IL-2-Dependent Regulation of Immune Homeostasis and Response to Infection

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Abstract

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Interleukin-2 (IL-2) is a critical regulator of immune homeostasis through its impact on both regulatory T (Treg) and effector T (Teff) cells. However, the precise role of IL-2 in the homeostatic maintenance and function of Treg cells in the adult peripheral immune system remains unclear. Here, we report that neutralization of IL-2 in mice abrogated all IL-2 receptor signaling in Treg cells but was well tolerated and only gradually impacted Treg cell function and immune homeostasis. Additionally, despite substantially reduced IL-2 sensitivity, Treg cells maintained selective IL-2 signaling and prevented immune dysregulation following treatment with the inhibitory anti-CD25 antibody PC61. Reduction of Treg cells with a depleting version of the same CD25 antibody permitted $CD8^+$ Teff proliferation before progressing to more widespread immune dysregulation. Thus, despite severely curtailed CD25 expression and function, Treg cells retain selective access to IL-2 that supports their anti-inflammatory functions *in vivo*. Antibody-mediated targeting of CD25 is being actively pursued for treatment of autoimmune disease and preventing allograft rejection, and our findings help inform therapeutic manipulation and design for optimal patient outcomes.

Treg cells play an essential role in return to homeostasis and maintenance of immunologic tolerance after response to infection, and the role of IL-2 in this context is unknown. In contrast to the minimal impact of short term IL-2 neutralization at homeostasis, antibody blockade of IL-2 during acute VSV infection results in reduced frequencies of Treg cells and increased frequencies of $CD4^+$ Teff cells at day 7 post infection. However, Teff cell responses to viral peptide stimulation were similar regardless of the level of IL-2 signaling. Mice harboring $CD4^+$ T cells that produce impaired levels of IL-2 displayed a similarly dysregulated balance of Treg and Teff cells after VSV infection. Our data suggest that viral infection under suboptimal IL-2 conditions may allow expansion of bystander $CD4^+$ T cells that could be a trigger of an autoinflammatory or autoimmune state.

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Dedication

For my parents

Chapter 1: Introduction

Immune tolerance

The immune system performs an essential role in protecting the host from invading pathogenic microorganisms. In order to accomplish this, the organism must be able to distinguish harmful antigens from those that are self-derived, commensal microorganisms or innocuous environmental antigens. The two major components of immunity, the innate and adaptive immune systems, work together to eliminate threats and build immunological memory while maintaining tolerance. The innate immune system acts quickly, using evolutionarily conserved molecular patterns on infectious agents to identify and respond with effector functions. However, if a pathogen is able to evade the innate response, the adaptive immune system can act with different mechanisms that will work to clear the threat and contribute to immune memory for future protection. In the adaptive immune system, lymphocytes, such as T cells, possess surface receptors which are generated by somatic gene rearrangement and are able to recognize a staggering breadth of antigenic diversity. T cell receptor (TCR) responses are highly selective and powerful. Therefore, many mechanisms exist to ensure these responses are targeted to harmful antigens, as responses toward self or other innocuous antigens can result in tissue damage, allergy or autoimmunity.

Self-tolerance is key to preventing aberrant immune responses. One aspect of this is central tolerance, a process that occurs during T cell development in the thymus. Newly formed T cells are tested for their ability to recognize peptide bound to major histocompatibility complex (MHC) molecules with appropriate affinity (positive selection) but without strong reactivity to self-antigens (negative selection) [1]. T cells that react too strongly are eliminated through clonal deletion. However, some overlap in specificity exists between self-peptides and those of harmful

antigens, so elimination of all weakly self-reactive T cells would diminish immune responses to pathogenic microorganisms. Additionally, not all self-antigens are thought to be expressed in the thymus, and commensal and food antigens are absent in the thymus. Thus, some autoreactive T cells inevitably 'escape' from the thymus and into circulation. Peripheral tolerance is important for controlling these autoreactive T cells and includes mechanisms such as elimination by apoptosis and functional hypo responsiveness (anergy) when self-antigen is recognized in the absence of costimulatory and cytokine signals. Finally, development of self-reactive regulatory T cells (Treg) in the thymus that are specialized to function in immune suppression are another tool the immune system has to maintain immune tolerance. A healthy immune system therefore requires a delicate and dynamic balance of these inflammatory and suppressive T cells in order to fend off outside threats, while simultaneously protecting the host's own tissues.

Initiation of T cell responses

Once their maturation in the thymus is complete, single positive (SP) CD4 and CD8 αβ T cells exit into the periphery in a naïve state. These cells circulate the body via blood and lymph sampling peptides presented by antigen presenting cells (APCs), in search of a peptide that matches their TCR specificity. Primary activation of a T cell typically occurs in secondary lymphoid organs, such as a lymph node or the spleen and consists of three signal components. Signal 1 is provided by engagement of the TCR with peptide presented by MHC molecules on APCs. Costimulation through receptors on T cells by corresponding ligands on APCs creates Signal 2, which is critical for cell survival and proliferation. Signal 3 guides the differentiation of the activated T cell through cytokines that are specialized for different immune functions.

Dendritic cells (DCs) are specialized APCs that capture, process, and present antigen to T

cells. DCs can be broadly categorized into two groups, plasmacytoid DCs and conventional DCs (cDCs), the latter of which is important in this dissertation. cDCs populate both non-lymphoid and lymphoid tissues, and within lymphoid tissues there are three main resident populations: $CD11b^{-}33D1^{-}CD8^{+}$ (referred to as cDC1), $CD11b^{+}33D1^{+}$ (cDC2), and $CD11b^{+}33D1^{-}$. Migratory DCs, which originate in peripheral tissues and traffic to lymph nodes (LN) loaded with antigen, also include the cDC1 and cDC2 subsets. These can be distinguished from resident DCs by higher expression of MHCII [2]. cDC1s are critical for activating naïve $CD8^{+}$ T cells, have increased cross-presentation potential, and are important for Th1 polarization, highlighting their importance in defense against intracellular bacteria and viruses [2]. Interestingly, this DC subset also seems to be important for deletional tolerance of self-reactive T cells and induction of peripheral Treg cells. cDC2s are thought to have a predominant role in MHC-II antigen presentation, and in particular for initiating Th17 responses and inducing T follicular helper cells that regulate the germinal center response [3]. However, these cells can also activate $CD8^{+}$ T cells, delete autoreactive T cells and induce Treg cell differentiation. The $CD11b^{+}33D1^{-}$ subset, which are highly inflammatory and monocyte-derived [4], is formed in response to inflammation.

DCs play an important role in determining the fate of naïve T cells, but antigen-experienced T cells can reciprocally affect DC responses [5]. For example, T cell 'licensing' refers to the requirement of DCs to receive a signal through CD40 from CD40L on $CD4^{+}$ Teff in order to generate cytotoxic $CD8^{+}$ T cells [6, 7]. On the regulatory side, Treg cells inhibit DC activation as a way of enforcing immune tolerance [8]. DCs can subsequently promote Treg cell homeostasis by presenting self-antigens and providing co-stimulatory molecules important for their maintenance, which connects DCs and Treg cells in a homeostatic loop [9, 10]. Importantly,

functional capacity for each subset is not limited to the actions listed, and significant redundancy occurs between subsets. The diverse functionality of these DC subsets clearly indicates their importance in both initiating inflammatory responses and enforcing tolerance.

Conventional T cells

Naïve $CD8^+$ T cells can differentiate into potent effector T cells (Teff) upon activation. $CD8^+$ Teff can efficiently kill pathogen-bearing target cells using pore-forming toxins such as perforin and granzymes and produce the inflammatory cytokines interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) as well [11]. These effector cells then proceed down distinct maturation paths with differing memory potential. Cells expressing high levels of KLRG1 and low levels of CD127 are short lived and called terminal effector cells (TECs), and $KLRG1^{lo}CD127^{hi}$ $CD8^+$ T cells are memory precursor effector cells (MPECs) which live much longer [12]. These MPECs further differentiate into multiple $CD8^+$ T cell memory subsets, including central memory (T_{CM}), effector memory (T_{EM}), and tissue resident memory (T_{RM}) [13]. CD8+ T_{CM} and T_{EM} are found mostly in circulation or in secondary lymphoid organs (SLOs), while T_{RM} are found in non-lymphoid tissues.

$CD4^+$ Teff cells, often referred to as helper T cells, guide many other cells in the immune system to provide diverse defenses against a wide variety of pathogens. Different subsets of $CD4^+$ Teff are formed in response to various cytokine cues, as well as the strength of TCR signal. In turn, these $CD4^+$ Teff subsets produce their own cytokines, propagating the immune response towards the cells and functions best suited to clear the specific threat. T helper 1 (Th1) cells are critical in response to intracellular bacteria and viruses by activating macrophages and $CD8^+$ T cell responses [14]. Th2 cells help regulate defense against extracellular parasites by

activating eosinophils and B cells [15]. Through production of IL-4 and IL-21, T follicular helper cells (Tfh) aid B cells in the germinal center [16]. Th17 cells are important in orchestrating responses against fungi and some extracellular bacteria [17]. If improperly regulated, all of these subsets can direct their effector functions toward self or environmental antigens, and cause damage to the host in the form of autoimmunity or allergy.

