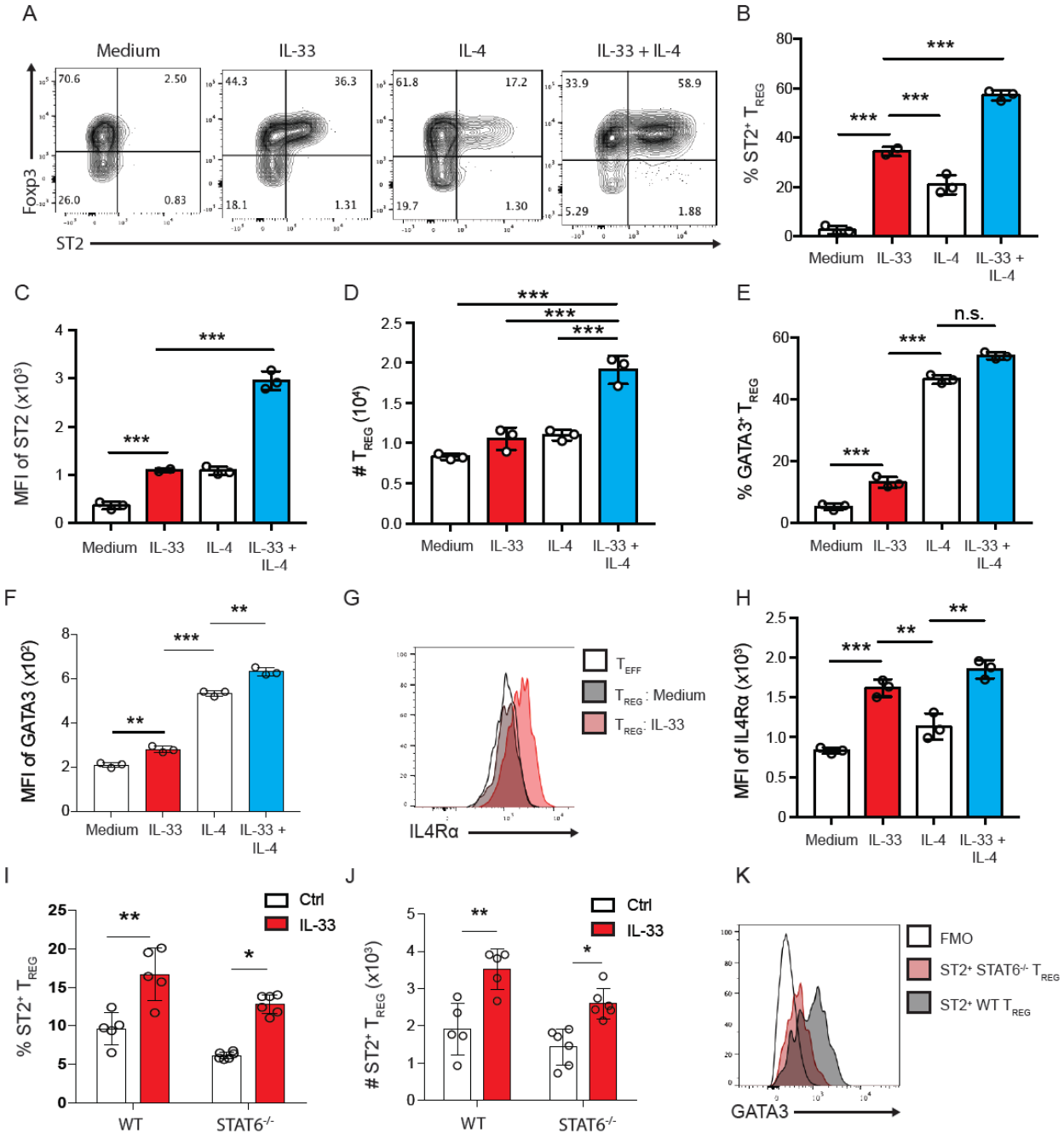


Figure 5

Figure 6. IL-4 potentiates Type-2 associated functions and disrupts the suppressive ability of ST2$^+$ T_{REG} cells *in vitro.*

(A-F) CD4$^+$GFP$^+$ T cells (B6.Foxp3^{GFPki}) were sorted and activated by plated αCD3 (5μg/ml) + αCD28 (2μg/ml) for 72 hours in the presence of IL-2 (200U/ml), IL-33 (10ng/ml) and/or IL-4 (10ng/ml).

(A) Expression of Foxp3 and IL-13 among T_{REG} cells in culture.

(B) Frequency of IL-13$^+$ among live T_{REG} cells after 72 hours. One-way ANOVA. Tukey correction. *p<0.05; **p<0.01; ***p<0.001.

(C-D-E-F) Multiplex ELISA (18-plex) from EVE technologies® of the supernatants of cultures from (A). Each point represents 1 distinct experiment, N=3. The quantities (pg/ml) were normalized per 10 000 cells. **(C)** Quantity of IL-5 (pg/ml); **(D)** IL-13 (pg/ml); **(E)** IFNγ (pg/ml); **(F)** IL-10 (pg/ml) One-way ANOVA. Tukey correction. *p<0.05; **p<0.01

(G) CD4$^+$GFP$^+$ T cells (B6.Foxp3^{GFPki}) were sorted and co-cultured at 1:2 (25 x10^4), 1:4 (12.5 x10^4) or 1:8 (6.75 x10^4) with 50 x10^4 CTV-labelled T_{EFF} cells and activated with αCD3 and irradiated APCs for 72 hours. Representative histogram of CTV dilution of the responder CD4+ T cells (T_{RESP}) and suppression percentage (100-(Div index/Div index of T_{RESP} alone *100)).

(H) CD4$^+$ CD25^{hi} (T_{REG}) and CD4$^+$CD25$^-$ (T_{RESP}) T cells were sorted from the spleen of BALB/c (WT) or STAT6$^{-/-}$ mice. Division index of the CTV$^+$ T_{RESP} cells at 72 hours. One-way ANOVA. Tukey correction. **p<0.01. Representative of 2 distinct experiments.

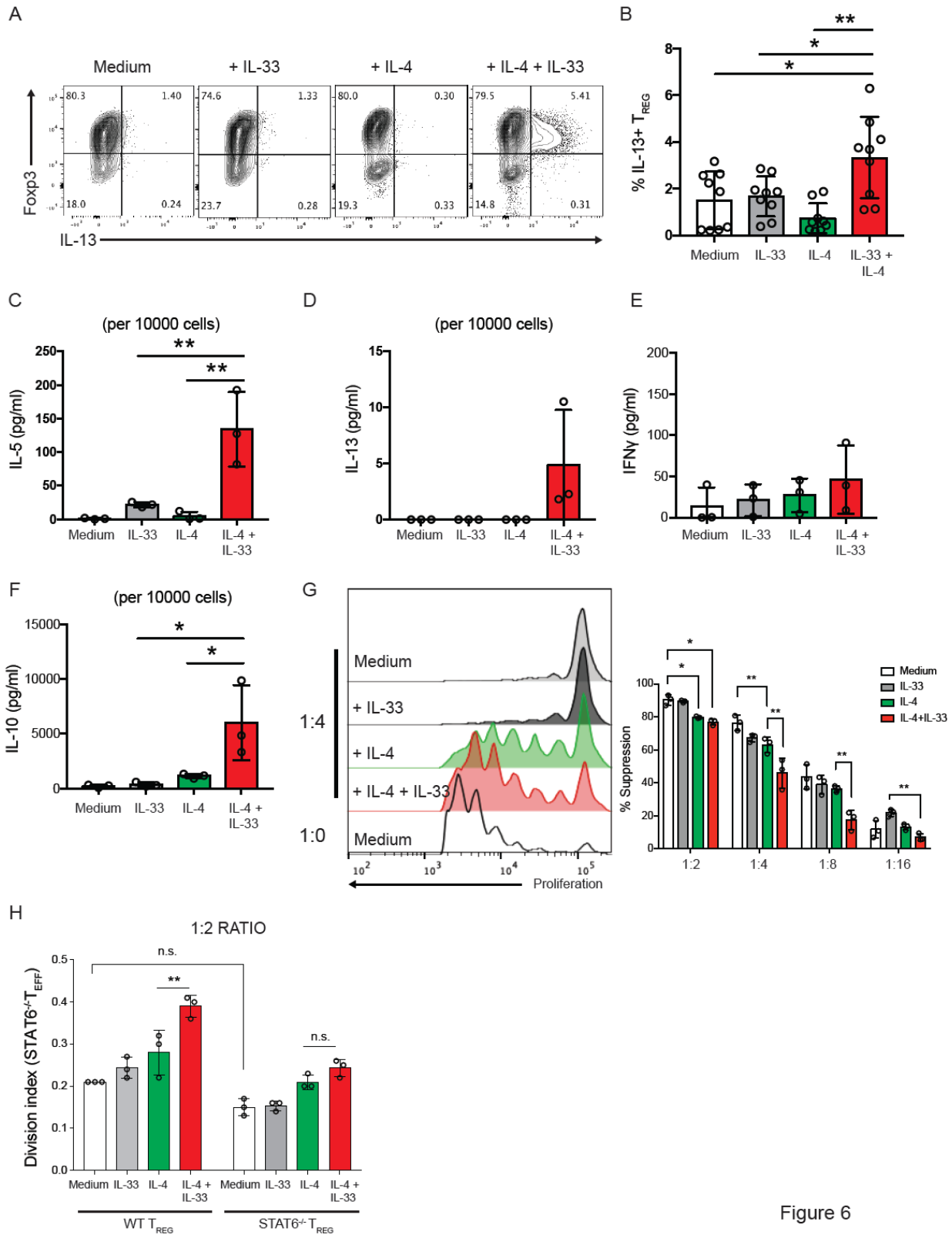

A
Medium
+ IL-33
+ IL-4
+ IL-4 + IL-33
Foxp3
IL-13
80.3
1.40
18.0
0.24
74.6
1.33
23.7
0.28
80.0
0.30
19.3
0.33
79.5
5.41
14.8
0.31
B
% IL-13+ T REG
Medium
IL-33
IL-4
IL-33 + IL-4
C
(per 10000 cells)
IL-5 (pg/ml)
D
(per 10000 cells)
IL-13 (pg/ml)
E
IFNγ (pg/ml)
F
(per 10000 cells)
IL-10 (pg/ml)
G
Medium
+ IL-33
+ IL-4
+ IL-4 + IL-33
1:4
1:0
Proliferation
% Suppression
1:2
1:4
1:8
1:16
IL-4+IL-33
H
1:2 RATIO
Division index (STAT6-/- T EFF)
n.s.
WT T REG
STAT6-/- T REG

Figure 6

Figure 7. Neonatal RSV infection potentiates IL-33-driven $ST2^+$ T_{REG} cells expansion in the lung in an antigen-independent manner.

(A) 5-7-day-old BALB/c pups were infected with 10^6 $TCID_{50}$/g of RSV A2 and left to recuperate while control BALB/c mice received PBS. The mice were left to recuperate. At 6 weeks, the mice received (0.25µg/mouse) of IL-33 I.N. and were sacrificed two days later. Compilation of 2 experiments

(B) Numbers of live $CD3^+$ $CD4^+$ T cells isolated from the lungs at day 2 post-IL-33. One-way ANOVA.

(C) Frequency of IL-4^+ $CD4^+$ T cells in the lungs. 3 hours PMA + Ionomycin.

(D) Frequency of $Ki67^+$ $CD4^+$ $Foxp3^+$ T_{REG} cells in the lungs. One-way ANOVA. Tukey correction. *p<0.05.

(E) Frequency of $ST2^+$ $CD4^+$ $Foxp3^+$ T_{REG} cells in the lungs. One-way ANOVA. Tukey correction. *p<0.05.

(F) Number of $ST2^+$ $CD4^+$ $Foxp3^+$ T_{REG} cells in the lungs.

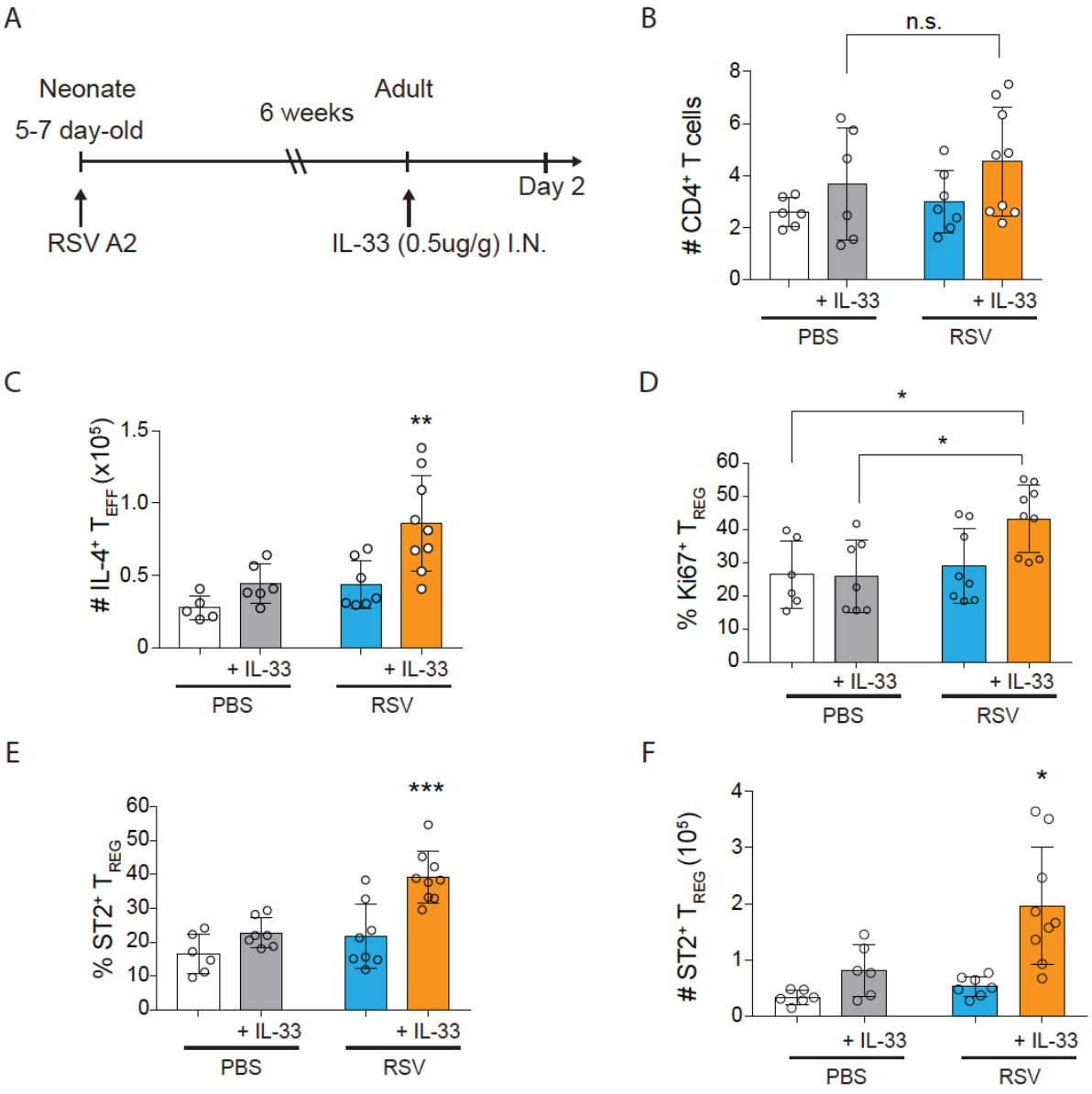
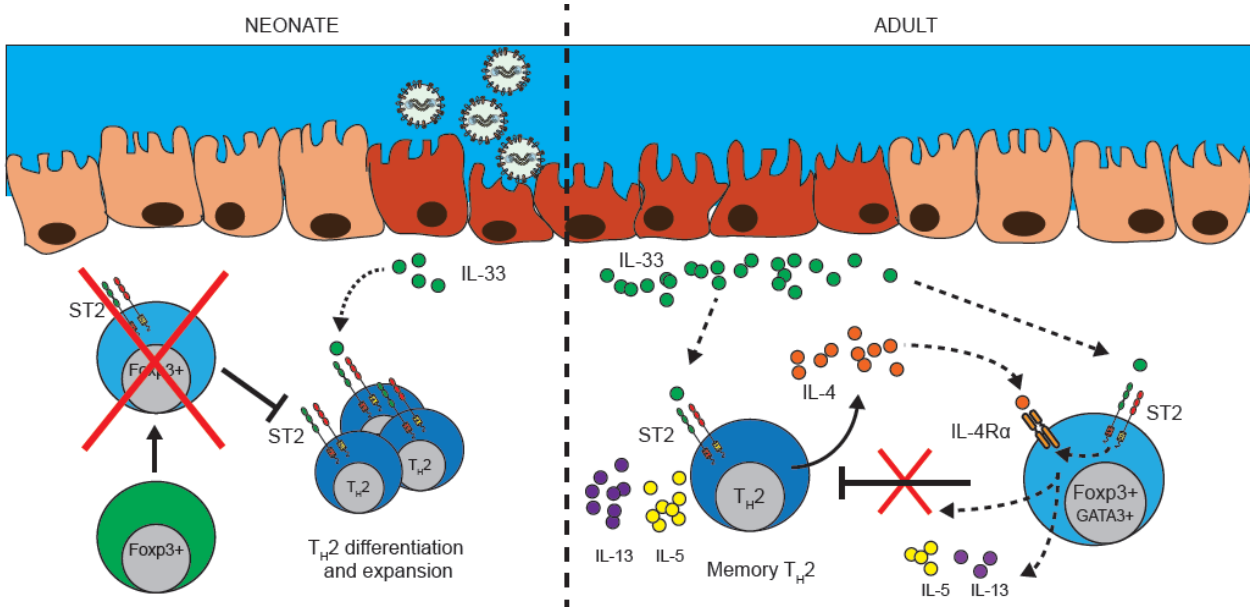

A
Neonate
5-7 day-old
6 weeks
Adult
Day 2
RSV A2
IL-33 (0.5ug/g) I.N.
B
n.s.
CD4+ T cells
+ IL-33
+ IL-33
PBS
RSV
C
IL-4+ TEFF (x10^5)
**
+ IL-33
+ IL-33
PBS
RSV
D
*
*
% Ki67+ TREG
+ IL-33
+ IL-33
PBS
RSV
E
***
% ST2+ TREG
+ IL-33
+ IL-33
PBS
RSV
F
*
ST2+ TREG (10^5)
+ IL-33
+ IL-33
PBS
RSV

Figure 7

Figure 8. Proposed model for the effect of aberrant neonatal memory T_H2 cell responses on the suppressive ability of T_{REG} cells.

Viral infections in the neonatal host drive the expansion of a memory T_H2 pool that persists in the lungs throughout adulthood. In this early infection, IL-33-responding $ST2^+$ T_{REG} cells fail to expand and prevent the clonal expansion of T_H2 cells. However, upon IL-33 release in adults (viral infection, allergen or physical damage), the now abundant local $ST2^+$ T_H2 cells respond in an antigen-independent manner by releasing high amounts of IL-4, in turn hindering the suppressive ability of the locally expanding $ST2^+$ $IL4R\alpha^+$ $GATA3^+$ T_{REG} cells.



Supplemental Figures

Figure S1. IL-4 and IL-33 enhance type 2 cytokine production by T_{REG} cells in a STAT6-dependent mechanism.

$CD4^{+}CD25^{high}$ T cells from BALB/c or $STAT6^{-/-}$ mice were sorted and activated by plated αCD3 (5μg/ml) + αCD28 (2μg/ml) for 72 hours in the presence of IL-2 (200U/ml), IL-33 (10ng/ml) and/or IL-4 (10ng/ml). The supernatants were collected and sent for Multiplex ELISA (Eve technologies®). The amounts were corrected to 10 000 cells.

(A) IL-5 (pg/ml), **(B)** IL-13 (pg/ml), **(C)** IL-10 (pg/ml) and **(D)** IFNγ (pg/ml) levels in the supernatants per 10 000 cells

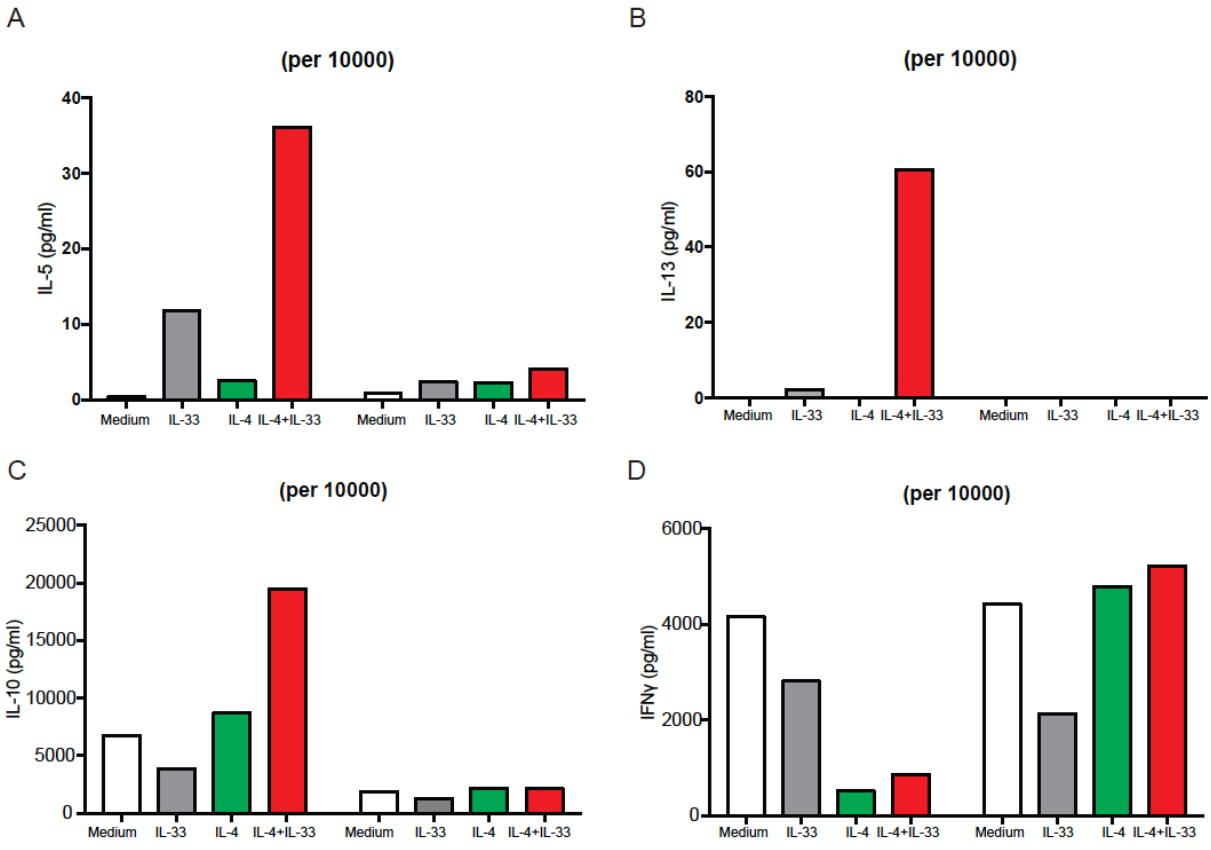
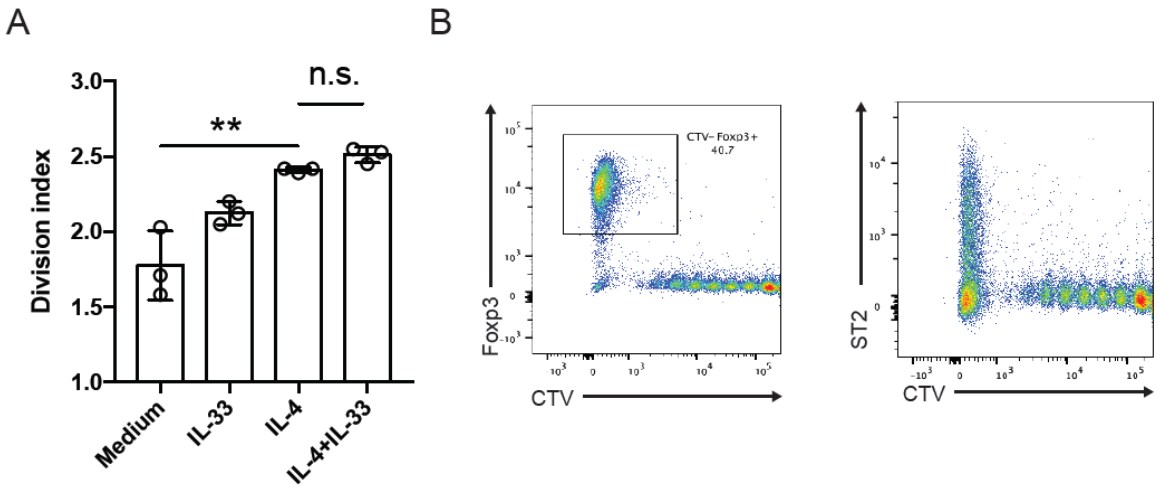


Figure S2. IL-4 reverses ST2$^+$ T_{REG} cell-mediated suppression by enhancing the proliferation of T_{EFF} cells in the presence of IL-33.