Regulatory T cells

Treg cells play an indispensable role in controlling autoreactive cells, and thus in maintaining immunological tolerance to self-antigens [18]. Sakaguchi and colleagues first described this population of $CD4^+CD25^+$ cells that could suppress autoreactive cells and prevent autoimmunity in thymectomized mice [19]. Subsequent discovery of Foxp3 [20-22], the master transcription factor of Treg cells that is required for their development, maintenance and function, led to rapid advancement in the understanding of this population. Both mice [23, 24] and humans [25] that lack Foxp3 develop severe widespread autoimmunity, demonstrating the profound impact of these suppressive cells. Most Treg cells are derived from the thymus, having recognized presented self-antigen. Treg cells can also develop in the periphery from conventional $CD4^+$ T cells in the appropriate cytokine environment (TGF-β) [26, 27]. Treg cells employ a variety of mechanisms to suppress immune responses, including production of anti-inflammatory cytokines, acting as a cytokine sink for vital survival signals effector cells need, and blunting DC and Teff activation through inhibitory receptors [8]. Since their initial discovery, Treg cells have been shown to have critical roles in modulating immune responses to infection, cancer, and transplantation, in addition to autoimmunity.

Interleukin-2 and interleukin-2 receptor

The role of interleukin-2 (IL-2) in immune responses has been extensively studied. After its discovery as a potent growth factor for T cells in culture [28, 29], subsequent studies showed that activation of T cells through TCR engagement and costimulation through molecules like CD28 led to T cell production of IL-2 and expression of the IL-2 receptor (IL-2R) components. This signaling pathway promoted clonal expansion and effector differentiation, suggesting that IL-2 was central to T cell-dependent immune responses. Surprisingly, inactivation of genes for IL-2, IL-2Rα (CD25), or IL-2Rβ (CD122) in mice led to severe lymphoproliferative and autoimmune phenotypes, instead of the predicted immunodeficiency [30-33]. We now appreciate that lack of IL-2 signaling causes a defect in the Treg cell population, and this is the cause of the autoimmunity observed in IL-2 and IL-2R deficient mice. This defect is cell-intrinsic, as transfer of purified $CD4^+CD25^+$ cells into IL-2 or IL-2R deficient mice completely rescued the animals from lethal autoimmune disease [34, 35].

IL-2 is produced predominantly by $CD4^+$ T cells, and to a lesser extent $CD8^+$ T cells. Upon engagement of TCR and costimulatory molecules, newly activated T cells rapidly make and secrete IL-2 [36]. Transcriptional induction and silencing of the IL-2 gene is tightly regulated, and this combined with rapid degradation of IL-2 mRNA leads to its short half-life [37-41]. A classic auto-regulatory feedback loop where IL-2 inhibits its own production contributes to its stringent regulation [42, 43].

The IL-2R consists of three subunits, CD25, CD122 and the common gamma chain (γ_c). All three subunits are required for formation of the high-affinity receptor, but since CD122 and γ_c possess the cytoplasmic tails involved in signaling, these two subunits can also function as an intermediate affinity receptor. When CD25 is present, it initially binds to IL-2 and then promotes

association with CD122 and γ_c (49, 50). Jak-3 (γ_c) and Jak-1 (CD122) phosphorylate key residues on CD122, triggering association of Shc and Stat5 or Stat3. Shc provides a platform to activate PI3K and MAPK pathways, while Stats are further phosphorylated to allow dimerization and translocation into the nucleus [36]. All downstream pathways involved by IL-2 signaling play important roles in activated T cells, but Stat5 is the main pathway by which IL-2 signaling contributes to Treg cell gene regulation. IL-2 binding to the receptor triggers rapid internalization, leading to degradation of CD122 and γ_c and recycling of CD25 to the cell surface [44-47].

Expression of IL-2R components differs between the various cell populations that respond to IL-2. The γ_c chain is widely expressed on hematopoietic cells and is shared by receptors for several cytokines [48, 49]. CD122 is shared between receptors for IL-2 and IL-15 and is expressed constitutively on natural killer (NK) cells, NKT cells, and naïve and memory conventional αβ T cells and γδ T cells. Activated Teff cells transiently express CD25 [50], but Treg cells are the only T cell population that constitutively express the high affinity IL-2R [51]. Thus, CD25 is a fairly faithful marker of Foxp3$^+$ Treg cells in the mouse, whereas in adult humans, conventional activated CD25$^+$ T effector cells are much more prevalent.

The role of IL-2 in immune regulation

Immune homeostasis depends in part on a healthy balance between Treg and Teff cells. It is well established that IL-2 signaling is critical for induction of Foxp3 and Treg cell development in the thymus [51-53], and normal thymic Treg cell production is required for maintenance of homeostasis in the periphery. The role of continued IL-2 signaling in peripheral

Treg cell maintenance is less well understood. $CD25^{-/-}$ mice contained near normal frequencies of Treg cells in the thymus and the periphery [51]. In contrast to scurfy mice, which lack all Treg cells due to a mutation in Foxp3, $CD25^{-/-}$ mice survive twice as long as a result of less severe autoimmunity, but clearly have dysfunctional Treg cells. Similarly, mice in which CD122 was only expressed in the thymus, allowing normal thymic development but no peripheral IL-2 signaling, did not succumb to the lethal autoimmunity observed in IL-2R$\beta^{-/-}$ mice [54]. Taken together, these studies showed that although IL-2 is required to maintain Treg cell-mediated immune homeostasis *in vivo*, peripheral maintenance of Treg cells appears to be at least partially IL-2 independent.

More recent work has addressed whether IL-2 signaling supports survival and maintenance, suppressive function, or a combination in Treg cells. Conditional knockout of CD25 or CD122 on Treg cells led to early onset and fatal autoimmunity in mice, establishing a clear role for IL-2 signaling in peripheral Treg function [55]. Induction of a constitutively active form of STAT5 in IL-2R deficient Treg cells was sufficient to restore Treg cell mediated control of immune homeostasis. These data were substantiated by studies using inducible conditional knockout of CD25 on Treg cells [56, 57]. Other work claiming a role for IL-2 signaling in peripheral Treg cell maintenance was done in settings that likely do not resemble normal physiology, such as immune reconstitution following bone marrow transplantatoion [58, 59], adoptive transfer [60], or autoimmunity [61-63]. Manipulation of the IL-2 signaling pathway using monoclonal antibodies has yielded confounding results; this will be discussed in detail in Chapter 3. Finally, different Treg cell subsets have been shown to have varying requirements for IL-2 in the periphery, with long-lived, quiescent central Tregs that populate lymphoid organs relying on IL-2 much more than their effector Treg cell counterparts [64, 65]. All in all, we still

do not have a precise understanding of the role of IL-2 signaling in Treg cells in the periphery, and its impact on immune homeostasis.

Conventional T cells are the primary producers of IL-2 *in vivo* [66, 67]. Treg cells cannot make their own IL-2 due to Foxp3 dependent repression of the *IL2* gene [68, 69], and thus rely on paracrine sources. Recently activated $CD4^+$ T cells in the lymphoid organs produce the highest amount of IL2 mRNA and protein [70]. In the steady state, it is autoreactive $CD4^+$ Teff cells primed with autoantigens that produce the IL-2 necessary to maintain resident Treg cell populations [71]. When an immune response to foreign antigen is mounted, IL-2 production by naïve and memory $CD4^+$ Teff occurs as soon as one hour after activation, and production by $CD8^+$ T cells is similarly rapid [36]. Secretion of IL-2 is directional and focused via an immunological synapse toward IL-2R bearing T cells in close proximity [72]. $CD4^+$ Teff cells that produce IL-2 do not consume it [73], therefore putting non-IL-2 producing $CD4^+$ Teff in competition with Treg cells and $CD8^+$ T cells. Lack of either IL-2 or Treg cells increases the size of the IL-2 producing $CD4^+$ Teff cell pool, whereas administration of exogenous IL-2 boosts Treg cells while reducing IL-2 producing cell numbers and their ability to produce IL-2 [74]. In this way, IL-2 appears to form a self-regulatory circuit by integrating homeostasis of activated $CD4^+$ Teff and Treg cells. In order to restrain $CD8^+$ Teff responses, Treg cells need to consume IL-2 to control consumption and subsequent proliferation and function of $CD8^+$ Teff cells [55]. IL-2 clearly plays a central role in how Treg and Teff cells reciprocally control each other to mount controlled immune responses while maintaining immune homeostasis, but precise understanding of this dynamic is still incomplete.

IL-2 signaling pathway in disease

When the IL-2-dependent balance of Treg and Teff cells is disrupted, autoimmunity and inflammation can occur. In addition to the systemic autoimmune disease observed in the absence of CD25, CD122, or IL-2 in mice [36], single nucleotide polymorphisms (SNPs) in the *IL2* and *IL2RA* genes are associated with multiple autoimmune diseases in both mice and humans [75, 76]. Therefore, manipulating the IL-2 signaling pathway therapeutically for treatment of autoimmune disease is an area of immense interest. Low dose IL-2 therapy, which enriches Treg cells, has shown efficacy in murine autoimmune models [77-82], and has also benefitted patients with graft versus host disease (GVHD) [83], Hepatitis C virus-induced vasculitis [84], alopecia areata [85], and lupus [86]. However, because IL-2 also acts on effector cells, high dose IL-2 can promote inflammatory responses and this is used for treatment of cancer [87]. As such, safety of therapeutic IL-2 remains a concern, and efficacy can vary widely depending on the current disease activity and immune history of the patient. Indeed, in two mouse models of type 1 diabetes, early intervention with IL-2 prevented disease, but initiation of treatment after loss of tolerance (but before overt hyperglycemia) accelerated disease progression [76, 82]. The fact that monoclonal antibodies against CD25 are also used as an immunosuppressive to treat organ transplant rejection [88] and demonstrated efficacy against multiple sclerosis (MS) [89] further highlights the complexity of targeting this signaling pathway.

Outstanding questions

The IL-2 signaling pathway has been an area of active investigation for several decades. This work has established an essential role for IL-2 signaling in thymic development of Treg cells. In the periphery IL-2 acts on a variety of cell types, particularly Treg and Teff cells, and is important in maintaining a healthy balance between these subsets to allow immune responses against threats while preserving immune tolerance. Despite the vast body of work aiming to characterize the precise role of IL-2 signaling in peripheral immune regulation, there is still much to discover.

Differences in IL-2R component expression on these different cells types contributes to the complexity of this signaling axis, as well as the dynamic and tightly regulated gene expression of IL-2. Genetic tools in mice have yielded much information, but often obscure results by potential defects in cell development. Experimental tools that better control for these defects are often not physiologically normal, such as bone marrow chimeras or adoptive transfer models. Therapeutic tools such as blocking antibodies have yielded studies with confounding results. This final tool is especially important to understand as therapeutics are used to treat human autoimmune disease.

In the following dissertation, we address these gaps in knowledge regarding the role of the IL-2 signaling pathway in peripheral immune regulation. In Chapter 3, we comprehensively examine how manipulating the IL-2/CD25 axis by different methods perturbs Treg cell maintenance, phenotype and function in maintaining normal immune homeostasis. In Chapter 4, we explore how changes in IL-2 bioavailability during acute infection disrupt immune regulation.

Chapter 2: Materials and Methods

Mice

C57BL/6 (B6) mice were purchased from The Jackson Laboratory. IL2-83E mice were provided by A. Wells (University of Pennsylvania, Philadelphia, PA). All mice were maintained at Benaroya Research Institute, and experiments were pre-approved by the Office of Animal Care and Use Committee of Benaroya Research Institute. Mice used in experiments were between 6-12 weeks of age at time of sacrifice. Female mice were used unless specified otherwise.

Intravascular labeling

Mice were anesthetized with 4% isoflurane and 3 ug of BUV395-conjugated CD45.2 (104) was injected into mice retro-orbitally (r.o.) in 200 uL PBS 3 min prior to sacrifice. Single cell suspensions were prepared for flow cytometry as described below, and localization of cells was determined by positive (blood-exposed) and negative (tissue-restricted) staining.

Cell isolation

Single cell suspensions from spleen, thymus and lymph nodes were prepared by tissue disruption with frosted glass slides into RPMI with 10% bovine calf serum (RPMI-10) and filtered through 70 μm strainers. For DC isolations, minced whole spleens were digested in basal RPMI supplemented with 2.5 mg/mL Collagenase D (Company) for 20 minutes under agitation at 37°C. Cell suspensions were then passed through 70 μm strainers into RPMI-10. Erythrocytes were lysed in ACK lysis buffer, and the remaining cells were washed in RPMI-10. DCs were

enriched using CD11c-microbeads (Miltenyi) according to the manufacturer's protocol. For lung cell isolation, lungs were finely minced with scissors and digested in basal RPMI with 50 ug/mL Liberase TM (Roche) and 10 U/mL DNase I (Roche) for 45 min under agitation at 37°C. Cell suspensions were then passed through 70 μm strainers into RPMI-10 and mashed using the plunger from a 3 mL syringe. Erythrocytes were lysed in ACK lysis buffer, and the remaining cells were washed in RPMI-10.

Flow cytometry

Cell surface staining for flow cytometry was performed in FACS buffer (PBS-2% BCS) using the following antibody clones: LiveDead, CD4 (GK1.5, RM4-5), CD8 (53-6.7), CD25 (PC61, 7D4), ICOS (C398.4A), CD44 (IM7), CD62L (MEL-14), NK1.1 (PK136), CD122 (5H4), CD132 (TUGm2), CD5 (53-7.3), CD19 (6D5), Gr-1 (RB6-8C5), CD11b (M1/70), CD11c (N418), MHCII (M5/114.15.2), DC marker (33D1), CD80 (16.10A1), CD86 (GL-1), CD40 (3/23), CD45.2 (104), CD73 (TY/11.8), KLRG1 (2F1/KLRG1), and CD127 (A7R34). Cells were incubated in the antibody mixture for 20 min at 4°C and then washed in FACS buffer before collecting events on an LSRII (BD Bioscience). For intracellular staining, surface antigens were stained before fixation and permeabilization with FixPerm buffer (eBioscience). Cells were washed and stained with antibodies to Foxp3 (FJK-16s), Ki67 (11F6), pSTAT5 (47/pStat5[pY694]), IFN-γ (XMG1.2), and CTLA-4 (UC10-4F10-11). Flow cytometry data was analyzed using FlowJo software (TreeStar). Polybead polystyrene nonfluorescent microspheres (15 mm, Polysciences) were used to determine absolute numbers of cells in flow cytometry samples. 100 uL of a fixed concentration of beads (C_B) was mixed with 100 uL of cells to be counted. Samples were acquired on a flow cytometer, with gates drawn on lymphocyte and bead

populations based on their forward and side scatter properties. The ratio of lymphocyte gate events (N_L) to bead gate events (N_B) was determined and used to calculate the concentration (C_L) of the original cell suspension as follows: $C_L = (N_L / N_B) \times C_B$

Phospho-flow staining

To assess pSTAT5 levels directly ex vivo, spleens were immediately disrupted between glass slides into eBioscience FixPerm. Cells were incubated for 20 min at room temperature, washed in FACS buffer, resuspended in 500 μL 90% methanol (MeOH), and incubated on ice for at least 30 minutes. Cells were stained with surface and intracellular antigens, including pSTAT5 (pY694) for 45 minutes at room temperature.

Tetramer staining

The VSV nucleoprotein (N) MHC class I (MHC-I) [H-2K^b/RGYVYQGL] tetramer was kindly provided by K. Klonowski (University of Georgia, Athens, GA). Single cell suspensions were stained with tetramer and CD8a antibody (clone) in FACS buffer for one hour at room temperature in the dark. Samples were then washed with FACS buffer and stained with remaining surface antibodies as described previously.

Restimulation for cytokine production analysis

Splenocytes were isolated from mice as described. To assess total cytokine potential, cells were incubated in 96 well plates at 1x10^7/mL in 200 uL RPMI-10 with PMA (50 ng/mL), ionomycin (1 ug/mL) and monensin (2 um) for 4-6 hours at 37°C. For VSV-specific cytokine production,

cells were incubated in 96 well plates at $1x10^7$/mL in 200 uL RPMI-10 with MHCII-restricted VSV-g peptide 416-433 [SKAQVFEHPHIQDAASQL] (GenScript) at 10 ug/mL for 2 hours before adding monensin (2 um) for an additional 4 hours at 37°C. Cells were then washed and stained as described above.

Fc.IL-2 proteins

Fc.IL-2 proteins (Fc.WT or Fc.Mut24) were generated and characterized as previously described [90].

In vitro assays

For *in vitro* CD25 blockade, splenocytes were isolated from untreated B6 mice as described. $5x10^5$ cells were plated per well into a 96-well round bottom plate. Commercially available PC61 (BioXcell) was added to designated wells at 1µg/mL final concentration, and samples were incubated at 37°C for 30 min and then washed. Meanwhile, 1000 U/mL recombinant IL-2 (eBioscience) was incubated with 50 µg/mL S4B6-1 (BioXcell) for 30 minutes at room temperature. rIL-2:S4B6 complexes were then serially diluted 10-fold to achieve all desired concentrations for the experiment. rIL-2 without S4B6 was subject to the same treatment. rIL-2 or rIL-2:S4B6 dilutions were then added to appropriate wells and samples were incubated at 37°C for 30 min. For experiments testing responses to IL-2 muteins, serially diluted Fc.WT or Fc.Mut24 were added to appropriate wells and otherwise experiments were set up in the same manner. Samples were then washed and fixed with FixPerm (eBioscience) for 20 min at room temp, washed and incubated in 500 µL MeOH on ice for at least 30 min, washed and finally stained with antibodies for 45 min at room temp. For *in vivo* CD25 blockade, animals were

injected intraperitoneally as described with 500 µg $PC61^{N297Q}$ or $PC61^{2a}$. Spleens were harvested 24 hours after injection, and *in vitro* response to IL-2 was measured as described above (without any incubation with commercial PC61).