(A) Division index of CTV-labelled CD4$^+$Foxp3$^-$ T cells activated in the presence or absence of IL-33 (10ng/ml) and/or IL-4 (10ng/ml) for 72 hours. One-way ANOVA. *p<0.05; **p<0.01; ***p<0.001.

(B) Expression of Foxp3 (left) and ST2 (right) among total CD4+ T cells. T_{REG} (CTV$^-$) and responder T cells (CTV$^+$) were plated at a 1:2 ratio and activated in the presence of soluble αCD3 (1μg/ml) and irradiated APCs for 72 hours. Representative of 3 independent experiments.



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CHAPTER 4 - The role of IL-18 in the functional adaptation of Foxp3^{+} regulatory T cells in T_H1-driven mucosal infections

Chapter 4 - The role of IL-18 in the functional adaptation of Foxp3^{+} regulatory T cells in T_H1-driven mucosal infections

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4.1 Bridging statements for Chapter 4

We have previously uncovered some key elements of the role of alarmins in the function and fate of T_{REG} cells during mucosal inflammation. In our respiratory infection models in **Chapter 2** we observed that IL-33 and IL-1 provide divergent signals to regulate the stability of Foxp3 expression and the fate and differentiation of T_{REG} cell populations in infected mucosal tissues. Collectively, our work revealed that IL-1 and IL-33 are key drivers of the functional adaptation of T_{REG} cells during infections. In **Chapter 3**, we uncovered that the functional outcome of IL-33 is conditioned by the type of inflammation, revealing a mechanism by which a given alarmin can provide either a positive or a negative signal for the effector differentiation and suppressive ability of T_{REG} cells. In **Chapter 4**, we pursued our investigation by detailing the role of another receptor we identified in our early microarray analysis in **Chapter 2** (1), namely the receptor for IL-18 (IL-18R1). Interestingly, although IL-18 has been suggested to enhance autoimmune, allergic and infectious diseases, there is very little information of its role on T_{REG} cells. In **Chapter 4**, we explored the functions of IL-18 in modulating the functional adaptation of T_{REG} cell during infectious disease and provide a rationale for the development of IL-18 targeted therapies.

4.2 Summary

Foxp3$^+$ regulatory T cells are a specialized subset of CD4$^+$ T cells involved in the maintenance of immune homeostasis. To migrate to non-lymphoid sites and adapt their suppressive functions, T_{REG} cells acquire tissue-specific phenotypes that drive their ability to respond to the evolving inflammatory conditions. In particular, members of the IL-1 family alarmins were shown to play a key role in dictating the fate of mucosal T_{REG} cells. In **Chapter 2** we used an unbiased screen to identify the alarmin receptors ST2 and IL-1R1 as being preferentially expressed between T_{REG} cells that maintain high Foxp3 expression and T_{REG} cells that have a propensity to acquire a pro-inflammatory T_H17-like phenotype *in vivo*. Using that database, we show in this report that pro-inflammatory adapted T_{REG} cells also express IL-18R1, another member of the IL-1 family of receptors. While T_{REG} cell have been shown to express IL-18R1 during disease, the role of IL-18 in T_{REG} cells remains to the understood. In this report, we investigated how IL-18 dictates the functional adaption of T_{REG} cells during immune challenge.

Through an acute Influenza A and a chronic *Leishmania major* T_H1-driven murine infection model, we show that IL-18 drives a T_H1-like adaptation of T_{REG} cells in the draining lymph nodes and mucosal barrier sites. At the peak of the T_H1 effector immune response, Helios$^+$ IL18R1$^+$ T_{REG} cells express the T_H1-promoting Tbet transcription factor, the CXCR3 homing receptor and secrete IFNγ. However, contrary to conventional T_H1 cells, IL-12, but not IFNγ, is required for the generation of Tbet$^+$ IL18R1$^+$ T_{REG} cells. Finally, IL-18 impairs T_{REG} cell-mediated suppression *in vitro*, revealing a mechanism by which T_H1 cell evade immune suppression to contribute to anti-pathogen responses.

Collectively, IL-18 facilitates the establishment of an effective T_H1 adaptive response by targeting the functional adaptation of T_H1-like T_{REG} cells. Understanding how T_{REG} cells modulate their adaptation to local tissue environments is key towards developing targeted therapies against T_H1-mediated diseases.

4.3 Introduction

Regulatory T cells are a specialized subset of $CD4^+$ T cells involved in the maintenance of systemic tolerance and mucosal immune homeostasis. They express the transcription factor forkhead-box P3 (Foxp3) that drives the transcriptional program required to exert their suppressive functions (2). T_{REG} cells are found in lymphoid and non-lymphoid tissues throughout the body, and display the ability to migrate, survive et proliferate during and between immune challenges. However, their role and fate during mucosal infections remain to be fully understood. For example, while early experiments involving Influenza A and *Leishmania* infections suggested T_{REG} cells to impair the ramping of the effector response (3-5), it was shown that these cells play a role in both orchestrating the response (6, 7) and facilitating a quick return to immune homeostasis (8, 9). Moreover, antigen specific T_{REG} cells were found to be rapidly recruited to the site of the infection (3, 10) (11), revealing that they are present throughout the immune response. As such, T_{REG} cells are thought to control their suppressive function during peak effector responses to facilitate pathogen clearance (12). Distinct mechanisms have been proposed that can modulate the suppressive function of T_{REG} cells during inflammation, including signaling pathways that drive the loss of Foxp3 expression (13-15), inhibit *de novo* Foxp3 expression (16) or favor immune evasion from T_{REG} cell-mediated suppression (17).

In order to acquire the ability to adapt to the tissue environment, T_{REG} cells modify part of their transcriptional program during infections (18). Notably, T_{REG} cells acquire the expression of secondary master transcription factors associated with specialized T_H cells subsets. For example, GATA3, IRF4, RORγT and Tbet expressing $Foxp3^+$ T_{REG} cells (bi-T_{REG}) have been identified at mucosal surfaces (19-21). The acquisition of these transcription factors was shown to confer T_{REG} cells with the ability to migrate, survive, proliferate and function in these sites (19-21). In recent years, alarmins, members of the danger-associated molecular patterns (DAMPs) family, were shown to modulate the transcriptional and functional adaptation of T_{REG} cells during an active infection (1, 22). In particular, IL-33 and IL-1, alarmins of the IL-1 family, were shown to facilitate the survival, proliferation and the production of cytokines by $GATA3^+$ and $ROR\gamma T^+$ T_{REG} cells,

respectively, in infectious disease (1, 22, 23). In **Chapter 2**, we observed that IL-33 facilitated the stable expression of Foxp3 and the suppressive function of T_{REG} cells in disease, while IL-1β favored a loss of Foxp3 expression and immune evasion by inflammatory T cells (1). When we compared the transcriptional profile of T_{REG} cells that maintained Foxp3 expression with those that downregulated it upon homeostatic expansion, we identified IL18R1 and IL18RAcP as being preferentially transcribed in *ex*Foxp3 cells that expressed pro-inflammatory cytokines (1), suggesting IL-18 may lead T_{REG} cells to become pro-inflammatory cells.

The role of IL-18 in modulating the functional adaptation of T_{REG} cells at mucosal surfaces, particularly during infections, remains largely unknown. IL-18 was identified more than 20 years ago as an interferon-gamma inducing factor (IGIF) in T cells (24). Since then, it has been particularly associated with T_H1 immune responses (25, 26). However, IL-18 was also described as an inducer of IL-13 and IL-5 in T_H2 cells (27, 28) and more recently in suppressing IL-17 production by T_H17 cells (29, 30). In T_{REG} cells, IL-18 promotes the production of amphiregulin both *in vitro* and in the lung of Influenza A infected mice in order to facilitate tissue repair mechanisms (23). Moreover, T_{REG} cells require IL-18 signaling to control T_H1 and T_H17-mediated colitis (29) and T_H2-driven asthma in mice (31), suggesting IL-18 is a critical signal for the mucosal migration and function of T_{REG} cells at mucosal sites. Nonetheless, the regulation of IL-18R on T_{REG} cells, the origin of IL-18 responding T_{REG} cells, their role in inflammation and their fate remained unclear. In this report, we investigated the role of IL-18 on the adaptation of T_{REG} cells to mucosal tissues during an infectious disease.

We show that IL-18R1$^+$ T_{REG} cells represent a significant proportion of thymic T_{REG} (tTreg) cells that play an prominent role in the control of the adaptive immune response at mucosal surfaces. IL-18R1$^+$ tT_{REG} cells express the Helios transcriptional factor, and display hallmark features of T_H1 cells in the course of both acute pulmonary H1N1/Ca Influenza A and chronic cutaneous *Leishmania major* infections. In both T_H1-driven infections, Helios$^+$ IL18R1$^+$ T_{REG} cells preferentially proliferate and express T-bet, CXCR3 and, in the case of a *Leishmania* infection, produce low amounts of IFNγ in the draining lymph node. When we addressed the polarizing capacity of Helios$^+$ and Helios$^-$ T_{REG} cells,

we observed that they differed in their response to IL-12, which in turn governed their ability to signal through IL-18. *In vitro*, IL-18 signaling impaired T_{REG} cell-mediated suppression and facilitated $CD4^+$ T cell proliferation. These results reveal that IL-18 provides a signal to promote the differentiation, migration and proliferation of $Tbet^+$ tT_{REG} cells, while contributing to an effective T_H1 cell response that facilitates pathogen clearance.

4.4 Results

1. IL-18R1 expression is increased in pro-inflammatory exT_{REG} cells and represent a significant population of Helios$^+$ T_{REG} cells at the steady-state in secondary lymphoid tissues.

In a previous report (**Chapter 2**), we used an unbiased screen to study transcriptional differences between T_{REG} cells that maintain Foxp3 expression in harsh homeostatic conditions and cells that lost Foxp3 expression and adopted an effector-like phenotype (*ex*Foxp3) (Figure 1A). In particular, we identified receptors for the IL-1 family of alarmins, IL-33 and IL-1, and proceeded in characterizing the role of these alarmins in dictating the function and fate of T_{REG} cells during inflammation (1). Using this database, we compared the genes directly associated with T cell differentiation (GO: GO:0030217; MGD) that were significantly (cut-off of $p<0.05$) expressed between stable and exT_{REG} cells. Here, we observed that the mRNA of *il18r1* and its accessory protein receptor (*il18rap*) were preferentially expressed in T_{REG} cells that lost Foxp3 expression and adopted an effector-like phenotype (*ex*Foxp3) (Figure 1B). Interestingly, while mRNA levels differed between the cells, assessment of IL18R1 expression by flow cytometry revealed that sT_{REG} cells also express the receptor (Figure 3C), suggesting that IL-18R1 is not restricted to unstable T_{REG} cells and may indicate a transitional state of T_{REG} cells upon loss of Foxp3 expression.

To understand the relative importance of IL-18 signaling on T_{REG} cells, we first assessed the expression of IL-18R1 in Foxp3$^+$ T_{REG} cells from naïve Foxp3^{GFPki} reporter mice relative to Foxp3$^-$ effector T (T_{EFF}) cells in homeostatic conditions. We observed IL-18R1 expression in both T_{REG} (GFP$^+$) and T_{EFF} (GFP$^-$) cells, with a greater frequency of these cells in the colon lamina propria (LP) compared to the spleen and peripheral lymph nodes (LN) (Figure 1D-E), suggesting that IL-18 contributes to the accumulation of T_{REG} cells at mucosal sites. A high frequency of GFP$^+$ T_{REG} cells expressed IL-18R1 (24.9 ± 6.3%), revealing lymphoid T_{REG} cells readily respond to IL-18. Additionally, IL-18R1 expression was expressed by both T_{EFF} and T_{REG} (Foxp3$^-$GFP$^+$) cells that lost the naïve T cell-associated homing receptor L-selectin (CD62L) and that concomitantly express the

activation-associated CD44 receptor on their surface, suggesting that IL18R1$^+$ T_{REG} cells are enriched in antigen-experienced cells and confirming a prior account (29) (Figure 1F). Interestingly, these results suggest a requirement for memory T_{REG} cells to respond to IL-18 in order to survive in the periphery.

We next investigated how IL-18R1 expression related to the expression of Helios, a transcription factor that was originally associated with thymic-derived T_{REG} cells (tT_{REG}) as it is not expressed by peripherally induced T_{REG} (pT_{REG}) (32). Moreover, Helios is plays a role in maintaining T_{REG} cell function and Foxp3 stability at mucosal surfaces (33). We observed that IL-18R1 expression was expressed exclusively by Helios$^+$ T_{REG} cells in the spleen and the peripheral LNs and by a subset of Helios$^-$ T_{REG} cells in the mesenteric LN (mesLN) and the colon (Figure 1G-H), suggesting that IL-18R1 expression on T_{REG} cells is not limited to Helios$^+$ T_{REG} cells. In all, these results suggest that IL-18 is an important signal for antigen experienced T_{REG} cell homeostasis in mucosal barrier sites.

2. Helios$^+$, but not Helios$^-$, IL-18R1$^+$ T_{REG} cells adopt a T_H1-like phenotype during both acute and chronic T_H1-driven infections.

While IL-18 is known to promote T_H1 cell differentiation *in vitro* (34), IL-18-deficient mice display faster clearance of Influenza A and enhanced activation of IFNγ$^+$ CD4$^+$ T cells (35), suggesting that IL-18 contributes to the immune evasion of the virus. Since T_{REG} cells play an important role in controlling pulmonary T_H1 cells during Influenza (11) and IL-18R1$^+$ T_{REG} were found in the lungs of mice infected with Influenza (23), we investigated how the infection influenced the accumulation and phenotype of IL18R1$^+$ T_{REG} cells. Using a model of H1N1/Ca Influenza A infection (Figure 2A) (1), we show that IL18R1$^+$ T_{REG} cells accumulate in the lungs and follows the peak T_{EFF} response (Figure 2B). At day 10, IL18R1$^+$ T_{REG} cells represent a large portion of the replicating CD4$^+$ T cells in the lungs (Figure 2C). We observed that the highly replicating (Ki67$^+$) T_{REG} cells in the mediastinal LN (MedLN) expressed increased levels of the T_H1-associated chemokine CXCR3 and transcription factor Tbet. In contrast to MedLN, both Helios$^+$ and Helios$^-$ Foxp3$^+$ T cells expressed IL18R1 in the lungs, although Helios$^+$ T_{REG} cells displayed increased expression of IL18R1, as determined by MFI (Figure 2M; Figure S1A).

Moreover, Helios^{+}Foxp3^{+} T cells expressed increased levels of CXCR3 when compared to Helios^{-} Foxp3^{+} cells in the MedLN (Figure 2D-E; Figure S1B) as well as increased levels of Tbet in the lungs (Figure 2F), suggesting that IL18R1^{+} Helios^{+}, but not Helios^{-}, T_{REG} cells are prone to develop a T_H1-like phenotype.

In order to confirm that this finding was not unique to an Influenza infection, we then characterized IL-18R1^{+} T_{REG} cells in the course of a sub-cutaneous *Leishmania major* infection. The importance of the T_H1 adaptive immune response in the control and clearance of *Leishmania major* has been demonstrated in both humans (36) and mice (37). Here, T_{REG} cell were shown to play a key role in suppressing the initial T_H1 response in an antigen-specific manner (3, 38). Importantly, since IL-18 deficient mice were shown to have increased protection through enhanced T_H1 responses (39), it may be possible that T_H1-adapted T_{REG} cells are active suppressors of the T_H1 response. To investigate the dynamics of IL-18R1^{+} T_{REG} cells, we infected C57BL/6 mice with *Leishmania major* in the right foot pad ($1x10^6$ promastigotes of in the right footpad (s.c.) and examined the frequency of IL-18R1 expressing T cells in draining popliteal lymph node (dLN) at weeks 0, 1, 5 and 10 post-infection (Figure 2G). We observed that T_H1 (CD4^{+}Foxp3^{-}IFNγ^{+}) cells actively expanded (Ki67^{+}) by weeks 5 and 10 of infection (Figure S2A-B). Interestingly, the frequency of Foxp3^{+} (T_{REG}) was significantly increased among the proliferating T_H1 cells (Figure 2H; Figure S2C), suggesting that Foxp3^{+} T_{REG} cells are selectively expanding in the course of infection. Among these T_{REG} cells, we observed a progressive increase in the frequency of IL-18R1^{+} cells that correlated with the frequency of T_H1 cells (Figure 2I), suggesting that the immune response facilitated the expansion and accumulation of IL-18 responding T_{REG} cells along with the T_H1 response in the dLN (Figure 2I) around the peak of parasite burden at week 5 in C57Bl/6 mice (40). Here, the expanding IL-18R1^{+} T_{REG} cells expressed the T_H1-associated transcription factor Tbet (Figure 2J-K) and a significant fraction of them produced low amounts of IFNγ (Figure 2L-M). Concomitantly, the expanding IL-18R1^{+} T_{REG} cells were Helios^{+} (Figure 2N; Figure S2D). These Helios^{+} IL-18R1^{+} T_{REG} cells expressed Tbet (Figure 2O; Figure S2D) and produced low amounts of IFNγ (Figure S2E-F). The co-expression of IL-18R1, Tbet and IFNγ in Helios^{+} T_{REG} suggests that the transcription factor Helios may be involved in the adaption of T_{REG} cells

to the T_H1 response.

3. Helios$^+$ IL-18R1$^+$ T_{REG} cells migrate to the colon and produce IFNγ, but not IL17A, during homeostatic proliferation.

Since T_{REG} cells can readily express IL-18R1 and pro-inflammatory cytokines during infections, we assessed if IL-18 can modulate Foxp3 expression in T_{REG} cells and drive their conversion towards a pro-inflammatory phenotype. We previously showed that T_{REG} cells undergoing homeostatic expansion in lymphopenic hosts readily lose Foxp3 expression (*ex*T_{REG}) and acquire an inflammatory phenotype (41). In order to understand the dynamic of IL-18R1 expression on T_{REG} cells during this process, we adoptively transferred T_{REG} cells ($2x10^5$ GFP$^+$CD4$^+$ T cells from Foxp3^{GFPki}) mice into lymphopenic TCRβ$^{-/-}$ mice (Figure 3A). In these mice, the majority of the donor T cells expressed IL-18R1, except for a small portion of sT_{REG} cells in the spleen (Figure 3B). Moreover, in that population of sT_{REG} cells, a small proportion of Helios$^+$, but not Helios$^-$, did not express IL-18R1 (Figure 4B). Importantly, we observed that all *ex*T_{REG} cells were IL18R1$^+$ and Helios$^-$, suggesting that IL-18 signaling could compromise Helios and Foxp3 expression in T_{REG} cells. Indeed, IL18R1$^+$ GFP$^+$ T cells, but not IL18R1$^-$ GFP$^+$ T cells, expressed higher levels of IFNγ but not IL17A (Figure 3C-D-E). To confirm that IL-18 favor a T_H1-like differentiation of splenic T_{REG} cells, we adoptively transferred FACS-isolated IL18R1$^+$ and IL-18R1$^-$ T_{REG} cells into TCRβ$^{-/-}$ recipient mice ($1x10^5$ cells; CD4$^+$ GFP$^+$ T cells from Foxp3^{GFPki} Ly5.1$^+$ mice) (Figure 3F). After 21 days, we observed that splenic Helios$^+$ IL-18R1$^+$ T_{REG} cells maintained Foxp3 expression when compared to sorted IL-18R1$^-$ (Figure 3G). In fact, IL-18R1$^+$ T_{REG} cells accumulated more in the colon than IL-18R1$^-$ T_{REG} cells (Figure 3H) and were more prone than IL-18R$^-$ splenic T_{REG} cells to become IFNγ-producing exT_{REG} cells (Figure 3I). These results suggest that IL-18 signaling does not facilitate the loss of Foxp3 in T_{REG} cells but facilitates their migration and differentiation into T_H1-like T_{REG} cells.

4. pT_{REG} but not *in vitro* generated iT_{REG} cells express IL-18R1

Since we observed that a significant portion of Helios$^-$ T_{REG} cells expressed IL-18R1 in the lungs during Influenza and in the gut at the steady-state, we hypothesized that Helios$^-$ IL-18R1$^+$ T_{REG} cells originate from peripherally-induced T_{REG} cells (pT_{REG}), as it was shown that, contrary to tT_{REG} cells, pT_{REG} cells do not express Helios (32). Indeed, when we transferred Foxp3$^-$ (GFP$^-$) CD4$^+$ T cells into lymphopenic TCRβ$^{-/-}$ mice, all of the *de novo* generated pT_{REG} expressed IL-18R1 (Figure 3B-C) and lacked Helios expression (Figure 3B). However, when we generated T_{REG} cells *in vitro* (iT_{REG}) by activating CD4$^+$ GFP$^-$ T cells from Foxp3^{GFPki} mice in the presence of TGFβ and IL-2 (42), we observed that iT_{REG} cells expressed very little IL-18R1 (Figure S3A). Moreover, addition of IL-18 did not affect the induction of Foxp3 in these cells (Figure S3B) Since a portion of Foxp3$^-$ T_{EFF} cells express IL-18R1, we hypothesized that the Helios-IL-18R1$^+$ T_{REG} cells originate from conventional T cells that expressed the receptor prior to their exposure to Foxp3-polarizing cytokines. To address this, we sorted CD4$^+$GFP$^-$ conventional T cells by further stratifying IL-18R1$^+$ and IL-18R1$^-$ subsets before inducing Foxp3 *in vitro*. We observed that, while IL-18R1$^-$ T_{conv} cells expressed Foxp3 under high concentrations of TGFβ, IL-2 and IL-18 (Figure S3C), IL-18R1$^+$ T_{conv} cells showed reduced expression of Foxp3 and even completely locked Foxp3 induction in the presence of IL-18 (Figure S3C). Collectively, this data suggests that the IL-18R1$^+$ Helios$^-$ T_{REG} cells observed *in vivo* at mucosal surfaces do not originate from T_H1 cells. However, while pT_{REG} cells express IL-18R1 *in vivo*, iT_{REG} cells do not, suggesting that additional tissue-specific signals drive IL-18R1 expression on T_{REG} cells.