In vivo antibody treatments

For CD25 blocking or depleting, mice were given 500 µg $PC61^{N297Q}$ or $PC61^{2a}$ by intraperitoneal injection every 7 days, or as otherwise specified. For IL-2 blocking experiments, mice were given 150 µg S4B6-1 (BioXcell) only, 150 µg JES6-1A (BioXcell) only, 150 µg S4B6-1 and 150 µg JES6-1A together, or 150 µg S4B6-1 and 500 µg JES6-1A together by intraperitoneal injection every 5 days, or as otherwise specified. For IL-2 complex treatments, 50 µg JES6-1A and 1.5 µg recombinant IL-2 (eBioscience) per mouse were mixed together and incubated at room temperature for 30 minutes. Volume was brought up to 100 uL per mouse with sterile PBS and mice were injected intraperitoneally on day 0 and day 2.

VSV infection

Mice were anesthetized with 4% isoflurane and injected with 10^4 plaque forming units (pfu) of VSV-Indiana-OVA in 50 uL sterile PBS intranasally using a P200 micropipet. Mice were visually monitored daily and sacrificed at day 7 post infection. VSV-OVA stocks were provided by P. Fink (University of Washington, Seattle, WA), and maintained and isolated by growth in VERO cells, and viral titers were determined by plaque assay.

Statistical analysis

All data are presented as mean values +/- SD and graphs were created and analyzed using Prism Software (GraphPad). Comparisons between treatment groups or genotypes were analyzed using one-way ANOVA, adjusted for multiple comparisons using Tukey's post-test. When multiple tissues were analyzed simultaneously, multiple t-tests with a Holm-Sidak correction or two-way ANOVA with Tukey's post-test were used.

Chapter 3: Treg cells maintain selective access to IL-2 and immune homeostasis despite substantially reduced CD25 function

Introduction

IL-2 is a critical regulator of immune homeostasis through its role in the development, maintenance and function of Treg cells and its impact on effector cell proliferation and differentiation [91, 92]. The IL-2R can be composed of 2 or 3 subunits: CD122 and γc chain together form the intermediate affinity receptor, and the addition of CD25 creates the high affinity receptor. Binding of CD25 to IL-2 induces a conformational change that decreases the energy needed to bind to the rest of the receptor, whereas CD122 and CD132 are the critical signaling chains [93]. Treg cells constitutively express CD25, which under homeostatic conditions allows them to outcompete $CD25^-$ Teff cells and natural killer (NK) cells for limiting amounts of IL-2. This is most important in the secondary lymphoid organs (SLOs), where pro-survival signals downstream of IL-2 signaling maintain Treg cells [94, 95]. Notably, Treg cells cannot make their own IL-2 [68, 69], and therefore depend on IL-2 produced mainly from autoreactive $CD4^+$ Teff cells [71, 96]. In this way, Teff and Treg cell populations are dynamically linked and reciprocally control each other to maintain immune homeostasis [74].

The inhibitory anti-CD25 antibody PC61 has been extensively used to examine the role of CD25 in IL-2 signaling in Treg cells in mice [97, 98], and model the impact of blocking IL-2 signaling *in vivo*. However, interpretation of results is difficult due to uncertainty of whether the observed *in vivo* effects are mediated by functional blockade of CD25, Treg cell depletion, or a combination [99-102]. Using PC61 derivatives with identical epitope specificity but divergent constant region effector function, a recent study showed that only depletion of $CD25^{hi}$ cells and not blockade of CD25 could disrupt immune homeostasis [103]. However, the fact that blockade

of CD25 for up to four weeks caused no disturbance in immune homeostasis is surprising, given the central role IL-2 is thought to play in the maintenance of Treg cells in SLOs. For instance, acute blockade of IL-2 using the IL-2 antibody S4B6-1 (S4B6) significantly reduces Treg cells, and when administered early in life causes Treg cell dysfunction sufficient to induce autoimmune gastritis in Balb/c mice [96]. However, in addition to blocking IL-2 binding to CD25 [104], this antibody forms superagonistic IL-2 immune complexes that are specifically targeted to $CD122^{hi}$ effector populations such as NK cells and memory T cells [105] and this may have contributed to disease development in these animals. These divergent results may reflect differences in the importance of IL-2 for the induction vs. maintenance of immune tolerance or may reflect idiosyncrasies in how the reagents used for IL-2 and CD25 blockade actually impact IL-2 availability and signaling in Treg and Teff cells.

In light of this confusion, we comprehensively examined how manipulating the IL-2/CD25 axis by different methods perturbs Treg cell maintenance, phenotype and function in maintaining normal immune homeostasis. We found that neutralization of IL-2 abrogated all STAT5 phosphorylation (pSTAT5) in Treg cells but did not immediately disrupt Treg cell function or immune homeostasis. However, sustained blockade of IL-2 led to mild dendritic cell (DC) activation and Teff cell proliferation and expansion. By contrast, Treg cells maintained normal IL-2 signaling in the presence of the inhibitory anti-CD25 antibody PC61 *in vivo*, despite substantially reduced sensitivity to IL-2. Continued IL-2 signaling was dependent on residual CD25 function, and we found that even $CD25^{lo}$ Treg cells that escape depletion after treatment with a strongly depleting IgG2a version of PC61 initially maintain IL-2 responsiveness and functionality *in vivo*. These findings demonstrate that even with severely curtailed CD25 function, Treg cells retain their selective access to IL-2 *in vivo*, and this is sufficient to maintain

normal Treg cell function and immune homeostasis. These data warrant re-examination of previous studies using the PC61 antibody [101-103], and have important implications for efforts to target the IL-2/CD25 axis therapeutically to dampen inflammation and induce immune tolerance.

Results

Complete antibody-mediated neutralization of IL-2 in vivo

When complexed with recombinant IL-2, the anti-IL-2 monoclonal antibodies S4B6 and JES6-1A12 (JES6) act as super-agonists for different leukocyte populations depending on the antibody used and the IL-2R component expression of the cell. There is evidence that injected antibody can complex with endogenous IL-2 as well [105], and thus, we first wanted to determine if the S4B6 and JES6 antibodies could be used alone or in combination to effectively neutralize IL-2 *in vivo*. To test this, we treated mice with either JES6 alone, S4B6 alone, equal amounts of S4B6 and JES6, or an excess of JES6 over S4B6, and assessed IL-2 signaling and upregulation of the proliferation marker Ki67 in Treg cells, $CD44^{+}CD62L^{+}$ $CD8^{+}$ central memory T cells ($CD8^{+}$ T_{CM}), $CD44^{+}CD62L^{-}Foxp3^{-}$ $CD4^{+}$ Teff cells and NK cells after seven days. Due to the qualitative difference in STAT5 phosphorylation response to IL-2 in Treg cells (bimodal) compared to NK and $CD8^{+}$ T_{CM} cells (a weaker unimodal shift) (Fig. S3.1A), we reported response to IL-2 as frequency $pSTAT5^{+}$ of Treg cells and geometric mean fluorescence intensity (gMFI) of the effector populations, respectively.