5. IL-12 drives the up-regulation of IL-18R1 on Helios$^+$ T_{REG} cells

Since polarizing cytokines that signal through the JAK/STAT pathway are key components of the differentiation of T_H cells (43), we then assessed the effect of some key cytokines on the expression of IL-18R1 on T_{REG} cells. IL-12 and type I and II IFNs have been described to induce IL-18R1 expression on T cells (26). Moreover, the expression of IL-18R1 on T_H1 cells was shown to be dependent on the transcription factor T-bet (44). Thus, we first hypothesized that IL-12 and Type I and II IFNs were responsible

for the induction of Tbet and IL-18R expression in T_{REG} cells. We first assessed the *in vitro* effect of polarizing cytokines by TCR-activating CD45.1$^+$ T_{REG} and CD45.2$^+$ T_{EFF} with soluble anti-CD3 stimulation (45, 46). IL-12, but not IL-18, induced IL-18R1 expression on T_{REG} cells (Figure 4A-B-C). Moreover, we observed an additive effect of IL-18 and IL-12 on the expression of the receptor (Figure 4B-C). Concomitantly, Helios$^+$ T_{REG} cells co-expressed the transcription factor T-bet and produced low levels of IFNγ (Figure 4D-E). To confirm that the production of IFNγ did not contribute to the induction of IL18R1 on T_{REG} cells, we assessed the effect of IL-12 and IL-18 on IFNγR$^{-/-}$ T_{REG} cells. Indeed, even when lacking IFNγR (IFNGR$^{-/-}$), T_{REG}, but not T_{EFF} cells, expressed comparable levels of IL-18R1 (Figure 4F), confirming that type II IFNs – contrary to T_{EFF} cells (47) - are not necessary to maintain Tbet and IL-18R1 expression in Helios$^+$ T_{REG} cells. Moreover, mice lacking the receptors for type I and II IFNs (IFNAR$^{-/-}$IFNGR$^{-/-}$ B6 mice) showed similar frequencies of IL-18R1$^+$ T_{REG} cells and expressions level of IL-18R1 (MFI) when compared to WT mice (Figure S4A-B) in the spleen, confirming that the homeostatic accumulation of IL-18R1$^+$ T_{REG} cells is not dependent on IFN signaling. On the other hand, when we polarized T_{REG} cells with IL-4, a T_H2 polarizing cytokine, we did not observe an effect on IL-18R1 expression (Figure 4H-I). Finally, we followed the effect of IL-6 and TGFβ, a combination known to favor T_H17 polarization (46), on the upregulation of IL18R1 on T_{REG} cells. We did not observe an increase in IL-18R1 MFI in T_{REG} cells stimulated with IL-6 and TGFβ in Helios$^+$ T_{REG} cells (Figure 4J-K). Nonetheless, these T_H17 polarizing conditions favored the expression of RORγT in a portion of T_{REG} cells that segregated as IL-18R1$^-$ (Figure 4K). This suggested that neither IL-6, nor TGFβ, induced the upregulation of IL-18R1 on RORγT$^+$ T_{REG} *in vitro*. Overall, these results reveal that T_H1 polarizing cytokines, but not T_H2 or T_H17 polarizing cytokines, promote the IL-18 signaling on T_{REG} cells.

Finally, in order to determine if IL-18R1$^+$ Tbet$^+$ T_{REG} cells originate exclusively/directly from Helios$^+$, we sorted Helios$^+$ and Helios$^-$ T_{REG} cells from a dual Foxp3/Helios-reporter mouse (B6.Helios^{iGFP}Foxp3^{iRFP}) and assessed the polarization of these cells (Figure S5). Here, we confirmed that the effect of IL-12 on the expression of IL-18R1$^+$ is specific to Helios$^+$ T_{REG} cells (Figure S5A-C), revealing that splenic Helios$^+$,

but not Helios$^-$, T_{REG} cells are poised to express Tbet and CXCR3 upon IL-12 signaling. On the other hand, IL-6 and TGFβ promoted the differentiation of Helios$^-$ T_{REG} cells into RORγT$^+$ T_{REG} cells (Figure S5B). We could not, however, replicate the Helios$^-$IL18R1$^+$ T_{REG} cells we observed *in vivo*, suggesting additional signals may drive the upregulation of IL-18R1 on these cells. Nonetheless, these results illustrate that Helios$^+$ and Helios$^-$ T_{REG} cells are unique subsets of T_{REG} cells that differ in the pathways that lead to the expression of the IL-18R1.

6. IL-18 promotes Helios$^+$ T_{REG} cells survival and stability but impairs their suppressive ability *in vitro*

Since we observed a significant increase in IL-18 responding T_{REG} cells during the course of infectious disease, we investigated the role of IL-18 on the proliferation of T_{REG} cells. We observed that IL-18 enhanced Helios$^+$ T_{REG} cell accumulation in a dose dependent manner (Figure 5A-B). When exposing IL-18R1$^+$ and IL-18R1$^-$ T_{REG} cells to IL-18 *in vitro*, we observed an IL-18 dependent increase in the expansion of T_{REG} cells that was dependent on the presence of the IL-18R1 (Figure 5C). Moreover, isolated IL-18R1$^+$ T_{REG} cells displayed a similar suppressive ability *ex vivo* to IL-18R1$^-$ T_{REG} cells (Figure 5D). Following these observations, we expected to observe an increase in the suppressive ability of T_{REG} cells when we added exogenous IL-18. However, although more T_{REG} cells accumulated in the culture, we observed a significant increase in the proliferation of the responder CD4$^+$ T cells (Figure 5E-F), which correlated with the expression of IL18R1 on the responder CD4$^+$ T cells. However, although IL-18 facilitates the evasion of responder T cells from suppression by T_{REG} cells *in vitro*, further investigation using IL-18R-deficient responder T cells will be required to confirm if this is a T_{REG}-independent effect.

Since T_{REG} cell are known to produce and respond to TGFβ, a potent repressor of T_H1 differentiation (48), we investigated how it impacted IL-18R1 expression in Helios$^+$ T_{REG} cells. We observed that, contrary to conventional Foxp3$^-$ T cells, T_{REG} cells maintain IL-18R1 expression in the presence of IL-18 (Figure S6A-B). Moreover, T_{REG} cells were able to maintain their numbers and proliferated in the presence of IL-18 (Figure S6C-D).

These results are in line with the fact that TGFβ does not directly inhibit IL-12 signaling but rather IFNγ production (49) and is further evidence that Helios^{+} T_{REG} cells become and maintain Tbet and IL-18R1 expression independently of STAT1 signaling. Collectively, these results suggest that IL-18 confers a proliferative advantage of T_{REG} cells in mucosal environments where the locally produced TGFβ, known as a critical mediator of immune homeostasis (50), favor the survival and proliferation of IL-18-adapted Tbet^{+} T_{REG} cells over T_H1 cells. These results suggest a mechanism by which T_{REG} maintain IL-18 signaling at mucosal surfaces at the steady-state and modulate their suppressive functions during peak production of IL-18.

7. IL-18 signaling is required for differentiation but not the accumulation of T_{REG} cells in the lungs in the course of an Influenza A infection.

In order to understand the functional consequence of IL-18 signaling on T_{REG} cells, we assessed how the lack of IL-18R1 modified the dynamics of T_{REG} cells in the course of an Influenza A infection. We reconstituted T cell-deficient (TCRβ-deficient) mice with CD4^{+} T cells (1x10^5/each) from either CD4^{CRE}IL-18R$^{fl/fl}$ (CRE^{+}) or IL-18R1$^{fl/fl}$ (CRE^{-}) in conjunction with CD45.1^{+} CD4^{+} (2x10^5/each) and CD8^{+} (1x10^5/each) T cells from Foxp3^{GFPki} Ly5.1^{+} mice and infected the mice intra-nasally with ¼ LD_{50} of H1N1/Ca (Figure 6A-B). We observed that CD45.2^{+} T cells accumulated in the lungs regardless of IL-18R expression (Figure 6C-D). Similarly, the frequency of Foxp3^{+} T_{REG} cells among CD4^{+} T cells lacking IL-18R1 did not differ between groups (Figure 6E), suggesting that IL-18 signaling does not specifically affect T_{REG} cell accumulation in the lungs during disease. Nonetheless, when we assessed the phenotypic differences between Foxp3^{+} T_{REG} cells in the same mouse (Figure 6F), we observed that T_{REG} cell that lacked IL-18 signaling expressed lower levels of CXCR3 (Figure 6G-H), suggesting that lack of IL-18 signaling influenced the phenotype of T_{REG} cells that accumulated in the lungs.

Importantly, we are currently evaluating how IL-18 signaling on T_{REG} cell impacts the outcome of disease. As stated above, studies made in IL-18$^{-/-}$ mice have revealed that this alarmin is detrimental to the establishment of a T_H1 cell response in both Influenza A and *Leishmania major* (35, 39), which, when combined with our early results, suggested

IL-18 promotes the specialization of tissue-localized T_{REG} cells. However, our *in vitro* experiments reveal that IL-18 facilitates immune evasion by T cells. To understand this discrepancy, we are in the process of generating a dual reporter $Foxp3^{GFP\text{-}ERT2}$ $Rosa26^{RFPloxP}IL18R1^{loxP}$ mouse that will allow for the specific deletion of IL-18R1 on tamoxifen-treated $Foxp3^{+}$ T_{REG} cells *in vivo*, while simultaneously allowing for the tracking of *ex*Foxp3 T cells. After a two-dose regimen of tamoxifen (I.P. injection), we plan to infect the mice with ½ LD50 of H1N1/Ca strain and follow the progression of disease through weight loss, T cell infiltration and survival. At days 3, 6 and 10, we will assess the effect of the specific tamoxifen-induced deletion of IL-18R1 on 1) T_{REG} cells-mediated suppression of T_H1 responses, 2) stability of Foxp3 expression, 3) the outcome of disease. This last experiment will reveal the specific effect of IL-18 signaling on T_{REG} cell adaptation and its role during Influenza.

4.5 Discussion

Regulatory $CD4^+$ $Foxp3^+$ T cells are an essential component of immune regulation and, as such, play a key role in modulating the mucosal immune response. Major advancements have been made in identifying how T_{REG} cells acquire the transcriptional program required to migrate, survive and adapt their suppressive function to non-lymphoid organs during and between immune challenge (51). Of particular interest are alarmins, a group of danger-associated molecules, that play a major role in orchestrating the nature and the extent of the immune response that unfolds at mucosal surfaces (52). Among these alarmins, our research aims to understand the role of the IL-1 family in T_{REG} cells function and fate. Interestingly, although T_{REG} cells have been shown to respond to prominent members of the IL-1 family, specifically IL-33 (53) and IL-1 (1, 54), the role of IL-18 on T_{REG} cell function and differentiation remained largely ill-defined despite showing important potential (29). Although the evidence remained scarce, T cell mediated colitis experiments (29), pulmonary allergy (31) and infection models (23) revealed that IL-18 plays a role in facilitating the suppressive function of T_{REG} cells in both the intestine and the lungs.

In this study, we show that IL-$18R1^+$ T_{REG} cells can be readily found in the spleen and lymph nodes of naïve C57Bl/6 mice. This distribution pattern is different from the tissue-specific distribution that we and others observed with the alarmin-receptors IL-1R1 and ST2 in murine T_{REG} cells (1, 22). We show that all IL-$18R1^+$ T_{REG} cells localized in lymphoid organs express the transcription factor Helios. Although the function of Helios remains ill-defined, it is associated with maintained Foxp3 expression (33). Importantly, Helios is not required for the *in vitro* suppressive function of T_{REG} cells (33), suggesting this transcription factor may play a role in dictating the specialized adaptation of T_{REG} cells to tissue environments (55) rather than modulate the function of T_{REG} cells. Since IL-18R1 expression was restricted to $Helios^+$ T_{REG} cells in lymphoid organs, we hypothesized that IL-18 signaling is a necessary signal for a subset of tissue-adapted T_{REG} cells.

To understand the dynamics of IL-18 responding T_{REG} cells in disease, we first investigated IL-$18R1^+$ T_{REG} cells in the course of an H1N1 Influenza A infection, where IL-$18R1^+$ T_{REG} cells were described (23), and observed a correlation between IL-18R1

and Tbet expression in Helios$^+$ T_{REG} cells in both the mediastinal lymph node and the lungs of infected mice. In this model, IL-18 signaling is associated with reduced viral clearance (35), and our data suggests that the preferential accumulation of IL-18 responding Helios$^+$ T_{REG} cells may play a role in controlling the T_H1 cell response. To observe if this association was specific to an Influenza A virus infection, we further investigated the dynamics of IL-18R1$^+$ T_{REG} cells in a chronic infection with *Leishmania major* on susceptible C57BL/6 mice. In this model, T_{REG} cells were found to favor the establishment of a chronic form of the disease by preventing an effective T cell response (3). Here, we observed that, among T_{REG} cells that readily expand during the infection, the vast majority express high levels of IL-18R1 and the transcription factors Tbet and Helios, suggesting that the *Leishmania*-driven T_{REG} cells adapt to be able to respond to IL-18 by acquiring a T_H1-like phenotype. Moreover, we observed the production of IFNγ by Helios$^+$ T_{REG} cells by week 5 and 10 of infection, suggesting that chronic inflammation potentiates the IL-18-associated T_H1-differentiation of T_{REG} cells. Collectively, these results illustrate a mechanism by which Helios$^+$ T_{REG} cells preferentially express Tbet and CXCR3 and gain the ability to respond to IL-18. Incidentally, while investigating the lungs of IAV infected mice, we uncovered another population of IL-18R1$^+$ T_{REG} cells that did not express high levels of either Tbet, CXCR3 or Helios. While we show that Helios$^-$ pT_{REG} cells can express IL-18R1, the polarizing conditions that favour *de novo* induction of Foxp3 do not favor the expression of receptor, suggesting additional signals are required. Thus, it remains to be understood how these cells differ from IL-18R1$^+$ Helios$^+$ T_{REG} cells.

Cytokines that signal through the STAT pathway play a key role in orchestrating the differentiation of T_{REG} cells *in vitro* and *in vivo* and their ability to acquire alarmin receptors (1, 20, 22, 54). In T cells, STAT4 signaling, downstream of IL-12, was shown to induce chromatin modifications to the *il18r1* locus whereas STAT6 signaling, downstream of IL-4, directly inhibit these modifications (56). Conversely, T-bet, a STAT1 and STAT4 target, binds the promoter of *Il18r1* (57). Here, we confirmed that IL-12 (STAT4), but not IFNγ (STAT1), IL-4 (STAT6) or IL-6 (STAT3), is a strong driver of IL-18R1 expression on T_{REG} cells *in vitro*. Importantly, whereas T_{REG} cells were shown to develop an abortive T_H1 polarization through decreased expression of the IL-12Rβ2 (58), we did not observe in our experiments a decrease in the intensity of IL-18R1 in T_{REG} cells

compared to T_H1 cells. Moreover, during the early phase of T_H1 polarization, STAT1 signaling through IFNγ was shown to provide the signal for the expression of Tbet and the subsequent expression of the IL-12Rβ2 chain on T cells (59, 60) and IL-18R1 (57). Indeed, we observed that, while conventional T cells lacking the IFNγR display impaired IL-18R1 expression in the presence of IL-12 and IL-18, T_{REG} cells lacking the IFNγR readily express IL-18R1, revealing a unique mechanism by which Helios$^+$Foxp3$^+$ T_{REG} cells differentiate to respond to IL-18. This mechanism is further confirmed by the ability of IL-18R1$^+$ T_{REG}, but not IL-18R1$^+$ T_H1 cells, to resist the IFNγ-inhibiting action of TGFβ *in vitro* (49, 61, 62). Moreover, we ensured that Helios$^+$, but not Helios$^-$, T_{REG} cells possess the ability to respond to IL-12 and IL-18 *in vitro*, suggesting a unique epigenetic background that differentiate these two populations of splenic T_{REG} cells and suggesting that Helios expression plays a role in the specialization of T_{REG} cells subsets.

To confirm the epigenetic fate of Helios$^+$ IL-18R1$^+$ T_{REG} cells, we used a T_{REG} cell transfer model in lymphopenic hosts (1, 41). We show that T_{REG} cells that express the IL-18R1 upon homeostatic expansion are prone to express IFNγ but no IL17A. Moreover, IL-18R1$^+$ but not IL-18R1$^-$ splenic T_{REG} cells more readily migrated to the colon, where they maintained high Foxp3 expression. Interestingly, the cells that ultimately lost Foxp3 (exT_{REG}) where more likely to produce IFNγ rather than IL17A, confirming the T_H1-like fate of Helios$^+$ IL-18R1$^+$ splenic T_{REG} cells. These experiments suggested that IL-18R1$^+$ Helios$^+$ T_{REG} cells possess an intrinsic ability to migrate and survive in inflammatory conditions and express T_H1-like characteristics.

In vitro, IL-18 drives the expansion of Helios$^+$ T_{REG} cells in a dose-dependent manner. However, when we cultured T_{REG} cells in the presence of IL-18, we observed that rather than improving their suppressive capacity, responder T cells more readily evaded T_{REG}-mediated suppression. It remains to be understood, however, if this effect is dose-dependent, and if other factors influence the suppressive ability of IL-18R1$^+$ T_{REG} cells *in vivo*.

When we assessed the role of IL-18 on the migration of T_{REG} cells in the course of an Influenza A infection, we observed that it is not necessary for the accumulation of T_{REG} cells to the lungs. Interestingly, we did observe that IL-18 signaling was required for the efficient accumulation of CXCR3$^+$ T_{REG} cells. Thus, a characterization of its role using

mice with a T_{REG}-specific knock-down of IL-18R1 will be essential in providing the causal effect of IL-18 on the outcome of the immune response to an Influenza and a *Leishmania major* infection.

Collectively, we report that IL-18 is a key signal for the proliferation and differentiation of Tbet$^+$ Helios$^+$ T_{REG} cells at mucosal surfaces (Figure 7). In turn, we hypothesize that IL-18 signaling provide local effector T cells the ability to evade the suppressive ability of T_{REG} cells in a concentration-dependent manner. However, it remains to be understood how IL-18 influences the function of tissue-localized Tbet$^+$ Helios$^+$ T_{REG} cells in the course of disease where IL-18 was found to be detrimental to the establishment of a T_H1 response. Importantly, since we also identified a subset of IL-18 responding Helios$^-$ T_{REG} cells, it is possible that IL-18 plays a distinct role in these subsets, and further investigation is required. Since its discovery, IL-18 was shown to enhance IFNγ on T cells (63) and promote T_H1 and T_H2 cell differentiation (64, 65), although classical T_H2 cells were shown to repress IL18R1 expression (56). Moreover, IL-18 is also a necessary signal to control IL17A production in T_H17 cells (29). As such, IL-18 drives multiple aspects of T cell responses. Future investigation on the effect of IL-18 must aim into understanding how this signal orchestrates the distribution of Tbet$^+$, GATA3$^+$ and RORγT$^+$ T_{REG} cells and how the distinct members of the IL-1 family of alarmins compete in balancing T_{REG} cell function during immune challenge (1). Collectively, these results provide a new model for the understanding of tissue-adapted T_{REG} cell function and fate and propose a novel rational for the development and use of therapies aimed at sequestering IL-18 (66, 67).

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Author Contributions

F.A., J.H.F. and C.P. established the conceptual framework of the study, designed experiments and wrote the paper. F.A. designed and performed all the experiments and analyzed the data. A.L.F. and M.O. prepared the *Leishmania major* promastigotes and performed the infections. I.R. and T.A. contributed to the *in vivo* Influenza experiments. D.H. and R.O. contributed to the *in vitro* experiments. All authors provided valuable input throughout the study and the writing of the manuscript.

Competing financial interests

All authors declare that they have no competing financial interests.

4.7 Material and Methods

Mice

WT (Ly5.2^{+}) TCR-β$^{-/-}$ C57BL/6 mice were obtained from Taconic Laboratories while B6.Foxp3^{GFP} reporter knock-in (Foxp3^{GFPki}) mice were provided by Alexander Rudensky and bred into the congenic background (Ly5.1^{+}) for more than 10 generations. Inbred BALB/c were originally purchased from Charles River. Foxp3/Helios dual reporter mice were kindly provided by Dr Ethan Shevach (NIH, Bethesda, MD). Briefly, Helios-IRES-eGFP mice (Thornton et al. 2019) were crossed with Foxp3-IRES-mRFP (008473, Jackson Laboratories, Bar Harbor, ME) as described previously (Thornton et al. 2019) and used as B6.Helios^{iGFP}Foxp3^{iRFP}. B6.IFNAR$^{-/-}$ (Ifnar1^{tm1Agt}) were donated by Dr J. L. Gommerman (University of Toronto, Toronto, ON) and crossed to B6.IFNGR$^{-/-}$ (kindly donated by Dr. Momar Ndao, originally from 003288, Jackson Laboratories, Bar Harbor, MA) to obtain homozygotes and bred in a specific pathogen-free facility at McGill University (Montreal, QC). B6.CD4^{CRE} IL18R2^{loxP} mice were kindly provided by Dr. Giorgio Trinchieri (MIH, Bethesda, MA).

B6.Foxp3tm9(EGFP/cre/ERT2)Ayr/J mice provided by Dr Suh (McGill University, Montreal, QC), were crossed with Rosa26RFP^{lox} dual reporter mice (provided by Dr Jeffrey Bluestone, UCSF, San Fransisco, CA) in our facility. The dual reporter mice were further crossed to B6.IL18R1^{loxP} mice (Dr Giogio Trinchieri (NIH, Bethesda, MA) to obtain a B6.Foxp3GFP/cre/ERT2 x Rosa26RFP^{loxP} x IL18R^{loxP}. All mice were used at 8-12 weeks of age. All mice were housed and bred under specific pathogen-free conditions and used according to institutional guidelines at McGill University.

Footpad infection with *Leishmania major*

Leishmania major Wild-type strain NIH S clone A2 (MHOM/SN/74/Seidman)(68) promastigotes were cultured at 26 °C in Schneider's Drosophila medium (Gibco, Thermo Fisher Scientific,Waltham, MA) supplemented with 10% heat-inactivated fetal bovine serum (Wisent, Saint-Bruno, Qc), 2 mM l-glutamine, 100 U/ml penicillin and 100 µl/ml streptomycin until reaching a stationary-phase (infectious form). Female C57BL/6 mice (6 to 8 weeks old) were infected with 5x10^{6} stationary-phase promastigotes in the right

hind footpad. Disease progression was assessed by measuring the swelling of the right footpad weekly with a metric caliper, up to 10 weeks post-infection. All experiments with mice were carried out in pathogen-free housing, in accordance with the regulations of the Canadian Council of Animal Care Guidelines, Institutional Animal Care and Use Committees at McGill University.

Intranasal infection with Influenza A

The mouse-adapted Influenza A virus H1N1 strain A/California/07/2009 was originally obtained from the National Microbiology Laboratory, Public Health Agency of Canada and provided by Dr Brian Ward (McGill University, Montreal, QC). The IAV was propagated and titrated by plaque assay on Madin-Darby canine kidney cells. The mice were anesthetized by isoflurane administration and received ½ LD50 as established by the Spearman-Karber calculation method of IAV intranasally. The mice were monitored daily for clinical score and weight loss. The experiments were done in 3 distinct experiments following the guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of McGill University.

Lymphocyte isolation

Isolation of T cells was performed, as previously described (Alvarez et al. 2019). After Isoflurane/CO_2 euthanasia, the lungs were perfused with a slow administration of cold PBS through the right ventricle. The lungs are collected and then digested in RPMI 1640 with 5% FBS (Wisent, Saint-Bruno, QC) containing collagenase IV (0,5mg/ml) in the presence of DNAse I (0.005uM) (Sigma-Aldrich, St-Louis, MO) for 45 minutes at 37°C and then mechanically through a 70µM cell strainer. The lamina propria T cells were obtained through the processing of the colon as previously described (Alvarez et al. 2019). The lungs and spleen red remaining blood cells were then lysed using an in-house made ammonium-chloride-potassium (ACK) buffer, as previously described (Alvarez et al. 2019). All the cells were then filtered through a 70µM cell strainer, counted using Trypan Blue (Gibco – Thermo Fisher Scientific, Waltham, MA) and kept in complete RPMI medium.