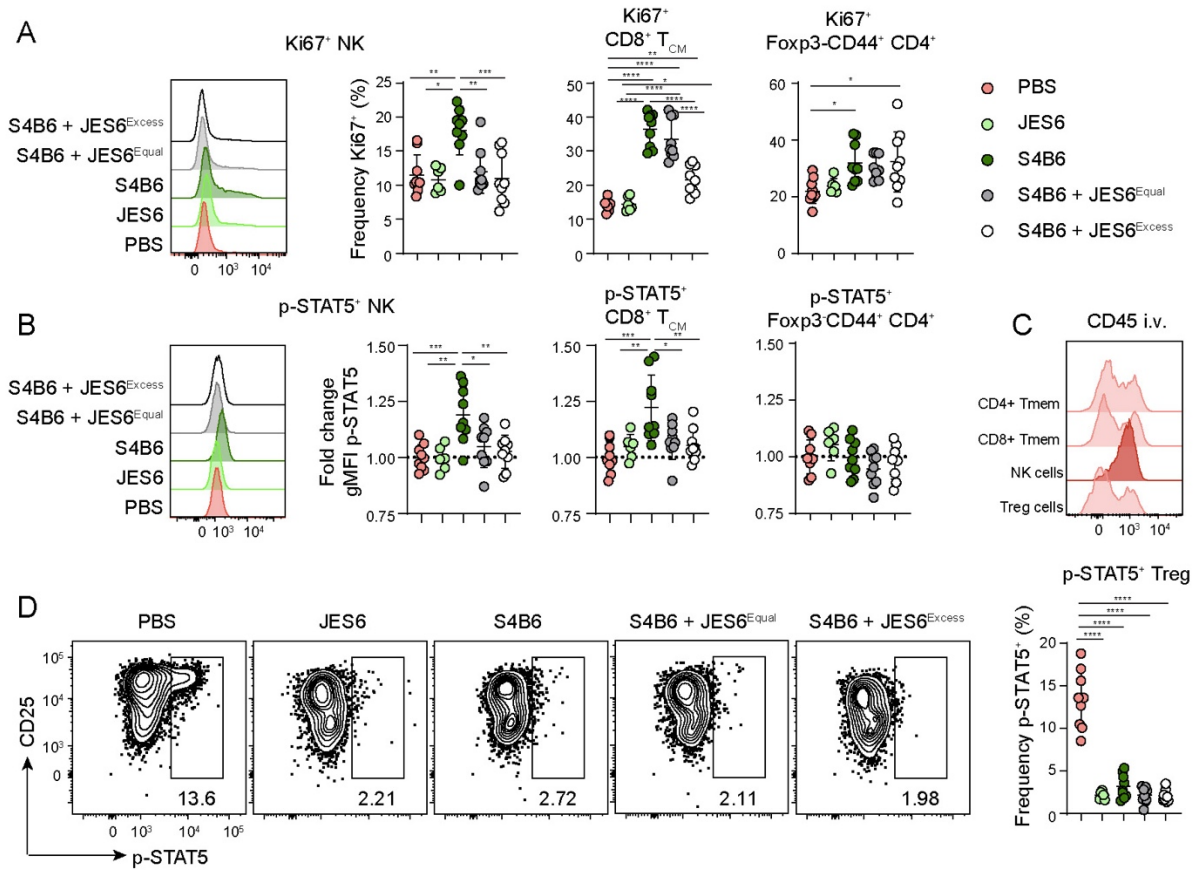

Figure 3.1. JES6 effectively neutralizes IL-2 *in vivo* from all leukocytes

WT B6 mice were treated IP with PBS, 150µg JES6 alone (no S4B6), 150µg S4B6 alone (no JES6), 150µg S4B6 and 150µg JES6, or 500µg JES6 and 150µg S4B6 on day 0 and day 5, and sacrificed on day 7 for analysis. (A) Representative flow cytometry histograms of Ki67 expression in gated NK1.1$^+$ splenic NK cells in each treatment group. Corresponding graphical analysis of frequency of Ki67$^+$ in splenic NK cells, CD8$^+$ T_{CM} cells, and Foxp3$^-$CD44$^+$ CD4$^+$ Teff cells. (B) Representative flow cytometry histograms of pSTAT5 expression in gated NK1.1$^+$ splenic NK cells in each treatment group. Corresponding graphical analysis of fold change gMFI over PBS controls of pSTAT5 in splenic NK cells, CD8$^+$ T_{CM} cells, and Foxp3$^-$CD44$^+$ CD4$^+$ Teff cells. (C) Representative flow cytometry histograms of CD45 IV labeling to assess relative location of splenic leukocyte populations in PBS treated controls. (D) Representative flow cytometry analysis of pSTAT5 and CD25 (clone 7D4) expression by gated splenic Foxp3$^+$ Treg cells. Right, graphical analysis of frequency of pSTAT5$^+$ Treg cells in each treatment group. Data is combined from three independent experiments, 6-9 mice per group total. Significance determined by one-way ANOVA with Tukey post-test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Injection of JES6, which inhibits IL-2 binding to CD122 and CD132, did not promote proliferation or phosphorylation of STAT5 in NK, CD8$^+$ T_{CM}, or CD4$^+$ Teff cells (Fig. 3.1A-B).

In contrast, S4B6 blocks IL-2 binding to CD25, and can target IL-2 to cells expressing high levels of CD122. Accordingly, S4B6 treatment induced robust proliferation of NK cells and Teff cell populations. Proliferation was associated with increased STAT5 phosphorylation in NK and $CD8^+$ T_{CM} cells but not $CD4^+$ Teff, which may be responding indirectly to S4B6 treatment (Fig. 3.1A-B). Addition of JES6 to S4B6 completely blocked proliferation of NK cells, and partially inhibited the proliferation of $CD8^+$ T_{CM} cells. These differential effects of JES6 on the effector populations may be due to their microenvironmental localization in the spleen. Indeed, intravascular labeling experiments showed that the $CD4^+$ and $CD8^+$ Teff reside predominantly in the white pulp (WP) where IL-2 is produced [64, 106], whereas NK cells are almost exclusively located the red pulp (RP) (Fig. 3.1C). Thus, the relative proximity of the $CD8^+$ T_{CM} cells to sites of IL-2 production may limit the ability of JES6 to block activation of these cells by locally produced S4B6/IL-2 complexes. Finally, consistent with the ability of S4B6 to effectively block IL-2 interaction with CD25, all treatments with S4B6 potently inhibited STAT5 phosphorylation in Treg cells (Fig. 3.1D). Surprisingly, even though JES6/IL-2 immune complexes generated *ex vivo* favor activation of $CD25^{hi}$ cells through a triggered exchange mechanism [104], treatment with JES6 alone also completely blocked IL-2 signaling in Treg cells and did not cause an increase in IL-2 signaling or cell proliferation in any of the cell populations examined (Fig 3.1A, B, D). Thus, we used treatment with the JES6 antibody alone for IL-2 neutralization in all subsequent experiments.

Differential impacts of targeting CD25 or IL-2 on $CD25^+$ Treg cells

To determine how inhibiting the IL-2/CD25 axis by targeting either CD25 or IL-2 impacts Treg cell abundance and immune homeostasis, C57BL/6 (B6) mice were treated intraperitoneally (IP) with an engineered isoform of PC61 ($PC61^{N297Q}$) that inhibits CD25

function but does not deplete CD25-expressing cells, an engineered isoform of PC61 ($PC61^{2a}$) that has strong depleting activity, or anti-IL-2 (JES6) as above. In line with previously reported findings [103], seven days after treatment Treg cells were reduced by ~50% in mice treated with $PC61^{2a}$ relative to PBS-treated controls (Fig. 3.2A). Surprisingly, mice treated with anti-IL-2 had only a slightly reduced frequency of Treg cells, and no significant change in the frequency or absolute number of Treg cells was observed in $PC61^{N297Q}$ treated mice. Treg cells can be divided into central (c)Treg and effector (e)Treg cells based on differential expression of CD62L and CD44. In $PC61^{2a}$-treated mice, there was a specific loss of $CD62L^{+}CD44^{lo}$ cTreg cells (Fig. 3.2B), which express the highest levels of CD25 and are the most dependent on IL-2 for their homeostatic maintenance within the spleen [64]. In contrast, there was little change in the ratio of Treg subsets in anti-IL-2 treated mice, and a slight increase in the prevalence of cTreg in $PC61^{N297Q}$ treated mice. Staining isolated cells with a fluorochrome-conjugated PC61 seven days after $PC61^{N297Q}$ and $PC61^{2a}$ treatment showed essentially complete coverage of the epitope (Fig. 3.2A and Fig. 3.2C), verifying that we used a saturating concentration of injected antibody. To assess CD25 expression in treated animals, we stained cells with the 7D4 anti-CD25 antibody, which recognizes a distinct epitope and does not compete with PC61 for binding. As expected, $PC61^{2a}$ treatment effectively depleted $CD25^{hi}$ cells, and the remaining Treg cells in these animals were $CD25^{mid/lo}$. CD25 expression was also significantly reduced in anti-IL-2-treated mice, which is likely due to the ability of IL-2 signaling and activated STAT5 to promote CD25 expression in a positive feedback loop [107]. Interestingly, despite lacking the ability to deplete $CD25^{+}$ cells, CD25 expression was also significantly decreased on Treg cells from mice that had been treated with $PC61^{N297Q}$ (Fig. 2C), indicating that this antibody may induce surface cleavage or internalization of CD25. Finally, $CD4^{+}$ and $CD8^{+}$ Teff cells can transiently express high levels

of CD25 upon activation, and thus could also be affected by the treatments administered. While very few $CD8^+$ Teff cells expressed CD25 in any treatment group (not shown), about 2% of $Foxp3^-CD44^+$ $CD4^+$ Teff cells were $CD25^+$ (Fig. 3.2D) and both $PC61^{N297Q}$ and $PC61^{2a}$ treatment significantly reduced the frequencies and absolute numbers of this population.