Purification of T cell subsets

For *in vitro* experiments, $CD4^+$ cells from splenocytes of WT or $Ly5.1^+$ $Foxp3^{GFPki}$ mice were first enriched using an autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany), and then $CD4^+GFP^+$ and GFP^- T cells (purity >99%) were sorted using a FACSAria™ (BD Biosciences). $IL18R1^+$ and $IL18R1^-$ $Foxp3^+$ T_{REG} cells were isolated form the spleen of $Foxp3^{GFPki}$ (unless otherwise stated) by FACSAria™ as $CD4^+GFP^+$ $IL18R1^+$ or $IL18R1^-$ T cells. For the T cells transfer, a PBS solution of T_{REG} cells ($2x10^5$; 200µl/mouse) was kept on ice until tail vein injection into age and sex-matched $TCR\beta^{-/-}$ mice. When working on the BALB/c background, $CD4^+CD25^{hi}$ (T_{REG}) and $CD4^+CD25^-$ cells (T_{EFF}) were sorted using a FACSAria™ the day of the transfer or the *in vitro* culture.

Adoptive transfer assay and Influenza infection

AutoMACS (Miltenyi Biotec) enriched $CD4^+$ and $CD8^+$ T cells (∽98% purity; $2x10^5$/each) from $Ly5.1^+$ $Foxp3^{GFPki}$ male mice (10 week old) and AutoMacs enriched $CD4^+$ T cells (∽98% purity; $1x10^5$) from $CD4^{CRE}IL18R^{loxP}$ or $IL18R^{loxP}$ male mice (10 week old) were co-transferred into $TCR\beta^{-/-}$ male recipient mice. The mice were left to rest for 24 hours before intra-nasal infection with ¼ LD50 of H1N1/Ca using the method described above. The mice were monitored for weight loss daily and sacrificed at day 7 post-infection

In vitro assays

FACS-sorted $CD4^+Foxp3^+$ T_{REG} cells (GFP^+, $50x10^3$) expressing IL18R1 or not were activated in 96-well flat-bottomed (0.2ml) plates previously coated with α-CD3 (3µg/ml) and α-CD28 (2µg/ml), and in the presence of IL-2 (100U/ml- or otherwise indicated) in RPMI (Wisent) supplemented with 10% FBS (Wisent) at 37°C for 72 hours. For *in vitro* stimulation assays, IL-18 (10ng/ml– or otherwise indicated, R&D biosystems, Minneapolis, MN), IL-6 (10 ng/ml - or otherwise indicated, R&D biosystems), mouse recombinant TGFβ1 (1ng/ml, Novoprotein Scientific, Summit, NJ) were added at the start of the culture unless otherwise stated. For suppression and polarization assays, $50x10^3$ FACS-sorted $CD4^+GFP^-$ (T_{RESP}) were plated in 96-well flat-bottomed plates together with $2.0x10^5$ irradiated feeder cells ($CD4^{neg}$ fraction of the MACs) and activated with soluble

α-CD3 (1µg/mL) in the absence of IL-2. Congenic, CD45.1^{+}, FACs sorted CD4^{+}GFP^{+} T_{REG} cells were added at decreasing distinct amounts of T_{REG} cells, starting at 1:2 ratio ($25x10^{3}$). The cells were labeled with CellTrace™ Violet (Thermo fisher Scientific). When assessing IFNGR$^{-/-}$ T_{REG} cells, the T_{EFF} cells plated in the same wells were IFNGR$^{+/+}$, unless stated otherwise.

Flow cytometry

After lymphocyte isolation, single-cell suspensions were stained with the following fluorescence-conjugated mAbs, purchased from Thermo Fisher Scientific (eBioscience or Invitrogen) unless otherwise stated: anti-CD4–Alexa700 (GK1.5), α-CD8-V500 (53-6.7) (BD Biosciences), α-IL18R1 PE or PECy7 (P3TUNYA), α-CD25-PECy7 (PC61, BD Biosciences), α-Foxp3-FITC or PE (FJK-16s), α-IL17A-APC (eBio17B7), α-IFNg-BUV737 or PECy7 (XMG1.2, BD Biosciences), α- RORγT-PE (AFKJS-9) or α-RORγT BV786 (Q31-378, BD Biosciences), α-GATA3-Alexa647 (BD Biosciences), α-CD45.1 PE (A20, PharMingen - BD Biosciences), α-Helios-Pacific Blue or PE (22F6, Biolegend), α-CD121a/IL1R1 (35F5, BD Biosciences) and CD45.2 APC-efluor 780 (104). Non-viable cells were excluded using fixable viability dye eFluor 780 or 506 reagent (Thermo Fisher Scientific). Data were acquired using a FACS Fortessa X-20 flow cytometer (BD Biosciences) and analyzed using FlowJo version 9 software (TreeStar- BD Biosciences).

Statistical analysis

For all experiments, the mean and standard deviation are shown. 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, N unpaired student T-test was used with the p-value expressed in the figure legend. All statistical analysis was performed with GraphPad Prism version 7 software (GraphPad Software)

4.8 Figures

Figure 1. Identification of IL-18R1 in *ex*T_{REG} cells that express Foxp3 and Helios in lymphoid organs at the steady-state

(A) $CD4^+GFP^+Ly5.1^+$ T cells from $Foxp3^{GFPki}$ mice were FACS-sorted and adoptively transferred into $TCR\beta^{-/-}$ mice and GFP^+ (sT_{REG}) and GFP^- (exT_{REG}) $Ly5.1^+$ were FACs sorted and sent for microarray analysis (see Chapter 2; (1)).

(B) Up and down-regulated mRNA transcripts between sT_{REG} and exT_{REG} cells. The data set obtained from **Chapter 2** (1) was re-analyzed by comparing the top 654 genes that significantly varied ($p<0.05$ cut-off) between sT_{REG} and exT_{REG} cells to the publicly available T cell Differentiation Annotation database (GO: 0030217; update 04/14/2020) of the Mouse Genome Database (MGD; Jackson Laboratory, Bar Harbor, Maine).

(C) Expression of IL-18R1 on sT_{REG} (green) and exT_{REG} (red) cells isolated from the spleen of $TCR\beta^{-/-}$ mice at day 21 post-transfer. Representative of 3 distinct experiments (n=3-4 per experiment).

(D-F) The spleen, peripheral LNs, mesenteric LNs (mesLN) and colon lamina propria (LP) from 8-10 week old $Foxp3^{GFPki}$ female mice were processed to isolate the T cells (see Methods). Representative of 3 separate experiments (n=3 mice per experiment).

(D) IL-18R1 expression among live CD4+ T cells in the spleen

(E) Frequency of $IL18R1^+$ among GFP^+ (Foxp3) T_{REG}; left) and GFP^- (T_{EFF}: right) per organ. One-way ANOVA. Tukey correction. *p<0.05; **p<0.01; ***p<0.001.

(F) Representative flow cytometry of the expression of CD44 and CD62L between $IL18R1^+$ and $IL18R1^-$ $CD4^+$ GFP^+ T_{REG} cells. Student T test. *p<0.05.

(G) Flow cytometry of Helios and IL18R1 expression in $CD4^+$Foxp3-GFP^+ T cells in a naïve mouse and frequency of $Helios^+$ cells among $IL18R1^+$ $CD4^+$Foxp3-GFP^+ T_{REG} cells.

(H) Frequency of $Helios^+$ among $IL18R1^+$ T_{REG}. One-way ANOVA. Tukey correction. *p<0.05; **p<0.01; ***p<0.001.

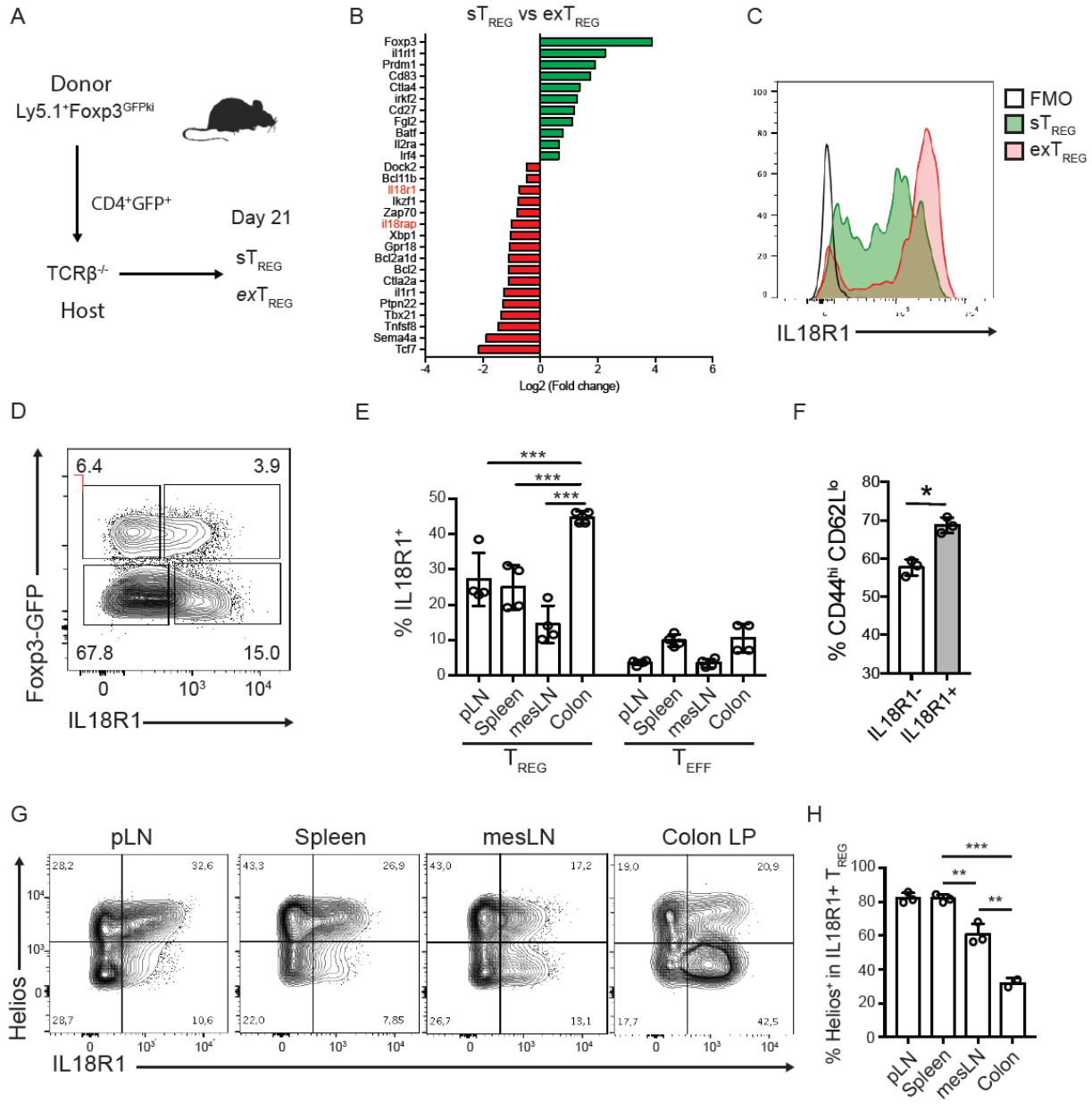

A
Donor
Ly5.1+Foxp3GFPki
CD4+GFP+
Day 21
TCRβ-/-
Host
sTREG
exTREG
B
sTREG vs exTREG
Foxp3
Il1rl1
Prdm1
Cd83
Ctla4
Irkt2
Cd27
Fgl2
Batf
Il2ra
Irf4
Dock2
Bcl11b
Il18r1
Ikzf1
Zap70
Il18rap
Xbp1
Gpr18
Bcl2a1d
Bcl2
Ctla2a
Il1r1
Ptpn22
Tbx21
Tnfsf8
Sema4a
Tcf7
Log2 (Fold change)
C
FMO
sTREG
exTREG
IL18R1
D
6.4
3.9
67.8
15.0
Foxp3-GFP
IL18R1
E
% IL18R1+
pLN
Spleen
mesLN
Colon
TREG
TEFF
F
% CD44hi CD62Llo
IL18R1-
IL18R1+
G
pLN
Spleen
mesLN
Colon LP
Helios
IL18R1
H
% Helios+ in IL18R1+ TREG

Figure 2. IL-18R1^{+} Helios^{+} T_{REG} cells adopt a T_H1-like phenotype in both acute and chronic T_H1-driven infections.

(A) 10 week-old male Foxp3^{GFPki} were infected intra-nasally with ½ LD50 of H1N1/Ca. The mice were monitored for weight loss and sacrificed at day 3, 6 and 10 post-infection. Representative of 4 distinct experiments. (n=3-4 per experiment)

(B) Absolute counts of live IL18R1^{+} CD4^{+} Foxp3^{GFP+} T cells in the lungs (black line) and live Foxp3^{GFP-}Tbet^{+}CXCR3^{+} T cells (red bars; dots). One-way ANOVA. Tukey correction. ***p<0.001 (black line); $^{###}$p<0.001 (red bars).

(C) Frequency of CD4^{+} Ki67^{+} T cells in the lungs at day 10 post-infection.

(D) Mean fluorescence intensity (MFI) of IL-18R1 on the surface of CD4^{+} GFP^{+} (Foxp3) T cells based on the expression of Helios (ΔMFI based as a ratio over FMO). Two-way ANOVA. Tukey correction. **p<0.01; *p<0.05.

(E) Helios^{+}, but not Helios^{-}, IL-18R1^{+} T_{REG} cells co-express CXCR3 and Tbet in the lungs during infection. Overlaid plot between Helios^{+} (red) and Helios^{-} (blue) CD4^{+}GFP^{+} (Foxp3) T cells in the lung at day 10 post-infection.

(F) Mean fluorescence intensity (MFI) of T-bet expression in CD4^{+}GFP^{+} T cells based on Helios expression. Two-way ANOVA. Tukey correction. **p<0.01; *p<0.05.

(G) C57BL/6 8-week old male mice were infected with 5x10^{6} promastigotes of *L. major* through a s.c. injection under the right footpad. The draining popliteal LN were isolated on weeks 0, 1, 5 and 10 after infection. Compiled data shown of 3 distinct experiments.

(H) Foxp3^{+} CD4^{+} T cells represent a significant frequency of replicating (Ki67+) CD4^{+} T cells at week 5. Student T-test. ***p<0.001

(I) Ki67^{+} T_{REG} cells accumulate as IL-18R1^{+} (black line) and follows the increase in the frequency of IFNγ^{+} T_H1 cells (red bar) in the draining lymph node.

(J) Expression of T-bet among IL-18R1^{-} (blue) and IL-18R1^{+} (red) CD4^{+} Foxp3^{+} T cells in the draining popliteal LN at week 5 post-infection.

(K) Mean fluorescence intensity (MFI) of Tbet expression between IL-18R1+ and IL-18R1^{-} T_{REG} cells in the draining LN at week 5 post-infection. Student T-test. *** p<0.001

(L) IL-18R1 and IFNγ production by $CD4^+Foxp3^+$ T cells in the popliteal LN at week 5.

(M) Frequency of IFNγ-expressing T_{REG} cells between IL-18R1$^+$ and IL-18R1$^-$ at week 5 post-infection. Student T-test. ***p<0.001

(N) IL-18R1$^+$ T_{REG} cells express Helios. Representative flow cytometry. Week 5 post-infection.

(O) Helios$^+$ T_{REG} express Tbet in the popliteal LN. Representative flow cytometry. Week 5 post-infection.

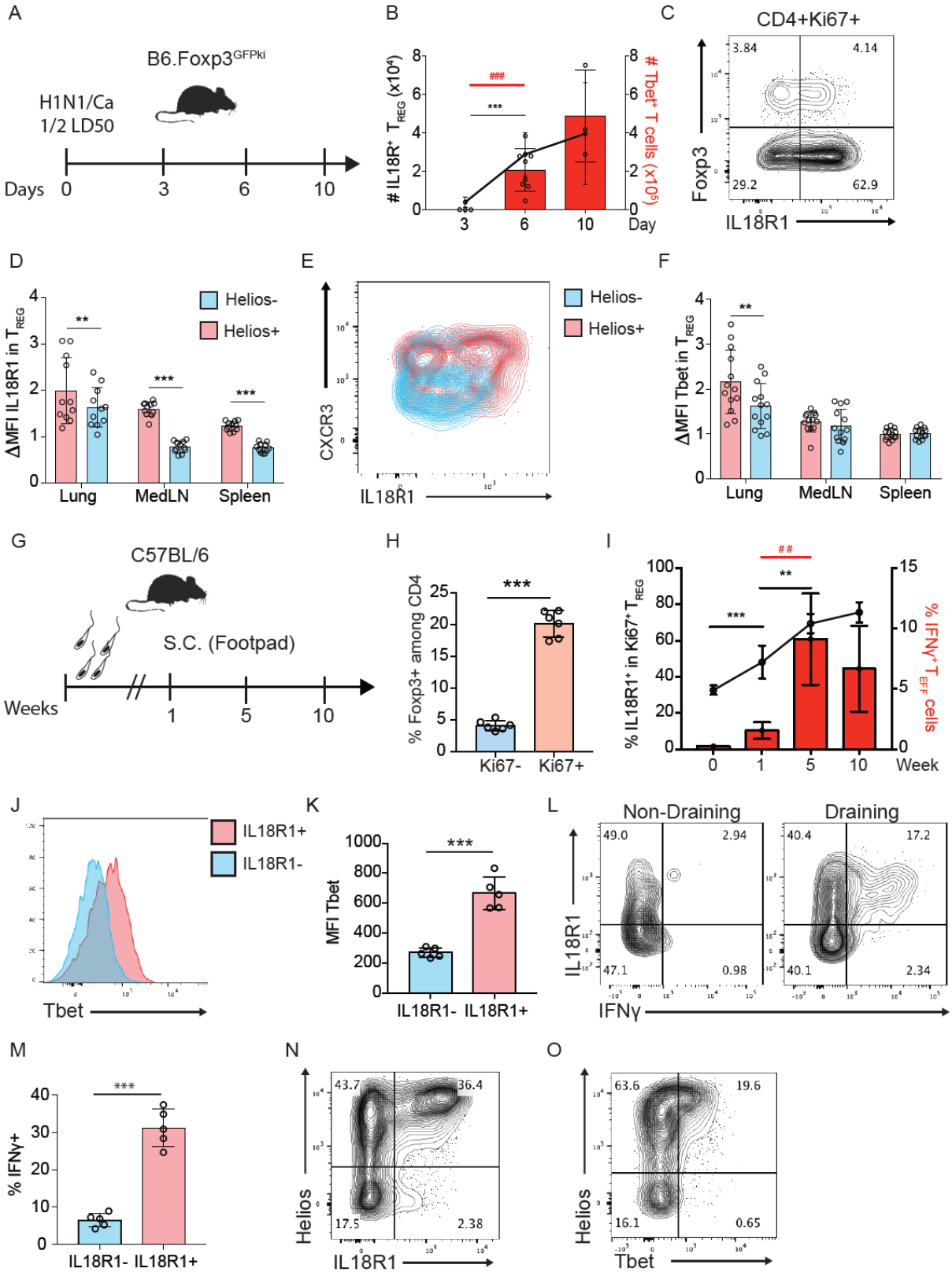

A
B6.Foxp3GFPki
H1N1/Ca
1/2 LD50
Days 0 3 6 10
B
IL18R+ TREG (x10⁴)
Tbet+ T cells (x10⁵)
###
***
3 6 10 Day
C
CD4+Ki67+
3.84
4.14
29.2
62.9
Foxp3
IL18R1
D
ΔMFI IL18R1 in TREG
Helios-
Helios+
**
***
***
Lung MedLN Spleen
E
Helios-
Helios+
CXCR3
IL18R1
F
ΔMFI Tbet in TREG
**
Lung MedLN Spleen
G
C57BL/6
S.C. (Footpad)
Weeks 1 5 10
H
% Foxp3+ among CD4
***
Ki67- Ki67+
I
% IL18R1+ in Ki67+ TREG
% IFNγ+ TEFF cells
##
**
***
0 1 5 10 Week
J
IL18R1+
IL18R1-
Tbet
K
MFI Tbet
***
IL18R1- IL18R1+
L
Non-Draining
Draining
49.0
2.94
47.1
0.98
40.4
17.2
40.1
2.34
IL18R1
IFNγ
M
% IFNγ+
***
IL18R1- IL18R1+
N
43.7
36.4
17.5
2.38
Helios
IL18R1
O
63.6
19.6
16.1
0.65
Helios
Tbet

Figure 3. Helios$^+$ IL-18R1$^+$ T_{REG} cells differentiate into T_H1-like cells upon losing Foxp3 expression in lymphopenic hosts.

(A) CD4$^+$GFP$^+$(Foxp3) T_{REG} cells and CD4$^+$GFP$^-$ T_{EFF} cells form Ly5.1$^+$ Foxp3^{GFPki} mice were isolated by FACsAria and adoptively transferred I.V. into TCRβ$^{-/-}$. The mice were necropsied 21 days later. Representative of 3 distinct experiments. n=4-5 mice per experiment.

(B) Expression of Helios and IL-18R1 in CD4$^+$GFP$^+$ T_{REG} (sT_{REG}) or peripherally-induced T_{REG} (pT_{REG}) (left) and frequency of IL-18R1$^+$ T_{REG} cells among the two populations in distinct organs following transfer (right). One-way ANOVA. Tukey correction. *p<0.05; **p<0.01;***p<0.001.

(C) IFNγ (left) and IL-17A (right) production and IL-18R1 expression among Ly5.1$^+$ sT_{REG} cells in the spleen. 3 hours PMA + Ionomycin + GolgiStop™.

(D) Frequency of IFNγ$^+$ between IL-18R1$^+$ and IL-18R1$^-$ T_{REG} cells. One-way ANOVA. Tukey correction. *p<0.05; **p<0.01;***p<0.001.

(E) Frequency of IL-17A$^+$ between IL-18R1$^+$ and IL-18R1$^-$ T_{REG} cells. One-way ANOVA. Tukey correction.

(F) IL-18R1$^+$ and IL-18R1$^-$ T_{REG} cells (CD4+GFP+) were isolated from the spleen of Ly5.1$^+$ Foxp3^{GFPki} female mice and transferred I.V. into TCRβ$^{-/-}$ female mice. After 21 days, the spleen, mesenteric LN and colon were collected and processed. Compiled results of 3 distinct experiments. (n=3-4 per experiment)

(G) Frequency of GFP$^+$(Foxp3) T cells among CD4$^+$Ly5.1$^+$ T cells in the colon and spleen. One-way ANOVA. Tukey correction. *p<0.05; **p<0.01;***p<0.001.

(H) Number of CD4$^+$Ly5.1$^+$ T cells recovered from the colon of mice at day 21 post-injection. Student T-test. *p<0.05.

(I) Frequency of IFNγ$^+$ *ex*T_{REG} (CD4$^+$Ly5.1$^+$GFP$^-$) T cells in the colon. Student T-test. *p<0.05.

(J) Frequency of IL-17A$^+$ *ex*T_{REG} (CD4$^+$Ly5.1$^+$GFP$^-$) T cells in the colon of mice. Student T-test. *p<0.05.