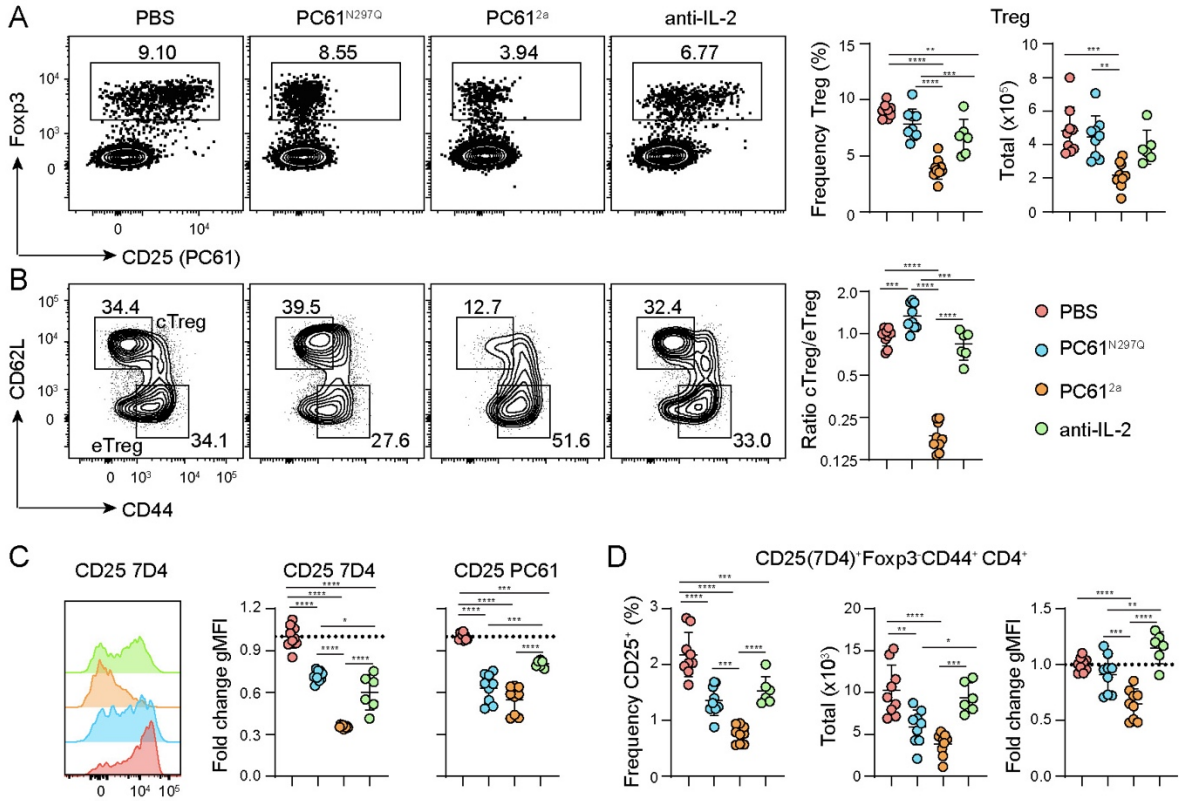

Figure 3.2. Impacts of targeting CD25 or IL-2 on Treg cells

WT B6 mice were treated IP with PBS, $PC61^{N297Q}$, $PC61^{2a}$, or anti-IL-2 (JES6), and sacrificed for analysis after seven days. (A) Representative flow cytometric analysis of Foxp3 and CD25 (clone PC61) expression by gated splenic $CD4^+$ T cells. $Foxp3^+$ Treg cells are gated as indicated. Right, Graphical analysis of frequency and total number of splenic Treg cells in each treatment group. (B) Representative flow cytometry analysis of CD44 and CD62L expression by gated splenic $Foxp3^+$ Treg cells showing gates used to define cTreg and eTreg populations. Right, graphical analysis of the ratio of cTreg cells to eTreg cells in the spleens of each treatment group. (C) Representative flow cytometry histograms of CD25 7D4 staining in Treg cells. Right, graphical analysis of fold change in gMFI over controls of CD25 PC61 and CD25 7D4 staining by Treg cells in each treatment group. (D) Graphical analysis of frequency, total number, and fold change gMFI of $CD25^+$ (7D4) $Foxp3^-CD44^+CD4^+$ splenic T cells in each treatment group. Data is combined from three independent experiments, 6-9 mice per group total. Significance determined by one-way ANOVA with Tukey post-test for multiple comparisons. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Short-term targeting of the IL-2/CD25 axis does not disturb immune homeostasis

Substantial cross-regulation occurs between Treg cells and DCs [9, 10], and therefore we examined how changes in Treg cell numbers in PC61^{N297Q}, PC61^{2a}, and JES6 treated mice impacted the three resident DC populations present in the spleen. No changes in the frequency or number of 33D1^{-}CD11b^{-}CD8^{+} type-1 conventional DCs (cDC1), 33D1^{-}CD11b^{hi}CD8^{-} monocyte-derived DCs (moDCs), or 33D1^{+}CD11b^{+} type-2 conventional DCs (cDC2) were observed (Fig. 3.3A, Fig. S3.1B). Thus, although the frequency and number of Treg cells are reduced in the PC61^{2a} and anti-IL-2 treated mice, this was not sufficient to drive expansion of DCs.

In addition to regulating their DC abundance, Treg cells also restrain DC activation and prevent excessive T cell priming. Therefore, to assess the functionality of Treg cells in the anti-CD25 and anti-IL-2 treated mice, we examined DC activation in the spleen by measuring expression of CD80 and CD86 [55, 108], and CD40 [10], three important costimulatory molecules that are upregulated in activated DCs. No changes in costimulatory molecule expression were observed on splenic DCs in any treatment group (Fig. 3.3B and Fig. S3.1C-D), although there was a trend towards increased expression of CD86 and CD40 in cDC2 of JES6-treated mice. However, expression of costimulatory molecules on cDC2s did increase when IL-2 was blocked by S4B6, even in the presence of JES6 (Fig. S3.1D). This illustrates that we can detect small changes in activation of DCs in this system, and that activation is truly absent with PC61 or JES6 treatment. Furthermore, it suggests that in contrast to total Treg depletion or deficiency [109], blocking IL-2 signaling in Treg cells is not sufficient, at least at this timepoint, to relieve Treg cell suppression of DCs, and that agonism of effector populations by endogenous IL-2/S4B6 immune complexes potentiates cDC2 activation. In contrast, promoting IL-2 signaling in Treg cells in mice with *ex vivo* generated IL-2/JES6 complexes robustly expanded

Treg cells, leading to increased cDC2 frequency and number, but decreased expression of costimulatory molecules (Fig. S3.2).

Interestingly, we observed increased proliferation of $CD8^+$ T_{CM} cells only in the $PC61^{2a}$ treated mice, whereas there were no significant changes in the proliferation of NK cell or $Foxp3^-$ $CD44^+$ $CD4^+$ Teff cell populations with any treatment compared to controls (Fig. 3.3C, D). As $CD8^+$ T_{CM} are more sensitive to increases in IL-2 than other effector cells [55, 110], proliferation of only this population likely reflects increased bioavailability of IL-2 in $PC61^{2a}$ treated mice, where there are fewer $CD25^{hi}$ Treg cells present to consume it.