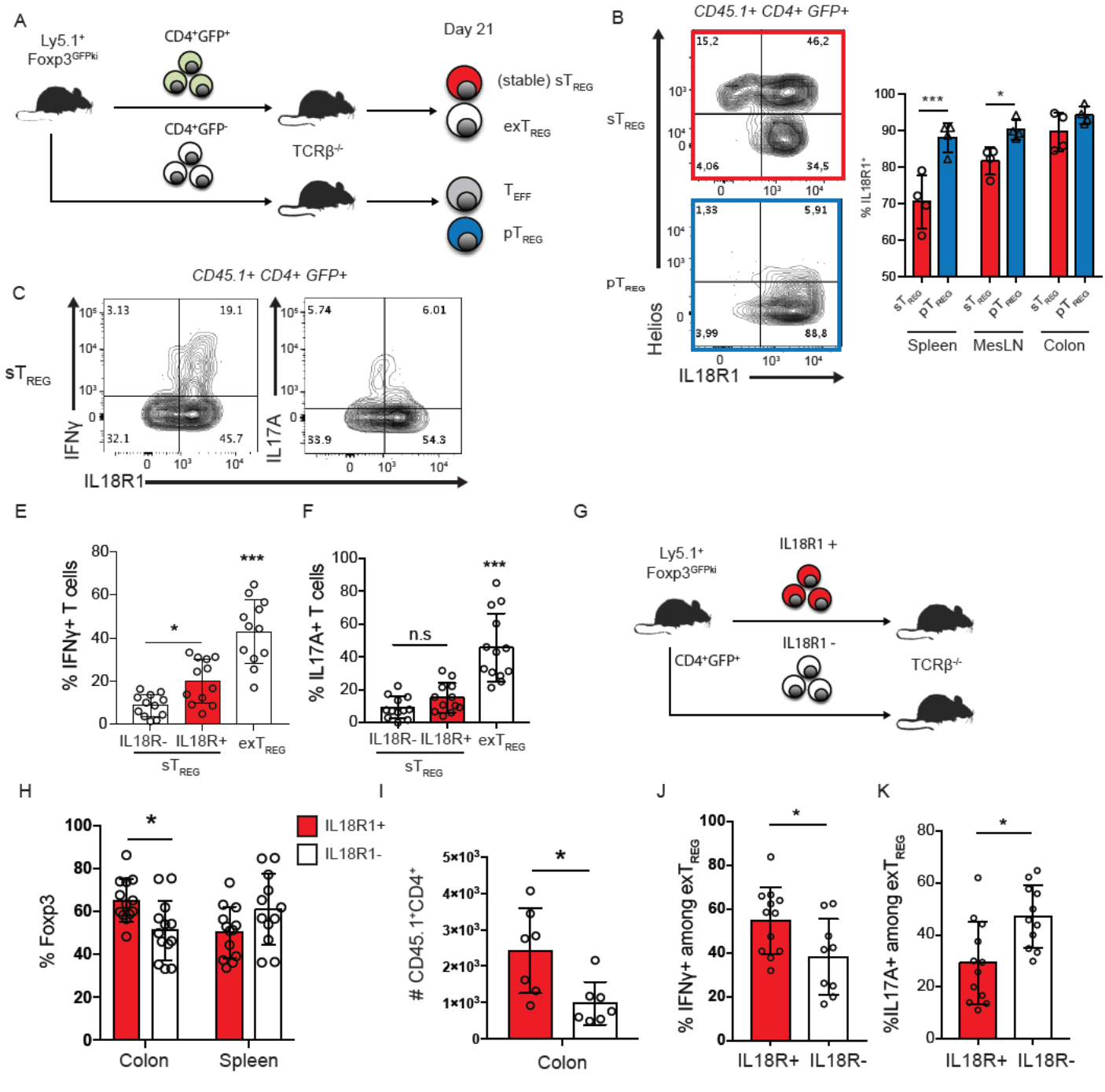

A
Ly5.1+ Foxp3GFPki
CD4+GFP+
CD4+GFP-
TCRβ-/-
Day 21
(stable) sTREG
exTREG
TEFF
pTREG
B
CD45.1+ CD4+ GFP+
sTREG
pTREG
Helios
IL18R1
15,2
46,2
4,06
34,5
1,33
5,91
3,99
88,8
% IL18R1+
***
*
Spleen
MesLN
Colon
C
CD45.1+ CD4+ GFP+
sTREG
IFNγ
IL17A
IL18R1
3.13
19.1
32.1
45.7
5.74
6.01
33.9
54.3
E
% IFNγ+ T cells
IL18R-
IL18R+
exTREG
sTREG
F
% IL17A+ T cells
n.s
G
Ly5.1+ Foxp3GFPki
IL18R1 +
IL18R1 -
CD4+GFP+
TCRβ-/-
H
% Foxp3
IL18R1+
IL18R1-
Colon
Spleen
I
CD45.1+CD4+
Colon
J
% IFNγ+ among exTREG
IL18R+
IL18R-
K
%IL17A+ among exTREG
IL18R+
IL18R-

Figure 4. IL-12, but not IL-4, IL-6 or IFNγ, favors the up-regulation of IL-18R1 on Helios$^+$ T_{REG} cells *in vitro*

$CD4^+$ GFP^+ T_{REG} cells from $Foxp3^{GFPki}$ mice were sorted and plated in the presence of $CD4^+GFP^-$ CTV-labelled T_{EFF} cells and irradiated APCs (CD4-negative fraction) in the presence of αCD3 (1µg/ml) for 72 hours. The cells were exposed to Medium (Ctrl), rIL-18 (10ng/ml), rIL-12 (10ng/ml) or a combination of both. Representative of 3 independent experiments performed in triplicates.

(A) Expression of Foxp3(GFP) and IL18R1 and after 72 hours.

(B) Frequency of IL-18R1-expressing cells among $CD4^+Foxp3^+$ T_{REG} cells. One-way ANOVA. Tukey correction. *p<0.05; ***p0.001. Compilation of 3 experiments

(C) Mean fluorescence intensity (MFI) of IL-18R1 on IL-18R1$^+$ T_{REG} cells after 72 hours. Representative of 3 distinct experiments.

(D) Expression of T-bet among CD4+Foxp3(GFP)+ T_{REG} cells in the presence of IL-12 or a combination of IL-12 and IL-18.

(E) Mean fluorescence intensity of IFNγ expression in T_{REG} cells when compared to T_{EFF} cells (dotted line) that were exposed to the same conditions (IL-12+IL-18) for 72 hours. One-way ANOVA. Tukey correction. P<0.001. Representative of 3 distinct experiments.

(F-G) $CD4^+CD25^{HI}$ T cells from C57BL/6 ($IFNGR^{+/+}$) and B6.$IFNGR^{-/-}$ mice were sorted and co-cultured in the presence of CTV-labelled WT $CD4^+CD25^-$ T_{EFF} cells and irradiated APCs (CD4-negative fraction) in the presence of αCD3 (1ug/ml) for 72 hours.

(F) Representative flow cytometry of the expression of Helios and IL-18R1 on $CD4^+$ $Foxp3^+$ T cells at 72 hours.

(G) Frequency of *de novo* generated IL-18R1$^+$ T cells among $IFNGR^{-/-}$ and WT ($IFNGR^{+/+}$) $CD4^+CD25^-$ plated alone (left) or $CD4^+CD25^{HI}$ (T_{REG}) plated with WT $CTV^+CD4^+CD25^-$ T cells (right). The cells were kept in medium alone or the presence of rIL-18 (10ng/ml) + rIL-12 (10ng/ml) (red) for 72 hours. Compilation of 3 independent experiments.

(H-K) $CD4^+$ GFP^+ T_{REG} cells from $Foxp3^{GFPki}$ mice were sorted and plated in the presence of $CD4^+GFP^-$ CTV-labelled T_{EFF} cells and irradiated APCs (Negative CD4 fraction) in the presence of αCD3 (1ug/ml) for 72 hours. The cells were exposed to Medium alone (Ctrl), IL-4 (10ng/ml), T_H17 conditions (rIL-6 (10ng/ml) + TGFβ (1ng/ml)) with or without rIL-18 (10ng/ml) or with Th1 conditions (rIL-18 (10ng/ml) + rIL-12 (10ng/ml)) for 72 hours. Representative of 5 independent experiments.

(H) Frequency of IL-18R1-expressing $CD4^+Foxp3(GFP)^+$ CTV^{neg} T cells in T_H2 polarizing conditions. One-way ANOVA. Tukey correction. *p<0.05; **p<0.01; ***p<0.001.

(I) Representative flow cytometry of $CD4^+Foxp3(GFP)^+$ CTV^{neg} T_{REG} cells in their expression of GATA3 and IL-18R1 in IL-4 conditions at 72 hours.

(J) Mean fluorescence intensity (MFI) of IL-18R1 on IL-$18R1^+$ T_{REG} cells. One-way ANOVA. Tukey correction. *p<0.05; **p<0.01; ***p<0.001.

(K) Representative flow cytometry of $CD4^+GFP^+$ (Foxp3) CTV^{neg} T_{REG} cells in their expression of RORγT and IL18R1 in T_H17 conditions at 72 hours.

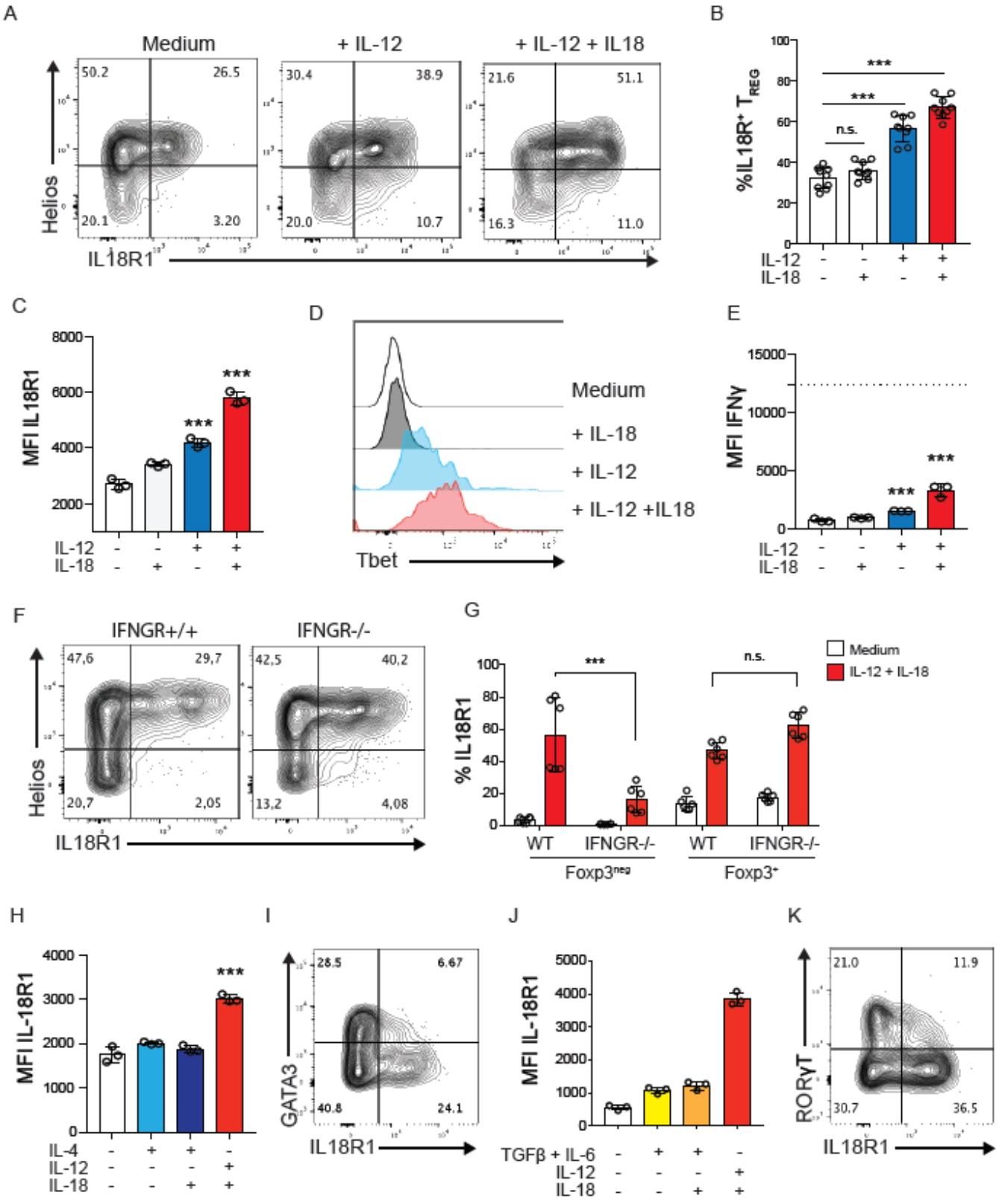

A
Medium
+ IL-12
+ IL-12 + IL18
50.2
26.5
20.1
3.20
30.4
38.9
20.0
10.7
21.6
51.1
16.3
11.0
Helios
IL18R1
B
%IL18R+ TREG
***
***
n.s.
IL-12
IL-18
C
MFI IL18R1
***
***
IL-12
IL-18
D
Medium
+ IL-18
+ IL-12
+ IL-12 +IL18
Tbet
E
MFI IFNγ
***
***
IL-12
IL-18
F
IFNGR+/+
IFNGR-/-
47,6
29,7
20,7
2,05
42,5
40,2
13,2
4,08
Helios
IL18R1
G
Medium
IL-12 + IL-18
% IL18R1
***
n.s.
WT
IFNGR-/-
WT
IFNGR-/-
Foxp3neg
Foxp3+
H
MFI IL-18R1
***
IL-4
IL-12
IL-18
I
28.5
6.67
40.8
24.1
GATA3
IL18R1
J
MFI IL-18R1
TGFβ + IL-6
IL-12
IL-18
K
21.0
11.9
30.7
36.5
RORγT
IL18R1

Figure 5. IL-18 signaling enhance Helios$^+$ T_{REG} cells survival and stability but impairs their suppressive ability *in vitro*

(A-B) CD4$^+$ GFP$^+$ T_{REG} cells from Foxp3^{GFPki} mice were sorted and activated in a αCD3 (5μg/ml) and αCD28 (2μg/ml) coated plate in the presence of rIL-2 (100U/ml) +/- rIL-18 (10ng/ml) for 72 hours. Representative of 3 distinct experiments performed in triplicates.

(A) Representative flow cytometry of CD4$^+$Foxp3(GFP)$^+$ T_{REG} cells on their expression of Helios and IL18R1.

(B) Helios$^+$ T_{REG} cells accumulate in the wells in a rIL-18 dose-dependent manner. One-way ANOVA. Tukey correction. *p<0.05; **p<0.01;***p<0.001.

(C) IL-18R1$^+$ T_{REG} cells respond to rIL-18 with enhanced proliferation. CD4$^+$ GFP$^+$ T_{REG} cells from Foxp3^{GFPki} mice were sorted based on the expression of IL-18R1 and labelled with cell trace violet (CTV) before being activated in a αCD3 (5μg/ml) and αCD28 (2μg/ml) coated plate in the presence of low amounts of IL-2 (100U/ml) +/- rIL-18 (10ng/ml) for 72 hours. Representative of 3 distinct experiments performed in triplicates.

(D) IL-18R1$^+$ and IL-18R1$^-$ T_{REG} cells have similar suppressive ability *ex vivo*. CD4$^+$ GFP$^+$ T_{REG} cells were sorted based on their expression of IL18R1 and plated at distinct ratios with CTV-labelled T_{EFF} in the presence of irradiated APCs (CD4^{neg} fraction) and soluble αCD3 (1μg/ml) for 72 hours.

(E-F) rIL-18 facilitates the evasion of responder T_{EFF} cells form T_{REG}-mediated suppression *in vitro*. Congenic Ly5.1+ CD4$^+$ GFP$^+$ T_{REG} cells and Ly5.2+ CD4$^+$GFP$^-$ T_{EFF} cells from Foxp3^{GFPki} mice were independently sorted. The Ly5.2$^+$ T_{EFF} were labelled with CTV and plated alone (50k/well) or in the presence of Ly5.1$^+$ T_{REG} (12.5k) in a 1:4 ratio in the presence of irradiated APCs (CD4^{neg} fraction) and soluble αCD3 (1μg/ml) for 72 hours. Representative of 5 distinct experiments.

(E) Representative division of Ly5.2$^+$ T_{EFF} alone (1:0), in the presence of Ly5.1$^+$ T_{REG} cells (1:4) with or without rIL-18 (10ng/ml).

(F) Division index of responding Ly5.2$^+$ T_{EFF} cells. One-way ANOVA. Tukey correction. *p<0.05; **p<0.01; ***p<0.001.

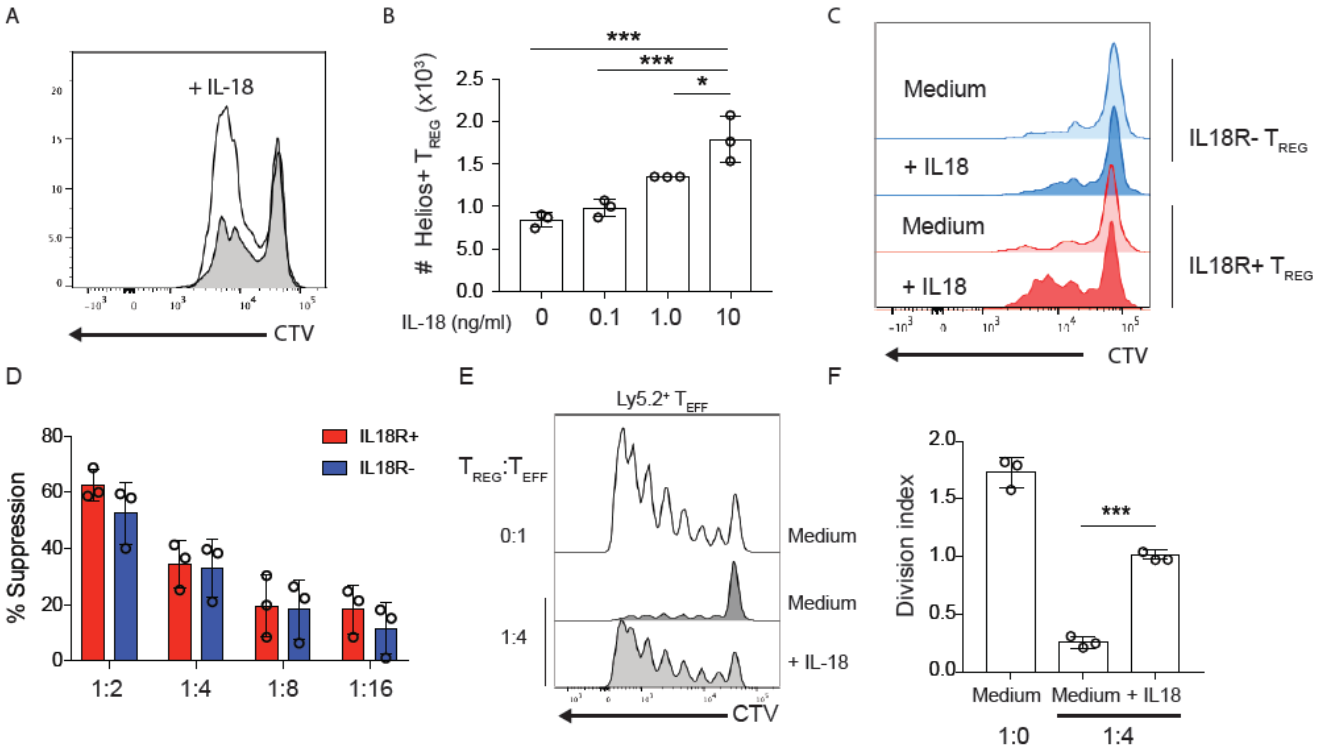

A
+ IL-18
CTV
B
Helios+ TREG (x10³)
IL-18 (ng/ml)
0
0.1
1.0
10
***
***
*
C
Medium
+ IL18
Medium
+ IL18
IL18R- TREG
IL18R+ TREG
CTV
D
% Suppression
IL18R+
IL18R-
1:2
1:4
1:8
1:16
E
Ly5.2+ TEFF
TREG:TEFF
0:1
1:4
Medium
Medium
+ IL-18
CTV
F
Division index
***
Medium
Medium + IL18
1:0
1:4

Figure 6. IL-18 signaling on T_{REG} cells is not required for their migration but is required for the establishment of an efficient T_H1 immune response

(A) FACs sorted (>99% purity) $1x10^5$ $CD4^+$ cells from either $CD4^{CRE}IL18R^{fl/fl}$ (CRE+) or $IL18R1^{fl/fl}$ (CRE-) were mixed with $2x10^5$ $CD4^+$ and $1x10^5$ $CD8^+$ T cells from $Foxp3^{GFPki}$ $Ly5.1^+$ mice and adoptively transferred I.V. into age-matched male $TCR\beta^{-/-}$. The next day, the mice were infected intra-nasally with H1N1/Ca at a ¼ LD50 (established on C57Bl/6 mice).

(B) Flow cytometry of the mixed cells displaying CD4 and CD45.2 at time of injection (Time 0) between mice that receive $CD4^{CRE}IL18R^{fl/fl}$ (CRE+) and $IL18R^{fl/fl}$ (CRE-) among the $CD45.2^+$ $CD4^+$ T cells.

(C) Frequency of $CD45.2^+CD4^+$ T cells among CD3+TCRβ+ T cells in the lungs at day 7 post-infection.

(D) Representative histogram of IL18R1 expression on T_{REG} cells in the lungs in CD45.2+ T cells CRE+ (69) and CRE- (white) at day 7 post-infection.

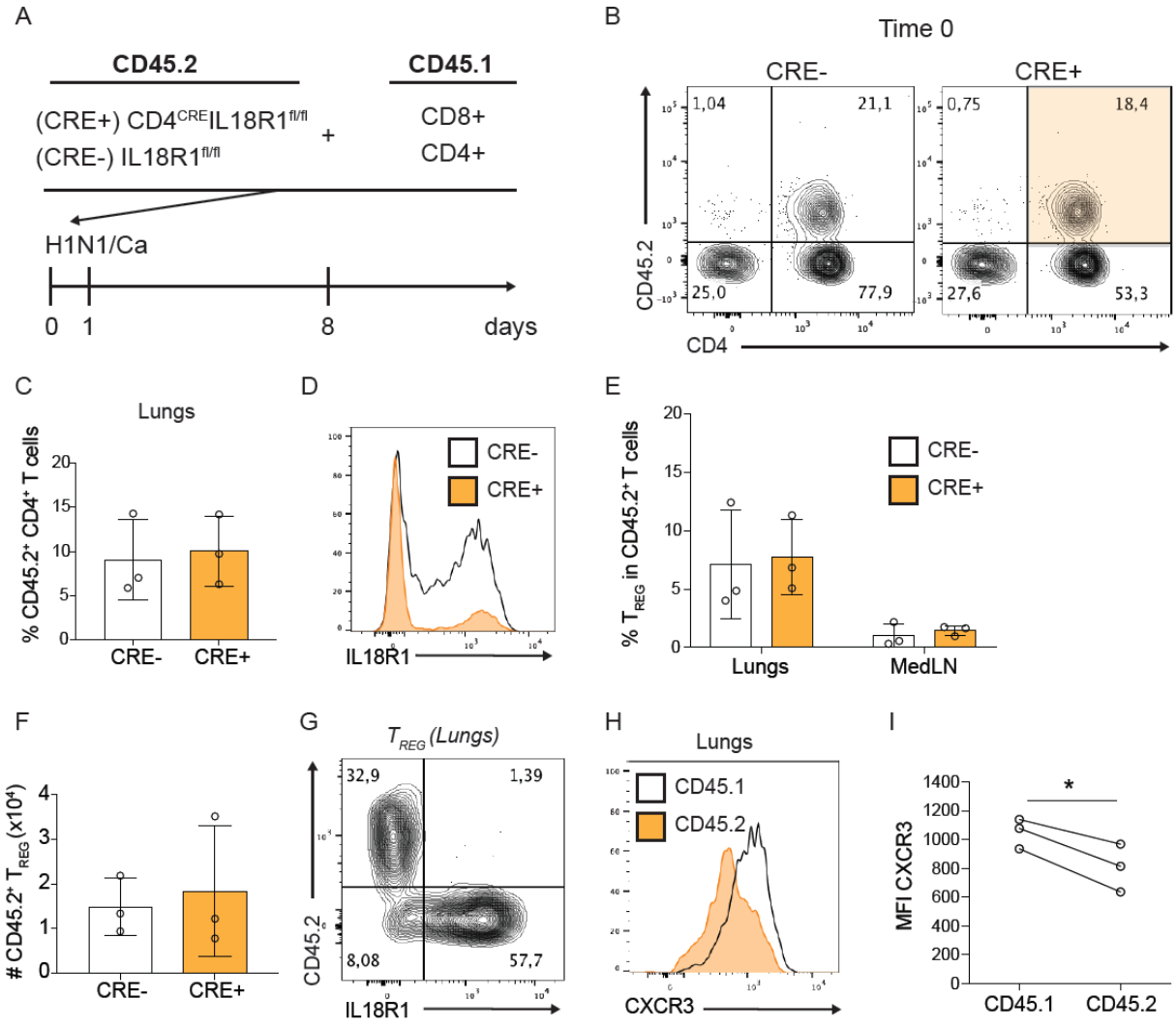
(E) Frequency of Foxp3+ T_{REG} among $CD45.2^+$ $CD4^+$ T cells in the lungs and MedLN.