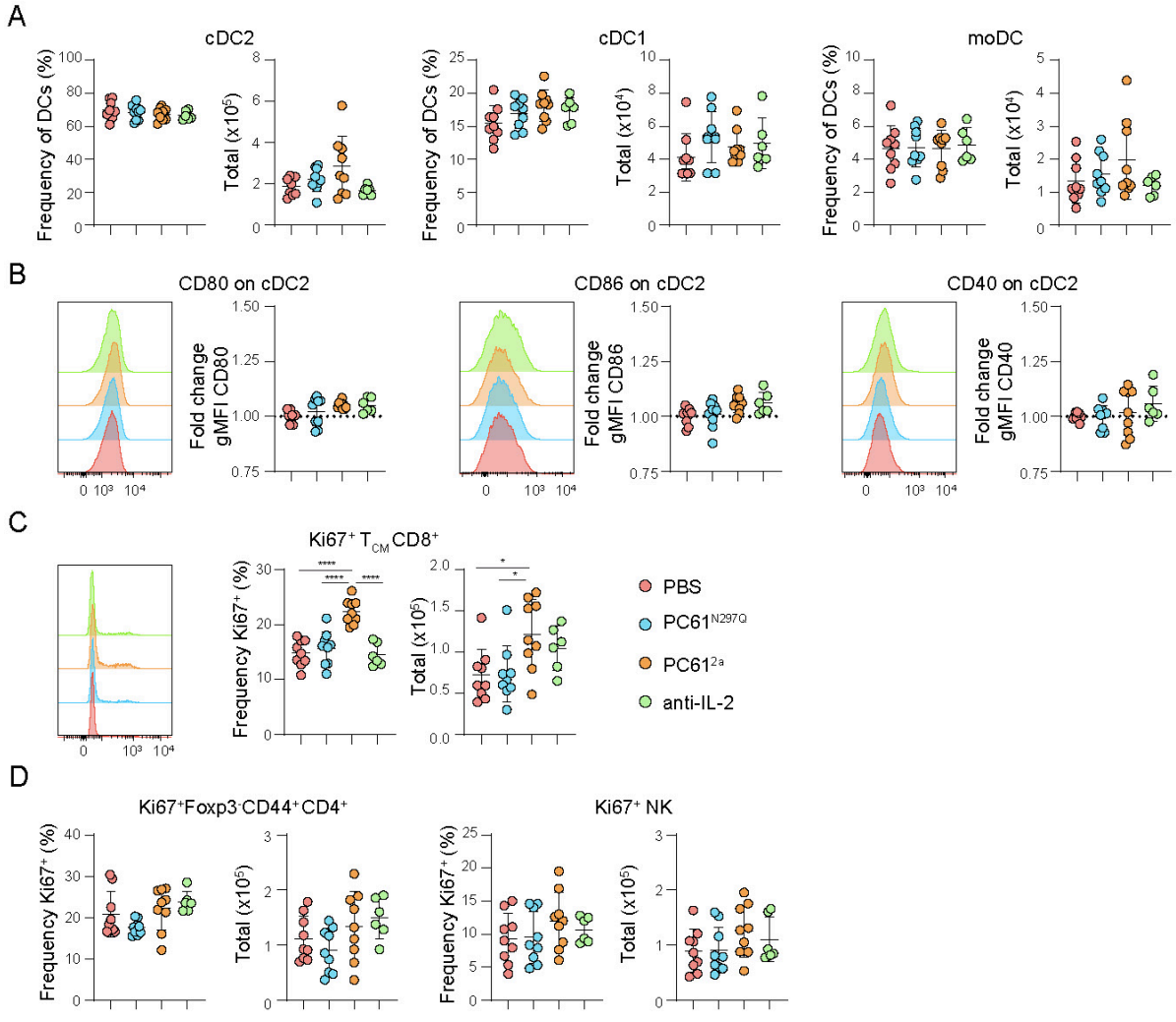

Figure 3.3. Impacts of targeting the IL-2/CD25 axis on DCs and Teff cells

(A-B) WT B6 mice were treated IP with PBS, PC61^{N297Q}, PC61^{2a}, or anti-IL-2 (JES6), and sacrificed after seven days for analysis. (A) Graphical analysis of frequency and total number of splenic MHCII$^+$CD11c$^+$33D1$^+$CD11b$^+$ cDC2, MHCII$^+$CD11c$^+$33D1$^-$CD11b$^-$CD8$^+$ cDC1, and MHCII$^+$CD11c$^+$33D1$^-$CD11b$^+$CD8$^-$ moDC. (B) Representative flow cytometry histograms of CD80, CD86 and CD40 expression by gated splenic cDC2. Corresponding graphical analysis of fold change gMFI over PBS controls of CD80, CD86 and CD40 in splenic cDC2s in each treatment group. (C) Representative flow cytometry histograms of Ki67 expression by gated CD44$^+$CD62L$^+$ CD8$^+$ T_{CM} cells. Graphical analysis of frequency and total number of splenic Ki67$^+$ CD8$^+$ T_{CM} cells in each treatment group. (D) Graphical analysis of frequency and total number of splenic Ki67$^+$Foxp3$^-$CD44$^+$CD4$^+$ Teff cells, and Ki67$^+$ NK cells in each treatment group. Data is combined from three independent experiments, 6-9 mice per group total. Significance determined by one-way ANOVA with Tukey post-test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Treg cells retain selective access to IL-2 in a CD25-dependent manner in the presence of PC61

The lack of acute immune dysregulation in mice treated with CD25 or IL-2 antibodies suggests that Treg cells in these animals retain significant functional capacity, in spite of alterations observed in Treg cell number and composition. This contrasts with Treg cell depletion from Foxp3-DTR mice, where loss of Treg cells results in significant expansion of DCs, NK cells, and Teff cells after seven days [10]. Therefore, we examined expression of various proteins important for suppressive function in Treg cells from the treatment groups. We found that the gMFI of Foxp3 was significantly reduced in anti-IL-2 treated mice, whereas PC61^{N297Q} and PC61^{2a} had only minor impacts on Foxp3 expression (Fig. 3.4A). Furthermore, there was a trend toward decreased CTLA-4 expression on Treg cells from anti-IL-2 treated mice compared to PBS and PC61^{N297Q} treated mice (Fig. 3.4B), while agonistic IL-2 immune complexes vastly increased CTLA-4 expression (Fig. S3.2B). Treg cells from PC61^{2a} treated mice had elevated expression of CTLA-4 compared to all other treatment groups, although this increase was at least partly driven by a higher proportion of eTreg cells in this group, which express more CTLA-4 than cTreg cells.

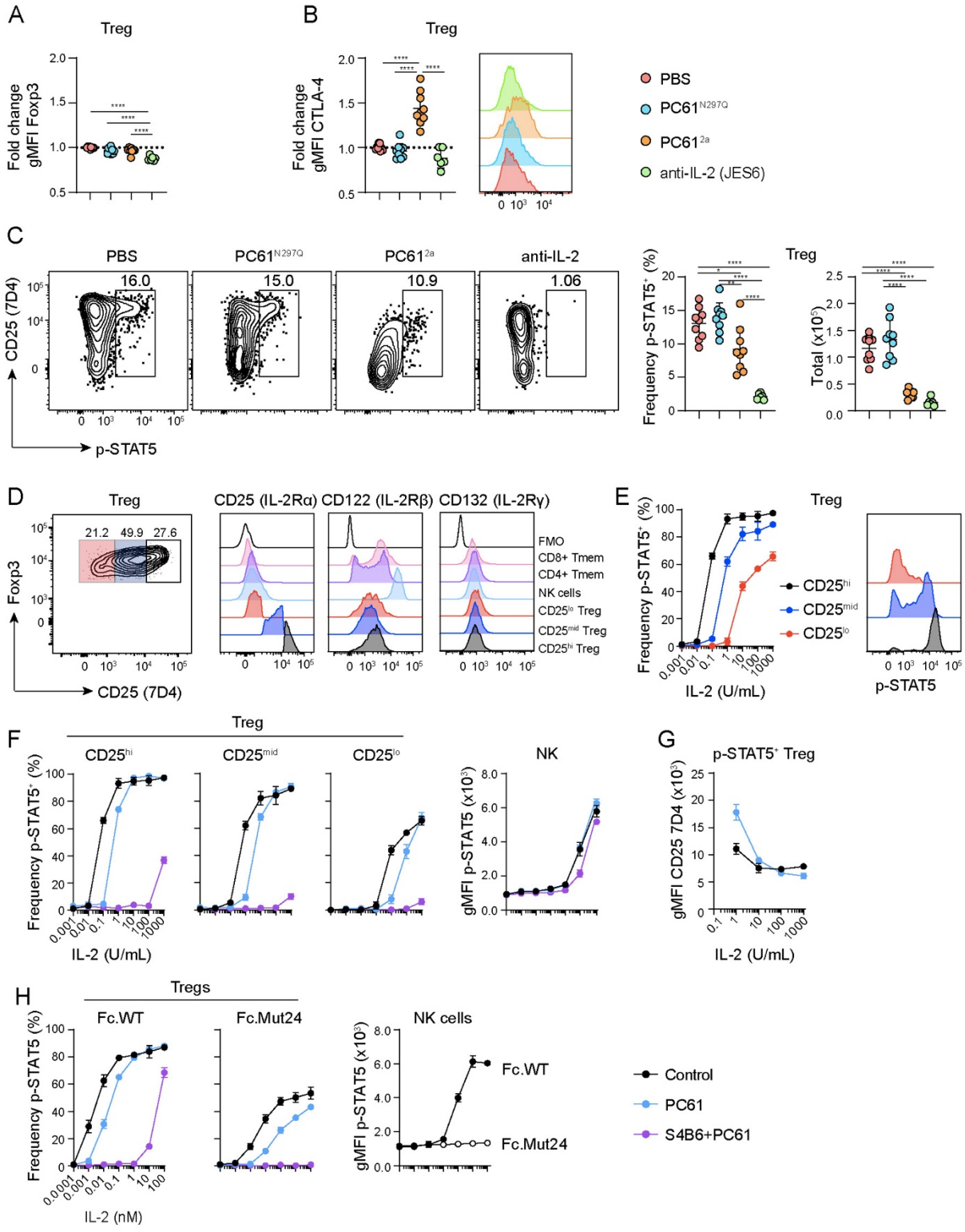

Figure 3.4. Treg cells retain selective access to IL-2 *in vivo* despite severely curtained CD25 function

(A-C) WT B6 mice were treated IP with PBS, PC61^{N297Q}, PC61^{2a}, anti-IL-2 (JES6), or anti-IL-2 (S4B6 + JES6^{Excess}) and sacrificed after seven days for analysis. (A) Graphical analysis of fold change gMFI over PBS controls of Foxp3 in gated Foxp3^{+} Treg cells in the spleens of each treatment group. (B) Graphical analysis of fold change in gMFI over controls of CTLA-4 staining by Treg cells in each treatment group. Right, representative flow cytometric analysis of CTLA-4 by gated splenic Treg cells. (C) Representative flow cytometric analysis of pSTAT5 and CD25 (7D4) by gated splenic Treg cells. Right, graphical analysis of frequency and total number of pSTAT5^{+} splenic Treg cells in each treatment group. (D) Representative flow cytometric analysis of CD25 (7D4) expression in untreated Treg cells, and gates defining CD25^{hi}, CD25^{mid} and CD25^{lo} cells are shown. Right, representative flow cytometric analysis of CD25, CD122 and CD132 expression by the indicated cell populations and fluorescence minus one (FMO) controls. (E) Graphical analysis of frequency of pSTAT5^{+} splenic Treg cells within each CD25 expression subset in response to rIL-2. Right, representative flow cytometric analysis of pSTAT5 staining in Treg cells in each CD25 subset in response to 1 U/mL rIL-2. (F) Graphical analysis of frequency of pSTAT5^{+} Treg cells in response to IL-2 after treatment *in vitro* with either PC61, or PC61 and S4B6 compared to controls. Right, graphical analysis of gMFI of pSTAT5 in NK cells under the same treatment conditions. (G) Graphical analysis of gMFI of CD25 (7D4) in pSTAT5^{+} Treg cells in response to IL-2 after treatment *in vitro* with PC61 compared to controls. (H) Graphical analysis of frequency of pSTAT5^{+} Treg cells in response to WT.Fc or Mut24.Fc after treatment *in vitro* with either PC61, or PC61 and S4B6 compared to controls. Right, graphical analysis of gMFI of pSTAT5 in NK cells in response to WT.Fc or Mut24.Fc. (A-C) Data is combined from three independent experiments, 6-9 mice per group total. Significance determined by one-way ANOVA with Tukey post-test for multiple comparisons. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. (D-H) Data is from one representative experiment, with three technical replicates per condition. Experiments were repeated independently at least three times.