(F) Number of $CD45.2^+$ T_{REG} cells in the lungs at day 7 post-infection.

(G) Expression of IL-18R1 and CD45.2 among all T_{REG} cells in the lungs.

(H) Expression of CXCR3 among lung infiltrating $Foxp3^+$ T_{REG} cells.

(I) Mean Fluorescence intensity (MFI) of the expression of CXCR3 in the lungs between CD45.2 (CRE+) and CD45.1 T_{REG} cells. Student T test. Paired analysis. *$p<0.05$.


A
CD45.2
CD45.1
(CRE+) CD4CREIL18R1fl/fl
(CRE-) IL18R1fl/fl
+
CD8+
CD4+
H1N1/Ca
0
1
8
days
B
Time 0
CRE-
CRE+
1,04
21,1
25,0
77,9
0,75
18,4
27,6
53,3
CD45.2
CD4
C
Lungs
% CD45.2+ CD4+ T cells
CRE-
CRE+
D
CRE-
CRE+
IL18R1
E
% TREG in CD45.2+ T cells
CRE-
CRE+
Lungs
MedLN
F
CD45.2+ TREG (x10⁴)
CRE-
CRE+
G
TREG (Lungs)
32,9
1,39
8,08
57,7
CD45.2
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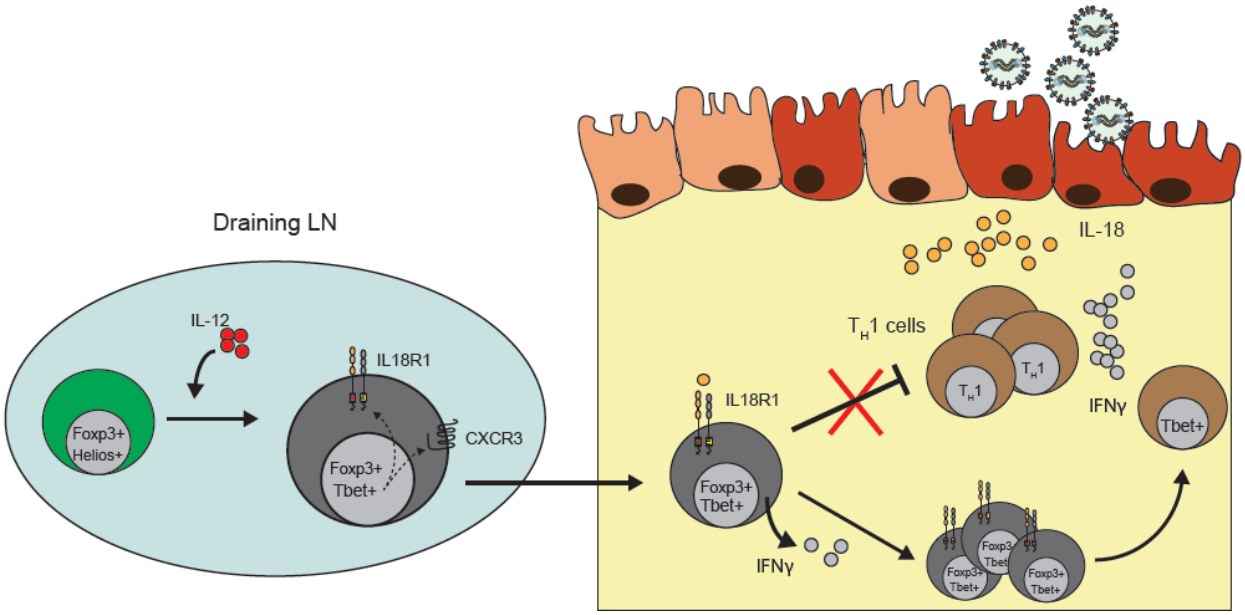
7. Graphical abstract of the role of IL-18 on T_{REG} cell adaptation

In this model, Tbet^{+}Foxp3^{+} T_{REG} cells are generated in the draining lymphoid organ upon IL-12 signaling, at which point they express CXCR3 and IL-18R1 and migrate to the inflammatory site, illustrated here by an Influenza A infection. There, they encounter IL-18 that temporarily impedes their suppression of T_H1 responses, all-the-while favoring IFNγ production and proliferation. While IL-18 does favors Foxp3 expression in these cells, the cells that ultimately lose Foxp3 expression become T_H1-like T cells and may contribute to the effector response.



Supplementary Figures

Figure S1. IL-18R1^{+} Helios^{+} but not Helios^{-} T_{REG} cells express CXCR3 prior to their localization to the lungs during an Influenza A infection.

Foxp3GFP^{ki} mice were infected with ½ LD50 of H1N1/Ca and the mediastinal lymph node (MedLN), spleen and perfused lungs were collected at days 6 post-infection. Representative of 3 distinct experiments.

(A) IL-18R1 expression correlate with Helios expression in T_{REG} cells in the mediastinal lymph node, but not in the lung. Representative flow cytometry plots of the expression of Helios and IL-18R1 on CD4^{+}GFP^{+}(Foxp3) T cells.

(B) Relative Mean fluorescence intensity of CXCR3 (relative to FMO) in T_{REG} cells based on the expression of Helios. One-way ANOVA. Tukey correction. ***p<0.001; **p<0.01

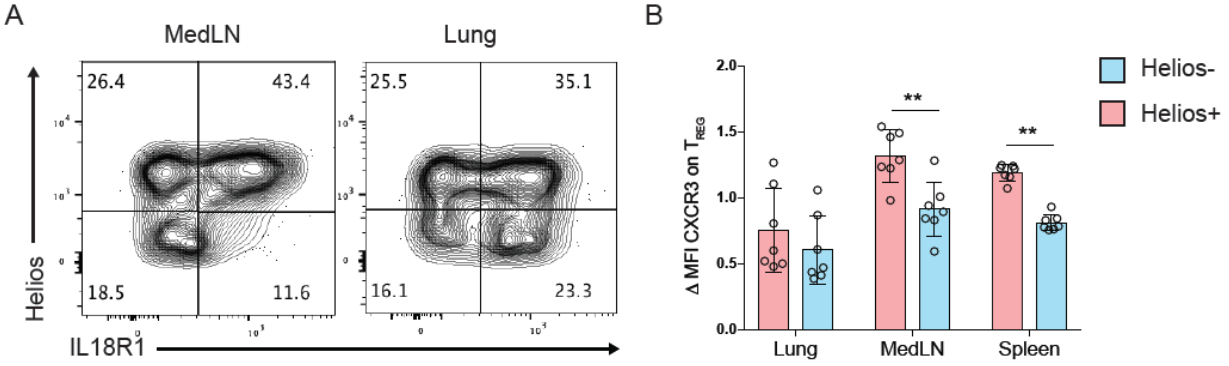


Figure S2. T_H1, but not T_H17, T cells accumulate in the draining popliteal lymph node in the course of a *L. major* infection.

C57Bl/6 mice were infected with $5x10^6$ promastigotes of L. major in the right footpad. The draining popliteal lymph node was collected from non-infected mice (week 0) and at weeks 1, 5 and 10 following infection. The T cell were isolated and activated with PMA, ionomycin and monensin (Golgi-stop®) for 3 hours.

(A) IFNγ production by live $CD4^+$ $Foxp3^-$ T cells. One-way ANOVA. Tukey correction. ***p<0.001; **p<0.01

(B) IL-17A production by live $CD4^+$ $Foxp3^-$ T cells. One-way ANOVA. Tukey correction

(C) Representative flow cytometry of Ki67 and Foxp3 among $CD4^+$ T cells in the draining and non-draining popliteal lymph nodes at week 5 post-infection.

(D) Representative flow cytometry of $Ki67^+$ and $Ki67^-$ T_{REG} cells in their expression of Helios and IL-18R1 at week 5 post-infection.

(E) Representative flow cytometry of IFNγ-producing T_{REG} cells based on Helios expression at week 5 post-infection in the draining LN.

(F) IFNγ+ $Helios^+$ $Foxp3^+$ T cells represent a significant frequency of the total T_{REG} pool in the draining LN at week 5 post-infection. Student T test. ***p<0.001.

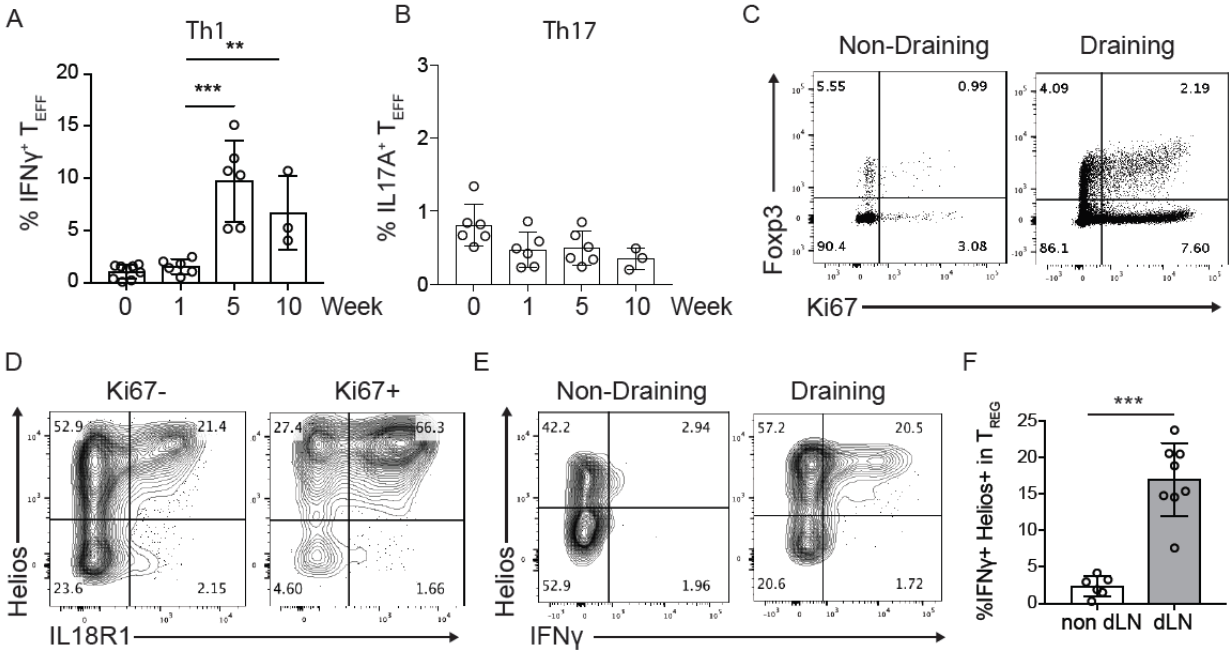


Figure S3. IL-18 does not potentiate iT_{REG} cell generation *in vitro*

$CD4^+$ GFP^- T cells were FACs sorted from the spleen of $Foxp3^{GFPki}$ male mice and activated in plated αCD3 and αCD28 in the presence of 5ng/ml TGFβ and 50U/ml IL-2 for 72 hours. Representative of 4 distinct experiments.

(A) *De novo* expression of Foxp3 is insufficient to promote the expression of IL18R1. Representative flow cytometry.

(B) Frequency of GFP^+ (Foxp3) T cells generated in vitro after 72 hours. One-way ANOVA. Tukey correction.

(C) IL-$18R1^+CD4^+$ GFP^- T cells resist Foxp3 expression in the presence of IL-18.

(D) Frequency of GFP^+ T cells generated *in vitro* after 72 hours between IL-$18R1^-$ (white) and IL-$18R1^+$ (red) effector T cells. ANOVA. Tukey correction. ***p<0.001

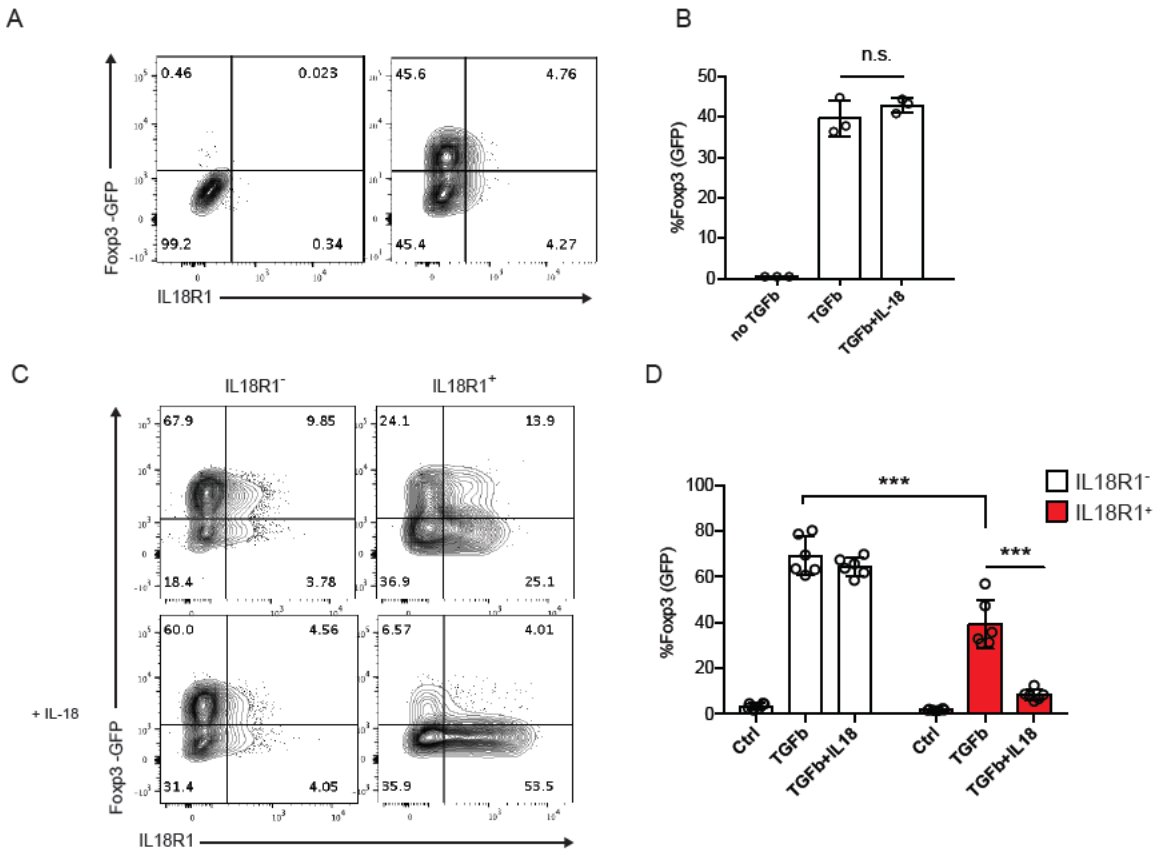


Figure S4. Absence of type I and II IFN receptors does not hinder the accumulation of IL18R1^{+} T_{REG} cells in the spleen at the steady state.

The spleen from 8-week-old male B6. IFNAR$^{-/-}$IFNGR$^{-/-}$ were collected and stained for flow cytometry. Each dot represents a single mouse.

(A) Representative flow cytometry of Foxp3 and IL-18R1 expression in the spleen.

(B) Frequency of IL-18R1^{+} T_{REG} cells in the spleen. Student T test.

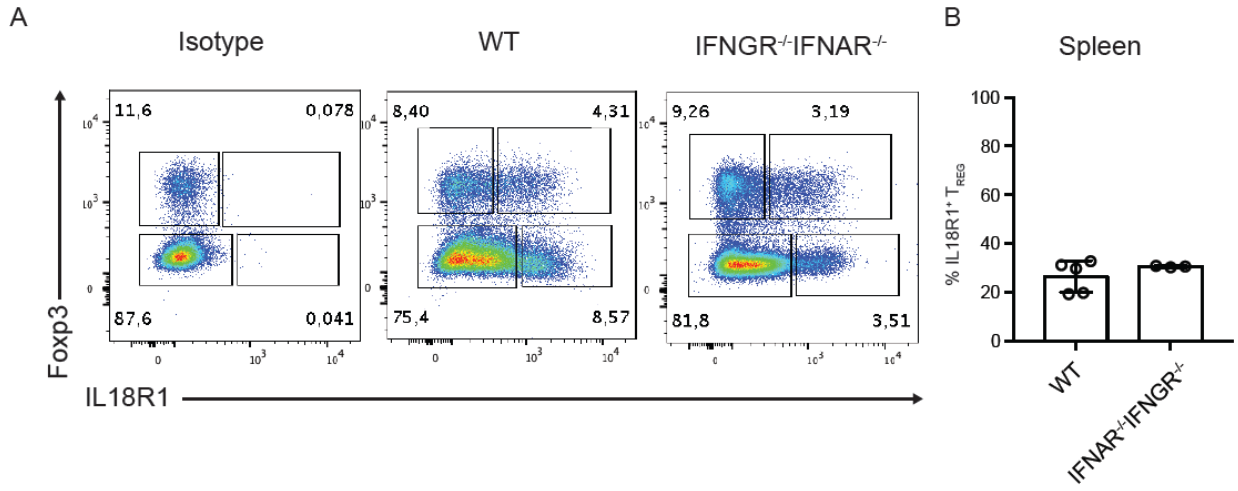


Figure S5. The ability of T_{REG} cells to express IL-18R1 or RORγT is linked to the expression of Helios.

The spleens and lymph nodes of 4-week-old Foxp3^{iRFP} Helios^{iGFP} were collected and CD4$^+$Foxp3$^+$ Helios$^-$ and Helios$^+$ T cells were FACs sorted prior to *in vitro* culture. Helios$^+$ and Helios$^-$ T_{REG} cells were co-cultured in the presence of CD4$^+$ GFP$^-$ T_{EFF} cells from Foxp3^{GFPki} Ly5.1$^+$ congenic mice at a ratio of 1:4 and activated in the presence of mitomycin-treated APCs (CD4$^-$ fraction from Ly5.1$^+$ mice) and soluble αCD3 (1μg/ml) for 72 hours.

(A) Helios$^+$, but not Helios$^-$, T_{REG} cells upregulate the IL-18R1 receptor in the presence of IL-12.

(B) RORγT is preferentially expressed by Helios$^-$ T_{REG} cells in TGFβ, IL-6 and IL1β *in vitro*.

(C) Mean Fluorescence Intensity (MFI) of IL-18R1 on the surface of Helios$^+$ (grey) and Helios$^-$ (white) in distinct polarizing conditions.

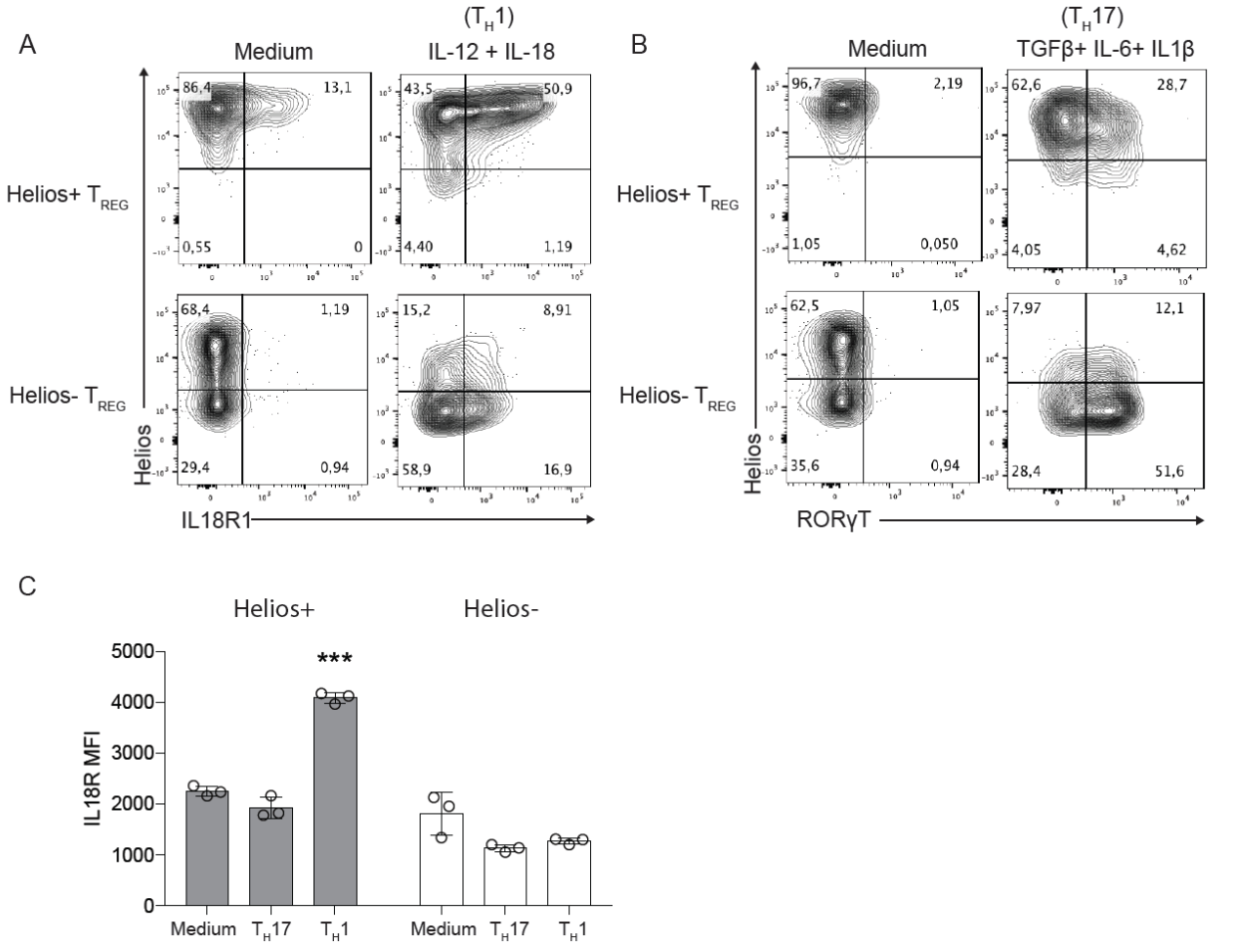


Figure S6. T_{REG} cells, but not T_{EFF}, resist the modulation of the receptor by TGFβ signaling in the presence of IL-18

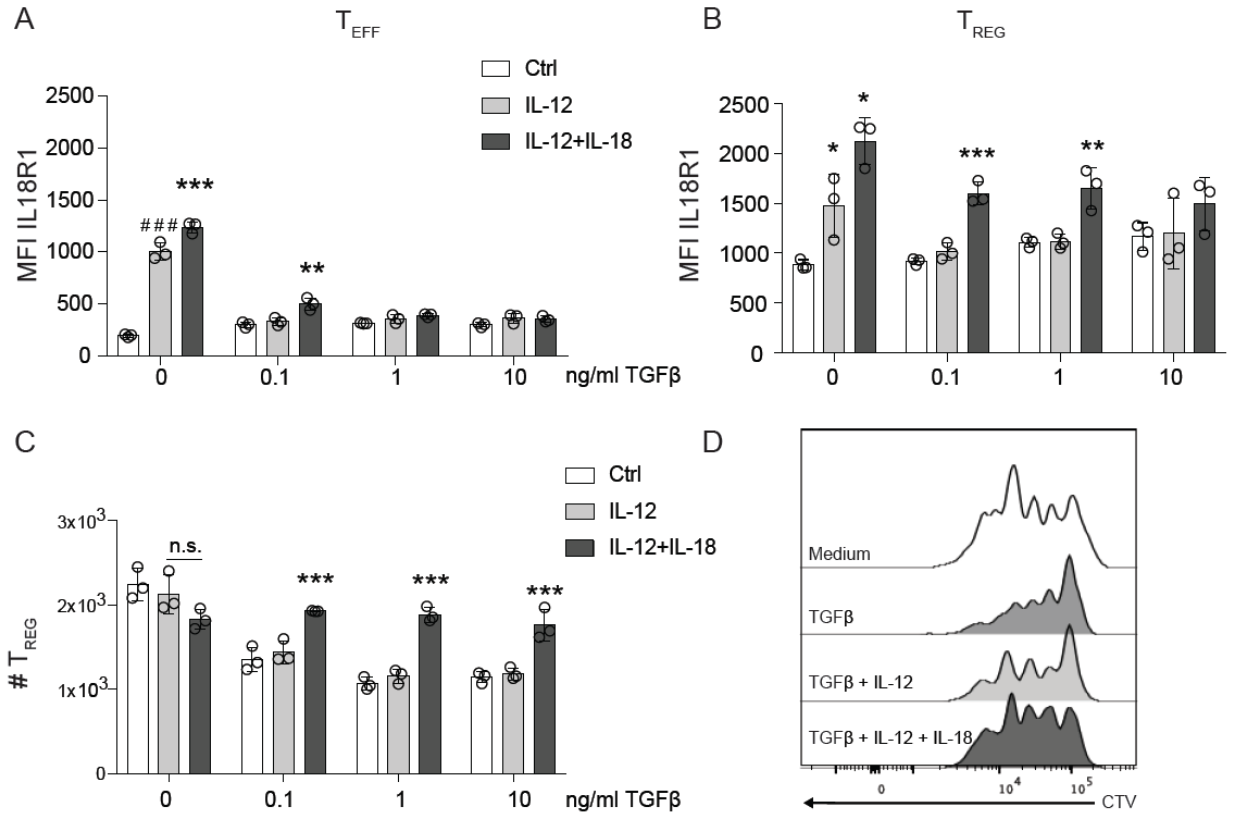
CTV-labelled $CD4^+$ GFP^+ from $Foxp3^{GFPki}$ mice and $CD4^+$ GFP^- from $Foxp3^{GFPki}$ $Ly5.1^+$ mice were FACs sorted and co-cultured at a ratio of 1 T_{REG}: 4 T_{EFF} cells in the presence of irradiated APCs ($CD4^-$ fraction) and soluble αCD3 for 72 hours. No exogeneous source of IL-2 was added to the culture. IL-12 (10ng/ml), IL-18 (10ng/ml) and 0, 0.1, 1 and 10 ng/ml of mTGFβ were added as indicated.

(A) Mean Fluorescence intensity (MFI) of IL-18R1 on $CD4^+$ $GFP^-Ly5.1+$ (T_{EFF}) cells in co-culture. Two-way ANOVA. Tukey correction. ***p<0.001; **p<0.01; *p<0.05

(B) Mean Fluorescence intensity (MFI) of IL-18R1 on $CD4^+$ GFP^+ (T_{REG}) cells in co-culture. Two-way ANOVA. Tukey correction. ***p<0.001; **p<0.01; *p<0.05

(C) Cell counts of T_{REG} cells after culture. Two-way ANOVA. Tukey correction. ***p<0.001; **p<0.01; *p<0.05

(D) Representative histogram of cell trace violet (CTV) on $CD4^+$ GFP^+ (T_{REG}) cells in the distinct conditions.



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CHAPTER 5 - General Discussion and Conclusions

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The role of Foxp3$^+$ regulatory T cells in peripheral and mucosal tolerance has been the focus of extensive research in the last two decades. This T cell subset possesses a unique self and non-self TCR repertoire and a multitude of suppressive functions, making them indispensable in the maintenance of mucosal homeostasis. Because of this, suppressive T_{REG} cells were considered to play a generally detrimental role in the establishment of a robust effective immune response during infections [reviewed in (361, 362)], while their role was thought to be limited to the control of prolonged immune and pathogen-induced pathology to favor a return to immune homeostasis (362). However, T_{REG} cells migrate and expand in the mucosa early during infection without blocking the expansion of the effector response, suggesting that local factors influenced their suppressive function (191). The mechanisms that govern the fate of T_{REG} cells during these early responses remained to be understood. While investigating these processes, T_{REG} cells were found to acquire additional transcriptional programs, that lead them to express new chemokines and receptors, produce pro-inflammatory cytokines and even lose Foxp3 expression (215, 218, 274, 363), in a process labelled “functional adaptation” of tissue-localized T_{REG} cells. As such, T_{REG} cells can develop strategies to modify their transcriptional program and adapt to the ongoing response.

In **Chapter 2**, we set out to identify signals that drive T_{REG} cells to lose Foxp3 expression and acquire a pro-inflammatory phenotype. This work was initiated through the prior demonstration, in our laboratory, that tT_{REG} cells can lose Foxp3 expression and become pro-inflammatory T_H1-like or T_H17-like T cells as they undergo homeostatic expansion into lymphopenic mice (218). By performing a genome-wide transcriptomic analysis on sorted stable Foxp3$^+$ and *ex*Foxp3$^+$ T cells, we identified mRNA transcripts that differed between T_{REG} cells that maintained Foxp3 (stable T_{REG}; sT_{REG}) and cells that lost Foxp3 (exT_{REG}). As we were looking for evidence of inflammatory signals that interfered with Foxp3 expression, our attention was drawn to receptors of the IL-1 family, specifically IL-33R(ST2), IL-18R1 and IL-1R1, that were differentially expressed between

Foxp3 expressing T_{REG} cells and *ex*T_{REG} cells, suggesting that these danger-associated alarmins dictated the differentiation and fate of tT_{REG} cells.

Here, we describe how tT_{REG} cells gain the ability to respond to IL-1, IL-18 and IL-33 alarmins in the course of infection, and how these alarmins dictate T_{REG} cell proliferation and function. We reveal that the ability to recognize local alarmins is closely associated to polarizing signals that favor the expression of T_H-associated master transcription factors (**Appendix Figure A1**). In **Chapter 2**, we describe that IL-33 facilitates GATA3$^+$ T_{REG} cells accumulation and suppressive function at mucosal sites, while IL-1β drives the pro-inflammatory conversion of T_{REG} cells to RORγT$^+$ *ex*T_{REG} cells. In **Chapter 3**, we demonstrate that the effect of IL-33 on T_{REG} cell function is context-dependent, since, in the presence of high amounts of IL-4, ST2$^+$ T_{REG} cells lose their suppressive ability and become pro-inflammatory. Finally, in **Chapter 4**, we describe how IL-18R1$^+$ T_{REG} cells express Tbet and IFNγ during IAV and *L. major* infections and lose their ability to control T_H1 cell expansion. Collectively, we demonstrate how IL-1 alarmins dictate the adaptation of T_{REG} cells in the mucosa and orchestrate the suppressive function of T_{REG} cells in the course of infectious disease.

5.1 IL-1β and IL-33 balance the pro- and anti-inflammatory functions of T_{REG} cells

In **Chapter 2**, we investigated the roles of IL-33 and IL-1 signaling in T_{REG} cells, with the hypothesis, based on our transcriptomic data, that IL-33, but not IL-1, favored a transcriptionally and functionally stable T_{REG} cell during inflammation. We confirmed, through various T_H1 and T_H17-driven inflammatory diseases, that these alarmins balance the suppressive and non-suppressive adaptation of T_{REG} cells. Moreover, we witnessed how the pool of mucosal T_{REG} cells changed in the course of disease and how these changes reflected the anti- and pro- inflammatory effect of IL-1 alarmins in the course of the infection (364). In particular, we confirmed that, while IL-1β promotes inflammation (365), IL-33 is a generally anti-inflammatory signal (351). It was particularly interesting to observe that the lack of IL-1R1 expression by T_{REG} cells facilitates the differentiation and expansion of ST2$^+$ T_{REG} cells, while a lack of ST2 signaling enhanced the differentiation

and expansion of pro-inflammatory RORγT$^+$ T_{REG} cells, revealing that these signals compete to direct the differentiation and expansion of T_{REG} cells (**Figure 6**). Moreover, *in vitro* work in **Chapter 2** suggested that, rather than favoring a subset of T_{REG} cells among a pool of T_{REG} cells, IL-1β and IL-33 competed to define the transcriptional adaptation in the same T_{REG} cell. However, further investigation using single cell cloning will be required to confirm these observations. Nonetheless, - if confirmed - this would be the first description that IL-1 alarmins contribute directly to the epigenetic adaptation of mucosal T_{REG} cells.

Importantly, while the antagonistic effects of IL-1β and IL-33 on the adaptation of T_{REG} cells may play a critical role in orchestrating immune homeostasis, we observed that their activity can also co-opted by pathogens to control the adaptation of T_{REG} cells. This is the case in a chronic disease caused by *C. neoformans*, where the pathogen directly skews the immune response to its benefit (366). In **Chapter 2**, we observed that during the first week of infection with *C. neoformans*, ST2$^+$ GATA3$^+$ T_{REG} cells accumulated preferentially in the lungs, while RORγT$^+$ T_{REG} cells slowly took over on the second and third week, during which time an effective T_H1/T_H17 immune response took place (366). This occurs because *C. neoformans* is able to co-opt the early immune response to favor T_H2 responses through, notably, enhanced IL-33 signaling (355) in order to allow for the establishment of the infection. However, in time, these mice are able to slowly establish an effective T_H1/T_H17 immune response and clear the fungal disease through IL-1 signaling (**Chapter 2)** (356). As such, this tug-of-war between the pathogen and the mucosal tissue provides a unique perspective into the timing and effect of IL-33 and IL-1β signaling in the lungs through the functional adaptation of T_{REG} cells. In particular, this model illustrates the dynamics of locally adapted T_{REG} cells populations and reveals why they respond to IL-1 by losing Foxp3 and tempering their suppressive capacity during disease. Indeed, in conditions that require an effective immune response to take place, IL-1 responding T_{REG} cells are beneficial, while IL-33 responding T_{REG} cells may negate an effective immune response and lead to pathogen evasion and death.

Collectively, these results suggest that the balance between IL-33 and IL-1β signaling in the mucosa plays a critical role in dictating the function of T_{REG} cells during disease. Moreover, preliminary data reveals that IL-18, similar to IL-33, can block the

generation of IL-1R1^{+} RORγT^{+} T_{REG} cells *in vitro* (*data not shown*), suggesting that the antagonistic nature of IL-1 alarmins extents to other members of this family and encouraging further investigation into this effect.

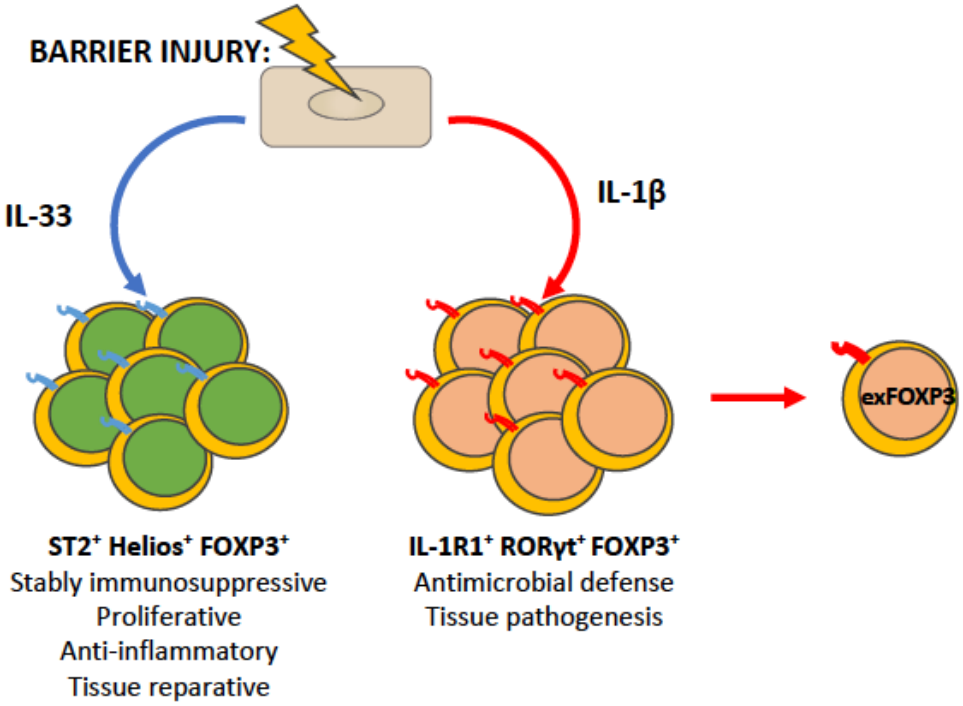


Figure 6. IL-33 and IL-1β balance the pro- and anti-inflammatory adaptation of T_{REG} cells. IL-33 drives the differentiation of GATA3^{+} T_{REG} cells while IL-1β facilitates the accumulation of RORγT^{+} T_{REG} and the immune evasion of T_H17 cells. The competing effect of IL-33 and IL-1β dictates the balance between highly stable, functionally suppressive GATA3^{+} T_{REG} and unstable, less suppressive, RORγT^{+} T_{REG} cells in the mucosa during infectious disease. Made using Adobe Illustrator® software.

5.2 The context-dependent effect of IL-33 on the function of ST2$^+$ T_{REG} cells

While low concentrations of IL-33 promote immune homeostasis, high concentrations of IL-33 drives a pro-fibrotic T_H2-driven inflammatory response (367). While our previous models of infection focused on T_H1 and T_H17-driven inflammation, it remained to be understood how IL-33 orchestrates the adaptation of T_{REG} cells to a T_H2-dominant disease. In this environment, a prior report suggested that IL-33 was responsible for the functional dysregulation of T_{REG} cells (350), suggesting that IL-33 could potentiate the pro-inflammatory conversion of T_{REG} cells. In **Chapter 3**, we assessed how a respiratory syncytial virus (RSV) infection orchestrates the IL-33 response of pulmonary T_{REG} cells. RSV is an endemic, RNA virus, that possesses the particularity of causing a significant, long-term, shift in the type of immune response in the lungs of infants, favoring type 2 over type 1 immune responses. Importantly, RSV infections are characterized by increased levels of pulmonary IL-33 that drives a dysregulated type 2 response (368). In **Chapter 3**, we hypothesized that a neonatal RSV infection generates a micro-environment that impairs the suppressive function of the locally adapted ST2$^+$ T_{REG} cells.

Through our investigation, we show that ST2$^+$GATA3$^+$ T_{REG} cells responded to IL-33 by upregulating IL4Rα, a key component of the IL-4 receptor complex. This, in turn, rendered them susceptible to STAT6 signaling and dampened their suppression of T cells. Interestingly, this effect did not require an active infection with RSV and depended solely on the presence of high levels of pulmonary IL-33, suggesting that a T_H2 memory response generated in infancy contributes to the long-term dysregulation of T_{REG} cells (**Figure 7**). These findings confirm the role of IL4Rα signaling in dampening the suppressive function of T_{REG} cells (233) and illustrates how IL-33 is a major contributor to this effect. Further investigation using tamoxifen-inducible T_{REG}-specific knock-down of ST2 (Foxp3-ERT2-CRE, ST2-floxed) will be important to determine the timing of this effect in the lungs of re-infected adult mice. Nonetheless, these experiments provide a novel rational for the development of therapies that inhibit STAT6 (369) and IL-33 signaling (353).

Collectively, these results highlight the importance of the immunological context where alarmin signaling occurs. Indeed, while IL-33 was considered to be a signal that favored the suppressive function in T_{REG} cells during T_H1 and T_H17 driven disease (**Chapter 2**), it is not the case during T_H2-driven diseases (**Chapter 3**). Thus, we can hypothesize that alarmins, like IL-1 or IL-18, may be favorable to T_{REG} cell function in T_H2-driven diseases. Indeed, murine work using a modified RSV virus (RSV-IL18) revealed that increased concentrations of IL-18 enhance T_H1 anti-viral immune responses that favor viral clearance (370) and reduce the skewed T_H2 response (371). In light of what we observed in **Chapter 4**, further investigation may reveal that local IL-18 production promotes the differentiation and expansion of T_H1-like T_{REG} cells that, in turn, enable the expansion of T_H1 cells and/or suppress T_H2 cells. Understanding the competing effects of IL-1 alarmins on the adaptation of T_{REG} cells to T_H2-driven immune responses may provide the grounds for novel therapies towards the control of oral allergy, asthma and atopic dermatitis.

5.3 Elucidating the plasticity of Foxp3$^+$ T_{REG} cells

Although T_{REG} cell can completely downregulate Foxp3 expression in harsh inflammatory conditions or in instances of lymphopenia, there is currently no evidence that they possess the ability to temporarily lose Foxp3 expression in order to relieve their suppressive program and allow immune responses to unfold (216). This capacity to switch lineage identity and effector functions is a concept referred to as “plasticity” and has been highly debated in the community for years, mostly because of the lack of proper tools to address the question.

It is now clear that Foxp3$^+$ T_{REG} cells can acquire sufficient epigenetic changes that drive them to completely lose Foxp3 expression and adopt an effector-like phenotype (215, 218). On this subject, it remained to be understood if T_{REG} cells from all origin, namely thymic (tT_{REG}) and peripherally induced T_{REG} cells(pT_{REG}), possess the potential to lose Foxp3. Indeed, up until recently, it was thought that the *ex*Foxp3$^+$ T cells were likely to originate from pT_{REG} cells that possess a highly methylated T_{REG}-specific methylated region (TSDR) and increased alloantigen specificity, rendering their

expression of Foxp3 promiscuous (372, 373). Yet, these experiments were based on *in vitro* generated T_{REG} cells (374) or by sorting out tT_{REG} cells form a pool of splenic T_{REG} cells based on proposed but unconfirmed markers of tT_{REG} cells like Neuropilin 1 (372). In **Chapter 2** and **Chapter 4**, we provide new evidence that tT_{REG} cells can effectively express new transcription factors and can, under alarmin signaling, lose Foxp3 expression. However, while we observed a loss of Foxp3 expression in T_{REG} cells upon adoptive transfer into lymphopenic mice, the presence of T_{REG} cells that lose Foxp3 expression in our infection models remain to be confirmed. Novel Foxp3 fate-mapping mice models have been used with success to reveal that *ex*Foxp3 are generated during parasite infections (233), suggesting these genetically engineered mice will be useful to confirm how IL-1 alarmins influence T_{REG} cell fate in our infection models.

Developing the Foxp3 fate-mapping mouse. In **Chapter 2** and **Chapter 4**, we refer to a Foxp3 fate-mapping mouse (215, 374) that allows for the unequivocal identification of *ex*Foxp3 T cells. In these mice, a bacterial chromosome (BAC) has been inserted that contains the Cre (tyrosine) recombinase (CRE) and a GFP protein under the Foxp3 promoter (374). Since this CRE targets a stop codon inserted in an RFP located in the non-coding locus Rosa26 (Rosa26-$RFP^{stop\text{-}loxP}$), it enables the tracking of T cells that have expressed Foxp3 at least once, as they will constitutively express RFP even after they stop producing Foxp3. While it is tempting to use these mice to confirm Foxp3 loss by mucosal T_{REG} cells, these mice display 10-15% *ex*Foxp3 (RFP^+) circulating T cells at the steady-state and, thus, cannot be used to specifically identify infection-induced *ex*T_{REG} cells (215). A variant of this model, using a tamoxifen-inducible CRE (ERT2-CRE), rather than a constitutive CRE, is currently being bred in our facility and promises to provide a better platform to confirm Foxp3 loss in disease since this model allows for the timely control of the expression of RFP in Foxp3-GFP^+ T_{REG} cells by tamoxifen injection (375). Crosses of Foxp3 fate-mapping mice with ST2-floxed, IL1R1-floxed or IL18R1-floxed mice (example: B6.$Foxp3^{CRE\text{-}ERT2\text{-}GFP}$ Rosa26-$RFP^{stop\text{-}loxP}$ x $ST2^{loxP}$) are currently underway and will be essential for the study of the specific role of alarmins on the development of *ex*Foxp3 cells in both infectious and non-infectious models.

Induction of *ex*Foxp3 to Foxp3$^+$ T_{REG} cells. Attempts to study if *ex*Foxp3 can re-gain Foxp3 expression has been made using the fate-mapping mouse that constitutively expresses CRE under the Foxp3 promoter. Interestingly, neither *in vitro* TCR activation of "*ex*Foxp3" nor transfer of these cells into lymphopenic mice (NOD TCRα$^{-/-}$) allowed for the upregulation of Foxp3 (215), suggesting T_{REG} cells that lose Foxp3 expression cannot gain it back. However, these studies are limited as they do not consider conditions that favor Foxp3 upregulation. While we illustrated in **Chapter 2** the importance of IL-33 signaling on the maintenance of Foxp3 expression by mucosal T_{REG} cells, mechanisms that induce Foxp3 expression from Foxp3$^-$ CD4$^+$ T cells include favorable metabolic pathways such as fatty acid oxidation (FAO), polarizing signals like TGFβ, retinoic acid (RA) and IL-2 and the presence of tolerogenic antigen-presenting cells (APCs) that provide Foxp3-inducible contact signals(113, 376, 377). As described in **Chapter 1** (section 2.3), these signals lead to molecular pathways that promote Foxp3 expression through, among others, SMADs (TGFβ), STAT5 (IL-2), IDO and c-Rel (NF-κB) signaling. As such, the *in vivo* demonstration of the conversion of *ex*Foxp3 to Foxp3 requires the development of more elaborate murine models.

Proposed models for the study of T_{REG} plasticity. To assess the *de novo* expression of Foxp3 in *ex*Foxp3 T cells, we propose to develop TCR-specific fate-tracking T_{REG} cells. First, by using a specific high affinity self-reactive TCR on T_{REG} cells (378) we remove the possibility of allo-antigenic recognition to favor tolerogenic interactions in the mucosa (379). As such, by sorting out self-specific *ex*Foxp3 and transferring them back into healthy mice, we assess if *ex*Foxp3 T cells re-gain Foxp3 expression as they interact with tolerogenic APCs that provide the required signals, notably TGFβ, Retinoic Acid and IDO (376) and are exposed to low concentrations of IL-33 at the mucosa(351). Second, to explore the effect of disease resolution on *ex*Foxp3 T cells, we propose to develop a murine model where all fate-tracking T_{REG} cells possess a non-self-affinity TCR, for example the influenza specific MHC-II-restricted TCR-HA mice (380). Upon transfer of TCR-HA specific *ex*Foxp3 (CD4$^+$GFP$^-$RFP$^+$) T cells into influenza-infected mice, we assess if there is *de novo* conversion of *ex*Foxp3 to Foxp3$^+$ cells in the lungs upon disease

resolution and, importantly, by crossing them to ST2-floxed, IL1R1-floxed or IL-18R1-floxed mice, we can confirm the role of these alarmins in the plasticity of T_{REG} cells.

Finally, we cannot discount the possibility that *ex*Foxp3 T cells cannot regain Foxp3. Indeed, preliminary experiments in our laboratory have revealed that even high concentrations of TGFβ and IL-2 fail to provide sufficient signal for the upregulation of Foxp3 in *ex*Foxp3 T cells *in vitro* (*data not shown*). This important concept, if proven, would suggest a linear fate of T_{REG} cells and put further importance into understanding the role of alarmins in dictating the epigenetic fate of tissue localized T_{REG} cells. Indeed, a better understanding of the development of pathogenic *ex*T_{REG} cells under alarmin signaling may reveal how modulating external signals can balance between a quick resolution of inflammation or a dysregulated immune response that leads to increased morbidity and even death.

5.4 The role of Helios in the adaptation of thymic T_{REG} cells to inflammation

T_{REG} cell immunologists are actively trying to understand the role of Helios, a transcription factor expressed almost exclusively in a subset of circulating Foxp3$^+$ T_{REG} cells (129). While pT_{REG} cells do not have the ability to express Helios (129), we and others have shown that splenic Helios$^-$ T_{REG} cells can gain Helios expression [**Chapter 4**, (130)], suggesting that splenic Helios$^-$ T_{REG} cells are a unique subset of tT_{REG} cells rather than pT_{REG} cells. Throughout this work, we observed that Helios$^+$ and Helios$^-$ T_{REG} cells do not possess the same ability to polarize in response to alarmins. Contrary to **Chapter 2**, where we observed a clear distinction between Helios$^+$ ST2$^+$ and Helios$^-$ IL1R1$^+$ T_{REG} cells, IL18R1$^+$ T_{REG} cells were largely a heterogeneous group comprised of both Helios$^+$ and Helios$^-$ T_{REG} cells at mucosal surfaces. Interestingly, in **Chapter 4,** we show that Helios expression confer T_{REG} cells the ability to gain T-bet, CXCR3 and IL18R1, revealing a novel pathway by which specialized T_{REG} cells adapt to the mucosa based on Helios expression. Moreover, we observed that Helios$^-$ T_{REG} cells upregulated RORγT and IL18R1 in the course of infection (*data not shown*) via a mechanism akin to IL18R1 expression by T_H17 cells (340), suggesting that Helios does not directly promote the expression of IL18R1. Finally, by using the dual reporter Foxp3^{RFP}/Helios^{GFP} mice in

Chapter 4, we confirm *in vitro* that while Helios$^-$ T_{REG} preferentially upregulate RORγT, Helios$^+$ T_{REG} can gain GATA3 or T-bet expression. These experiments provide further confirmation that Helios plays a role in determining the specialized adaptation of T_{REG} cells in tissues. Indeed, a prior report revealed Foxp3-specific deletion of Helios does not to impair T_{REG} cell accumulation but rather induce late-life mucosal autoimmunity (133, 381), suggesting that rather than impairing their suppressive functions, the lack of Helios hinders the ability of tT_{REG} cells to adapt to the tissue.

Since we found that the expression of Helios is intrinsically linked to the *in vitro* polarization of T_{REG} cells, Helios may play a direct role in suppressing or favoring the molecular pathways of polarizing cytokines. Through Helios-targeted chromatin immunoprecipitation, Helios was shown to promote important genes involved in the transcriptional program of T_{REG} cells, notably *STAT5b*, *Bcl2* and *IL2Rα* (130). Importantly, the IL-2 signaling pathway has been shown to promote the differentiation of T_H1 and T_H2 cells (reviewed in (382)), but to antagonise T_H17 cell induction (383). As such, Helios may favor the IL18R1$^+$ Tbet$^+$ and ST2$^+$ GATA3$^+$ polarization of T_{REG} cells but antagonise the IL1R1$^+$ RORγT$^+$ differentiation of T_{REG} cells.

Although further investigation is required to confirm these hypotheses, our results provide new evidence that Helios$^+$ and Helios$^-$ tT_{REG} cells are unique in their ability to adapt their transcriptional program and respond to IL-1 alarmins.

5.5 Therapeutic potential of modulating alarmin signaling in T_{REG} cells

It is now well established that Foxp3$^+$ T_{REG} cells are critical in preventing autoimmunity and ameliorating disease upon cellular transfer (21), suggesting therapies targeting T_{REG} cell function to be a viable avenue in the fields of autoimmunity and transplantation (384). Importantly, our discoveries have implications beyond the scope of infectious disease. Indeed, alarmin-responding T_{REG} cells have now been identified in models of cancer (385), diabetes (386) and other autoimmune disorders [reviewed in(295)], suggesting that our findings impact global mechanisms of T_{REG} cell mediated regulation in non-lymphoid tissues. As such, understanding the outcome of T_{REG} cells to alarmins may pave the way towards novel therapies.

Autoimmunity, injury and transplantation. During autoimmunity, injury or transplantation, a dysregulated inflammatory response leads to enhanced immunopathology. As such, alarmin targeted therapies to enhance the suppressive function of T_{REG} cells has become an attractive avenue to prevent further damage. In particular, the development of a chimeric IL-2/IL-33 protein (IL-2-33) in the treatment of kidney injury has been proposed in order to amplify GATA3$^+$ T_{REG} cell expansion *in situ* and control the effector immune response (387). Moreover, adoptive transfer of ST2$^+$ T_{REG} cells was proposed to facilitate muscle repair after injury, suggesting that non-infectious injury can benefit from T_{REG}/IL-33 targeted therapy (388). In turn, these results conveyed the possibility of using this therapy in organ transplantation (389), where T_{REG} cell prevent organ rejection (390). Indeed, adoptive transfer of ST2$^+$ T_{REG} cells were found to favor cardiac allograft survival (389) and prevent graft-versus-host disease in mice (391). From these results, a method of *in vitro* expansion of T_{REG} cells with IL-33 was developed to generate highly purified autologous suppressive T_{REG} cells and validate the use of ST2$^+$ T_{REG} cell based therapy in men (229).

On the other hand, targeting IL-1 signaling on T cells has not yet proved to be so successful. Although the use of an IL-1 receptor antagonist, a drug called anakinra, Kineret™ (*Swedish Orphan Biovitrum*, Stockholm, Sweden), is currently approved for the treatment of rheumatoid arthritis (392), a condition characterized by the presence of IL-17-producing T cells (393), it provides only moderate benefits (394). In **Chapter 2,** we have shown that anakinra efficiently rescues T_{REG} cells from adopting a RORγT$^+$ T_{REG} cell phenotype, suggesting that the drug can target T_{REG} cell *in vivo*. Future examination of the effect of an IL1R1 antagonist on T_{REG} cell *in vivo* may provide a novel rationale for the use of anakinra in transplantation and auto-immune diseases. Importantly, the use of anakinra may potentiate the effect of therapeutic IL-33 administration in the accumulation of suppressive T_{REG} cells.

Finally, IL-18 is involved in the exacerbation of many autoimmune diseases, including type 1 diabetes, multiple sclerosis, rheumatoid arthritis, psoriasis, systemic lupus erythematosus and atopic dermatitis (reviewed in (395)), suggesting that IL-18 may impair mechanisms of immune regulation. Surprisingly, the role of IL-18 on T_{REG} cells has

yet to be thoroughly investigated in these diseases. Although there is currently no commercially available IL-18 antagonist, some molecules have recently been proposed (396) that could enable the study and development of T_{REG}-targeted therapies.

Cancer. Exploiting strategies to block the suppressive function of T_{REG} cells in cancer have been proposed as a viable therapeutic avenue (397, 398). There is currently no immunotherapy targeting IL-1, IL-33 or IL-18 alarmins in order to enhance the anti-tumor response. This is likely due to the complexity of the role of these cytokines in both promoting and inhibiting tumor growth, depending on the type of tumor (399). There is evidence, however, that IL-33 signaling by tumor-infiltrating T_{REG} cells contributes to their immunosuppressive effect (348, 400), suggesting that targeting IL-33 may favor an anti-tumor response and potentiate the effect of immune checkpoint inhibitors. On the other hand, intra-tumor IL-18 production by genetically engineered T cells was shown to favor anti-tumor responses (401), suggesting that competing IL-33 and IL-18 signals may directly target the suppressive function of tumor infiltrating T_{REG} cells. Collectively, while the role of IL-1 alarmins in tumor infiltrating T_{REG} cells remain to be understood, targeting their signal on T_{REG} cells could lead to important novel therapies that may further potentiate the effect immune checkpoint inhibitors aimed at enhancing the anti-tumor adaptive response.

Applications in vaccinology. Although it remains to be confirmed, targeting T_{REG} cells function was proposed to improve vaccine immunogenicity as they are through to be largely detrimental to the development of a protective humoral and cellular memory response (402). Although there is currently no direct evidence that targeting alarmin signalling in T_{REG} cells may provide adjuvant properties, our work suggests that it may be a viable target for future vaccine development.

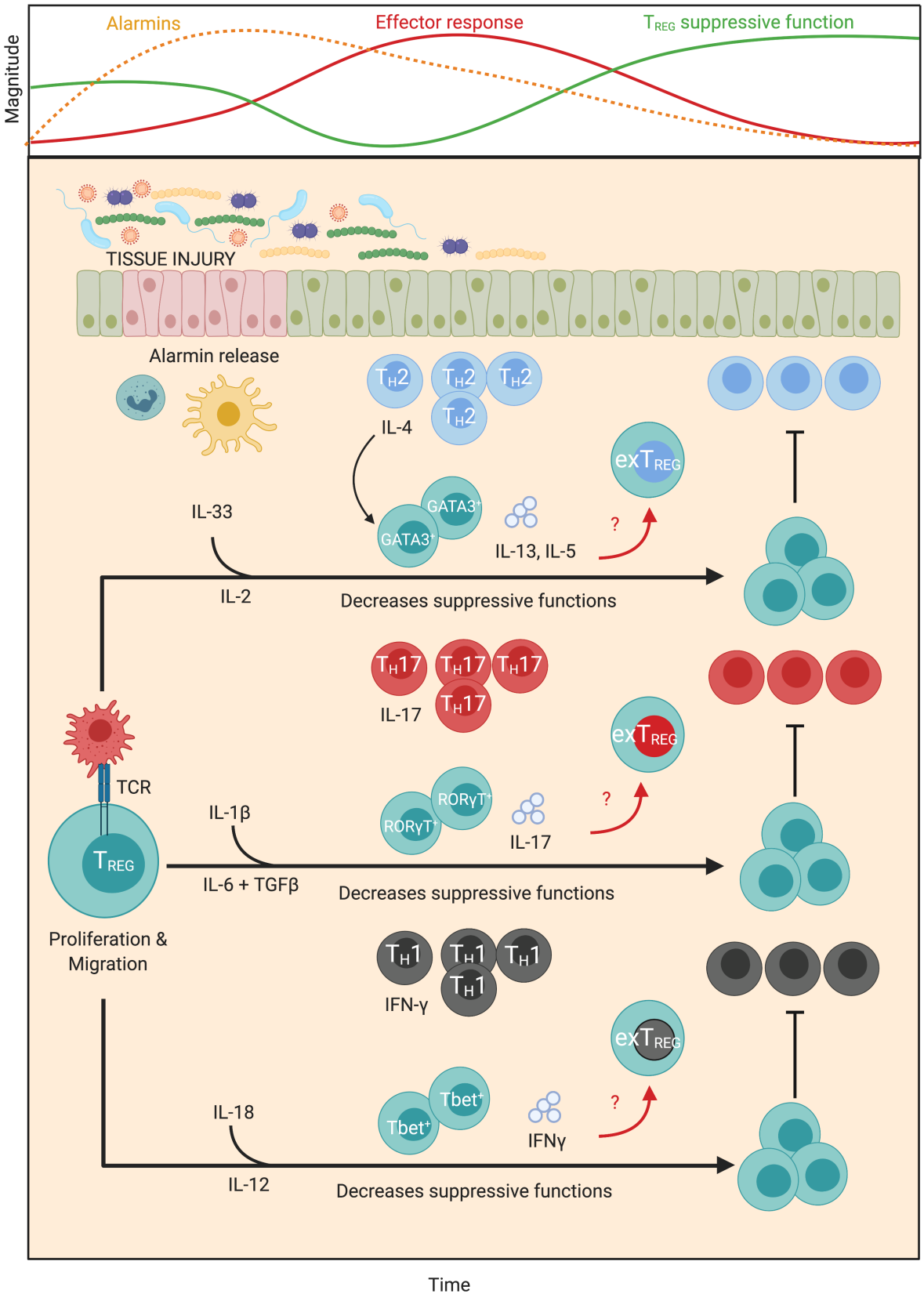
Applications in the treatment of infectious disease. While regulatory elements of our immune system can prevent acute immunopathology, infectious diseases can still induce, in some individuals, an inflammatory spiral that leads to a cytokine storm and, ultimately, death. To prevent this cytokine storm, favoring the suppressive function of T_{REG} cells has

been proposed as a viable solution (403) and alarmin-targeted therapy could provide a novel therapeutic avenue (404). As such, targeting IL-1 alarmin signaling on T_{REG} cells to enhance their suppressive functions while dampening their pro-inflammatory conversion may represent the future for the development of intensive care therapy.

5.6 Concluding remarks

In this work, we demonstrate that danger signals are a major evolutionary mechanism by which T_{REG} cell adapt their suppressive functions in order to favor an adequate immune response. Although the signals provided by IL-1 alarmins can be co-opted by pathogens in order to favor their replication, it remains highly efficient, as it is directly conditioned by the level of cellular damage (405). Although it remains to be understood if IL-1, IL-18 and IL-33 responsive T_{REG} cells regain their suppressive functions upon resolution of the disease, our results reveals that IL-1R1$^+$ and IL-18R1$^+$ T_{REG} cells are highly suppressive *in vitro* in the absence of IL-1 or IL-18, respectively, suggesting that removal of their respective alarmin may bring back their suppressive functions. Indeed, in order to appreciate the roles of IL-1 alarmins on T_{REG} cell homeostasis, it is important to consider when they are released and activated. This is dictated the complex interaction that occurs between the pathogen and the host and fluctuates in time. We propose the following model for the temporal effect of IL-1, IL-18 and IL-33 on the suppressive functions of tissue adapted T_{REG} cells in the course of infections (**Figure 7**). We are now starting to appreciate the high level of flexibility of T_{REG} cells and their ability to “sense” multiple biological and physical signals (406). Importantly, the way alarmins regulate T_{REG} cell function and fate are but an example of the various strategies developed by these cells to interact with a hostile inflammatory environment where adaptation is key.

Figure 7. Proposed model for the temporal effects of IL-1 alarmins on T_{REG} cells in the course of inflammation. Upon infection, cumulative cellular damage and innate cell activation drives the extracellular concentration of IL-33, IL-1β and IL-18. In turn, these alarmins accelerates the generation of a T_H2, T_H17 and Th1 responses, respectively, while promoting the survival and proliferation of specialized T_{REG} cell subsets that can even become pro-inflammatory exT_{REG} cells to contribute to the effector response. Upon resolution of the danger, the concentration of alarmin decreases. The proliferating T_{REG} cells can then rapidly suppress the effector response and engage the contraction of the adaptive immune response. Made using ©Biorender Software (biorender.com).


Magnitude
Alarmins
Effector response
TREG suppressive function
TISSUE INJURY
Alarmin release
TH2
IL-4
GATA3+
IL-33
IL-13, IL-5
IL-2
exTREG
?
Decreases suppressive functions
TH17
IL-17
RORγT+
TCR
TREG
IL-1β
IL-6 + TGFβ
Proliferation & Migration
TH1
IFN-γ
Tbet+
IL-18
IL-12
IFNγ
Time

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APPENDIX

Figure A1. Graphical abstract: Description of the signals that lead Foxp3$^+$ T_{REG} cells to respond to IL-1 alarmins. T_{REG} cells undergo a phenotypic adaptation through the action of polarizing cytokines of the JAK/STAT pathway. Under IL-2 and IL-33, T_{REG} cells acquire GATA3 and IL4Rα expression, while TGFβ and IL-6 drive the expression of RORγT and IL1R1 and IL-12 to promote the expression of IL-18R1. In turn, IL-2 and IL-33 promote the proliferation and function of T_{REG} cells, while IL-4 potentiates their conversion to T_H2-like cells and impairs their suppressive functions (*red*). Similarly, IL-1β, in the presence of IL-6 and TGFβ, drives the expansion and conversion of T_H17-like cells, while IL-18, in the presence of IL-12, potentiates the conversion to T_H1-like cells, all-the-while impairing their suppressive functions. Made using Adobe Illustrator® software.

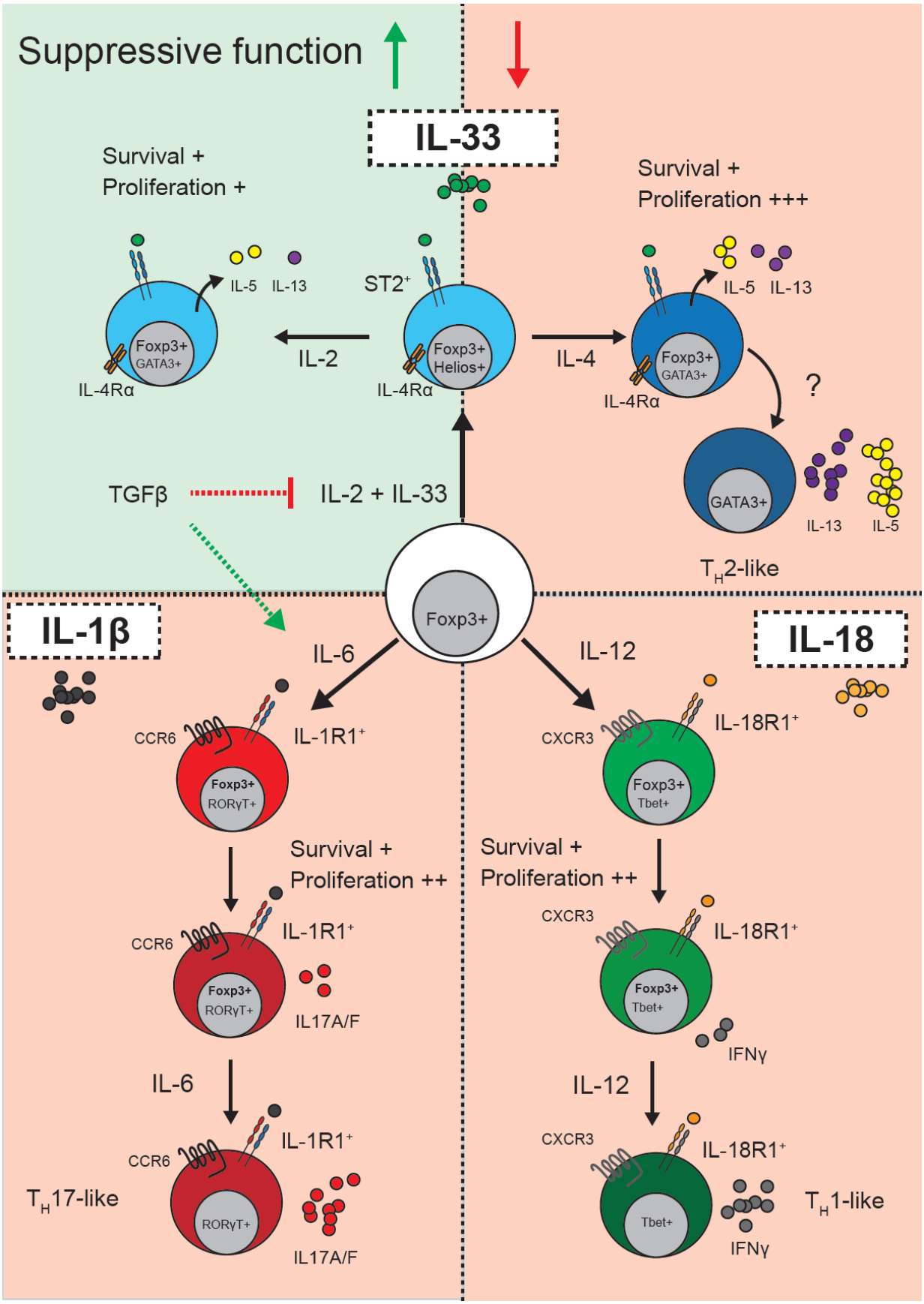
Suppressive function
IL-33
Survival +
Proliferation +
Survival +
Proliferation +++
IL-5
IL-13
ST2+
Foxp3+
GATA3+
IL-4Rα
IL-2
Foxp3+
Helios+
IL-4Rα
IL-4
IL-5
IL-13
Foxp3+
GATA3+
IL-4Rα
?
GATA3+
IL-13
IL-5
TGFβ
IL-2 + IL-33
TH2-like
Foxp3+
IL-1β
IL-6
IL-12
IL-18
CCR6
IL-1R1+
Foxp3+
RORγT+
CXCR3
IL-18R1+
Foxp3+
Tbet+
Survival +
Proliferation ++
Survival +
Proliferation ++
CCR6
IL-1R1+
Foxp3+
RORγT+
IL17A/F
CXCR3
IL-18R1+
Foxp3+
Tbet+
IFNγ
IL-6
IL-12
CCR6
IL-1R1+
RORγT+
IL17A/F
CXCR3
IL-18R1+
Tbet+
IFNγ
TH17-like
TH1-like

Table A1. List of abbreviations

Ag	antigen
AICD	activation-induced cell death
ANOVA	analysis of variance
AP1	adaptor protein 1
APC	antigen-presenting cells
APECED	autoimmune polyendocrinopathy, candidiasis and ectodermal dystrophy
ATP	adenosine tri-phosphate
cAMP	cyclic adenosine monophosphate
CFSE	carboxyfluorescein succinimidyl ester
ChIP	chromatin immunoprecipitation
CNS	conserved non-coding sequences
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CTV	cell trace violet
DC	dendritic cell
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalitis
Ebi3	Epstein-Barr virus induced gene 3
FACS	fluorescence assisted cell sorting
FasL	Fas ligand
FBS	fetal bovine serum
FOXP3	forkhead winged helix protein 3
GFP	green fluorescent protein

GITR	tumor necrosis factor receptor superfamily, member 18
GVHD	graft versus host disease
IAV	Influenza A virus
IBD	inflammatory bowel disease
IDO	Indoleamine 2, 3-dioxygenase
IFN	interferon
IKZF2	Ikaros zing-finger family 2
ILC	innate-like lymphoid cell
IL-1R1	interleukin 1 receptor 1
IL-1R2	interleukin 1 receptor 2
IL-1RA	interleukin 1 antagonist
IL-1RAcP	interleukin 1 receptor accessory protein
IL-2Rα	interleukin 2 receptor alpha
IL-6R	interleukin 6 receptor
IL-7R	interleukin 7 receptor
IL-10R	interleukin 10 receptor
IL-18R1	interleukin 18 receptor 1
IL-18RAcP	interleukin 18 accessory protein
IL-18RBP	interleukin 18 binding protein
IL-23R	interleukin 23 receptor
IPEX	Immuno-deficiency, Polyendocrinopathy, enteropathy, X-linked
IRES	internal ribosome entry sequence
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
iT_{REG}	in vitro induced regulatory T cells
JAK	Janus Kinase
LAG	lymphocyte-activation gene
LAP	latency-associated peptide

LCMV	Lymphocytic Choriomeningitis Virus
Log logarithm	logarithm
LZ	leucine zipper domain
mAb	monoclonal antibody
MACS	magnetic bead assisted cell sorting
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MHCII	type II major histocompatibility complex
mRNA	messenger RNA
MS	multiple sclerosis
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
NFAT	nuclear factor of activation in T cells
NFkB	nuclear factor kappa B
NK	natural killer
PAM	pulmonary alveolar macrophages
PBMC	peripheral blood mononuclear cells
PD1	programmed cell death 1
Pi3K	phosphatidylinositol 3-kinases
PMA	phorbol 12-myristate 13-acetate
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
RA	retinoic acid
RA	rheumatoid arthritis
RAR	retinoic acid receptor
RSV	Respiratory Syncytial Virus
RSV A2	Respiratory syncytial Virus strain A2
rhIL-2	recombinant human IL-2

ROR	RAR-related orphan receptor
RUNX	runt-related transcription factor
SCID	severe combined immune deficiency
SD	standard deviation
SLE	systemic lupus erythematosus
SMAD	small body size and mothers against decapentaplegic homolog
SNP	single nucleotide polymorphism
STAT	signal-transducer and activator of transcription protein
T1D	type-1-diabetes
Tbet	T-cell-specific T-box transcription factor
TCR	T-cell receptor
Tconv	conventional (non-Foxp3) T cells
T_{EFF}	effector T cells
TGFβ	transforming growth factor beta
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
Tr1	type-1 regulatory T cells
T_{REG}	regulatory T cells
TSDR	Treg-specific demethylated region
WT	wild type
ZF	zing finger