IL-2 signaling helps maintain Treg cell function by promoting high expression of Foxp3 [111] and other inhibitory molecules like CTLA-4 [112], and we speculated that the different expression levels of these molecules could be due to differences in IL-2 signaling in mice given PC61 compared to JES6. Therefore, we examined pSTAT5 directly *ex vivo* in different cell populations one week after antibody administration, when PC61 epitopes on CD25 were still completely saturated (Fig. 3.2A, C). Whereas neutralization of IL-2 blocked all pSTAT5 as expected, Treg cells from animals treated with PC61^{N297Q} maintained normal levels of pSTAT5, and even treatment with PC61^{2a} had only a modest impact on the frequency of pSTAT5^{+} Treg cells (Fig. 3.4C). The pSTAT5 staining we observed in the treated animals does not simply reflect prolonged IL-2 signaling that occurred prior to treatment initiation, as we have previously

shown that injection of IL-2 antibodies as little as 30 minutes prior to sacrifice ameliorates all detectable pSTAT5 in Treg cells [71]. We could not detect increased IL-2 signaling in effector populations, as the gMFI of pSTAT5 in both NK cells and $CD8^+$ T_{CM} was not increased by any of the treatments (Fig. S3.3A). However, we speculate that this is due to the limited sensitivity of our assay, and that treatment with $PC61^{2a}$ does result in more IL-2 consumption by $CD8^+$ T_{CM} that drives their enhanced proliferation in these mice.

The ability of Treg cells to maintain IL-2 responsiveness in the presence of the PC61 antibodies led us to two competing hypotheses. Either the CD25 remaining on the cell surface was still functional and mediating IL-2 signaling, or residual IL-2 signaling was CD25-independent. CD25-independent signaling could result from either upregulation of the other IL-2R components on Treg cells, or from increased sensitivity to IL-2 resulting from changes in the IL-2R signaling pathways such as downregulation of the negative regulator protein phosphatase 2A (PP2A) [113]. Co-staining with the 7D4 anti-CD25 antibody clearly showed that as in control mice, pSTAT5 was enriched among Treg cells expressing the highest amounts of CD25 in both $PC61^{N297Q}$- and $PC61^{2a}$-treated mice (Fig. 3.4C). We therefore compared the expression of the other IL-2R components from untreated splenic Treg cells divided into three subsets based on their expression of CD25 by 7D4 staining. Expression of CD122 and CD132 was similar between all three subsets of Treg cells (Fig. 3.4D). Furthermore, CD122 expression by Treg cells was much lower than expression by memory T cells or NK cells. Thus, enhanced expression of the intermediate affinity IL-2R does not explain the ability of $CD25^{hi}$ Treg cells to selectively respond to IL-2 in the presence of the PC61 antibodies.

To directly assess the impact of PC61 on CD25 function and the sensitivity of splenic Treg cells to IL-2, we performed *in vitro* stimulations in the presence of PC61 and the anti-IL-2

clone S4B6, which directly blocks interaction between IL-2 and CD25 but has minimal impact on IL-2 signaling via the intermediate affinity CD122/CD132 complex [104]. For analysis, Treg cells were subsetted based on their expression of CD25 by 7D4 staining as in Fig 4D. $CD25^{hi}$ Treg cells achieved maximal pSTAT5 at a relatively low dose of rIL-2 (1 U/mL), while $CD25^{mid}$ Treg cells were approximately 10-fold less sensitive and $CD25^{lo}$ Treg cells were more than 100-fold less sensitive (Fig. 3.4E). Pre-treatment with PC61 for 30 min prior to IL-2 stimulation reduced IL-2 sensitivity by ~10-fold in all three Treg cell populations (Fig 3.4F), but all were still able to achieve the maximal level of pSTAT5 observed in untreated cells. However, further addition of S4B6 severely curtailed IL-2 sensitivity in all Treg cells, indicating that they do not efficiently signal through the intermediate-affinity IL-2 receptor. By contrast, in NK cells, which lack CD25 but have high levels of CD122, IL-2 responses were completely unaffected by the addition of PC61 (Fig. 3.4F, right), and S4B6 had only a small effect on signaling which is due to minor steric inhibition *in vitro* [104]. Thus, we conclude that CD25 retains significant functionality when bound by PC61. Indeed, PC61 does not directly occlude IL-2 binding, but rather inhibits CD25 function by inducing a conformational change in the IL-2 binding pocket [114]. Interestingly, at low doses of IL-2 (1 U/mL), treatment with PC61 actually makes Treg cells more CD25 dependent, as evidenced by the increased CD25 gMFI of $pSTAT5^{+}$ Treg cells treated with PC61 compared to untreated control cells (Fig. 3.4G).

To further explore residual CD25 function in PC61-treated Treg cells, we stimulated cells with an IL-2 'mutein' (Mut24) that cannot signal in the absence of CD25 due to mutations that reduce its affinity for CD122 [90]. Here, we used the IL-2 mutein as a fused Fc-homodimer (Fc.Mut24) and compared it to Fc-fused WT IL-2 (Fc.WT). As before, splenocytes from B6 mice were treated *in vitro* with PC61 before stimulation with a range of doses of either Fc.WT or

Fc.Mut24 IL-2. Treg cells responded to both Fc.WT and Fc.Mut24 in a dose-dependent manner, and addition of PC61 resulted in a ~10-fold decrease in sensitivity to both (Fig. 3.4H). Furthermore, addition of S4B6 completely blocked signaling in response to Fc.Mut24, demonstrating that Fc.Mut24 stimulation even in the presence of PC61 is CD25 dependent. Treg cells could still respond to high concentrations of Fc.WT in the presence of PC61 and S4B6 through the intermediate affinity receptor. In contrast, NK cells did not respond at all to Fc.Mut24 but responded normally to Fc.WT with little impact of either antibody, consistent their exclusive use of the intermediate-affinity receptor (Fig. 3.4H, right). Taken together, these data show that even with substantially reduced CD25 function, Treg cells selectively access IL-2 when in competition with effector cells.

To examine how *in vivo* treatment with either $PC61^{N297Q}$ or $PC61^{2a}$ affected IL-2 sensitivity, we performed similar dose-response experiments on cells isolated from mice 24h after *in vivo* antibody treatment. Even at this early timepoint, PC61 had saturated all detectable epitopes in mice treated with $PC61^{N297Q}$ or $PC61^{2a}$ (Fig. S3.3B), and by 7D4 staining we observed reduced CD25 expression in $PC61^{N297Q}$-treated mice, and nearly complete depletion of $CD25^{hi}$ Treg cells in $PC61^{2a}$-treated animals (Fig. S3.3C). IL-2 sensitivity of $CD25^{lo}$, $CD25^{mid}$ and $CD25^{hi}$ Treg cell populations from treated mice was reduced by about 50-fold (Fig. S3.3D). However, these Treg cells were still more IL-2 responsive than both $CD8^+$ T_{CM} and NK cells (Fig. S3.3E). Again, addition of S4B6 to further block IL-2/CD25 interaction severely curtailed IL-2 signaling in treated cells. Together, these data show that although PC61 does substantially reduce the sensitivity of Treg cells to IL-2, sustained CD25 expression and function in $PC61^{N297Q}$ and $PC61^{2a}$ treated mice maintains the Treg cell-dominated hierarchy of access to IL-2 *in vivo*.

Extended treatment with PC61^{2a} or JES6 disrupts Treg cell mediated immune homeostasis differently

Whereas minor immune dysregulation evidenced by elevated proliferation of CD8$^+$ T cells was only apparent in the PC61^{2a} treated mice after one week of treatment, we wondered if long-term inhibition of CD25 with the PC61^{N297Q} or neutralization of IL-2 would ultimately result in compromised Treg cell function. As we observed after one week, the frequency of Treg cells was significantly decreased in mice treated with PC61^{2a} for four weeks, and this predominantly impacted CD25^{hi} cTreg cells (Fig. 3.5A). Interestingly, prolonged treatment also resulted in a small but significant decrease in Treg cell frequency in PC61^{N297Q} treated mice, and a large decrease in the anti-IL-2 treated mice (Fig. 3.5A). However, absolute numbers of Treg cells in the spleen were diminished only in the PC61^{2a}-treated mice. Endogenous STAT5 phosphorylation in Treg cells was strikingly similar at four weeks compared to one week, but we now observed activation by enhanced costimulatory molecule expression of cDC2 and moDC in PC61^{2a} and anti-IL-2 treated animals (Fig. 3.5B). Levels of CD86 expression were highest on DCs from anti-IL-2 treated mice, while levels of CD40 were comparable between this treatment group and PC61^{2a}. Accordingly, we detected increased frequencies and numbers of CD44^{hi}CD62L^{lo} CD4$^+$ (Fig. 3.5C) Teff cells in PC61^{2a} and anti-IL-2 treated mice. In contrast, as we observed at 1 week post-treatment, CD44^{hi} CD8$^+$ Teff cells were only expanded in the PC61^{2a} treated mice (Fig. 3.5D). The expanded Teff cell populations were associated with enhanced production of the pro-inflammatory cytokine IFN-γ by both CD4$^+$ and CD8$^+$ T cells (Fig. 3.5E, F). Therefore, continued IL-2 signaling after Treg cell depletion with PC61^{2a} can preserve immune homeostasis only in the short term, while even extended treatment with PC61^{N297Q} does not lead to immune dysregulation. Neutralization of IL-2 by JES6 in the

periphery does not acutely disrupt immune homeostasis, but loss of Treg functionality contributes to dysregulation after several weeks.

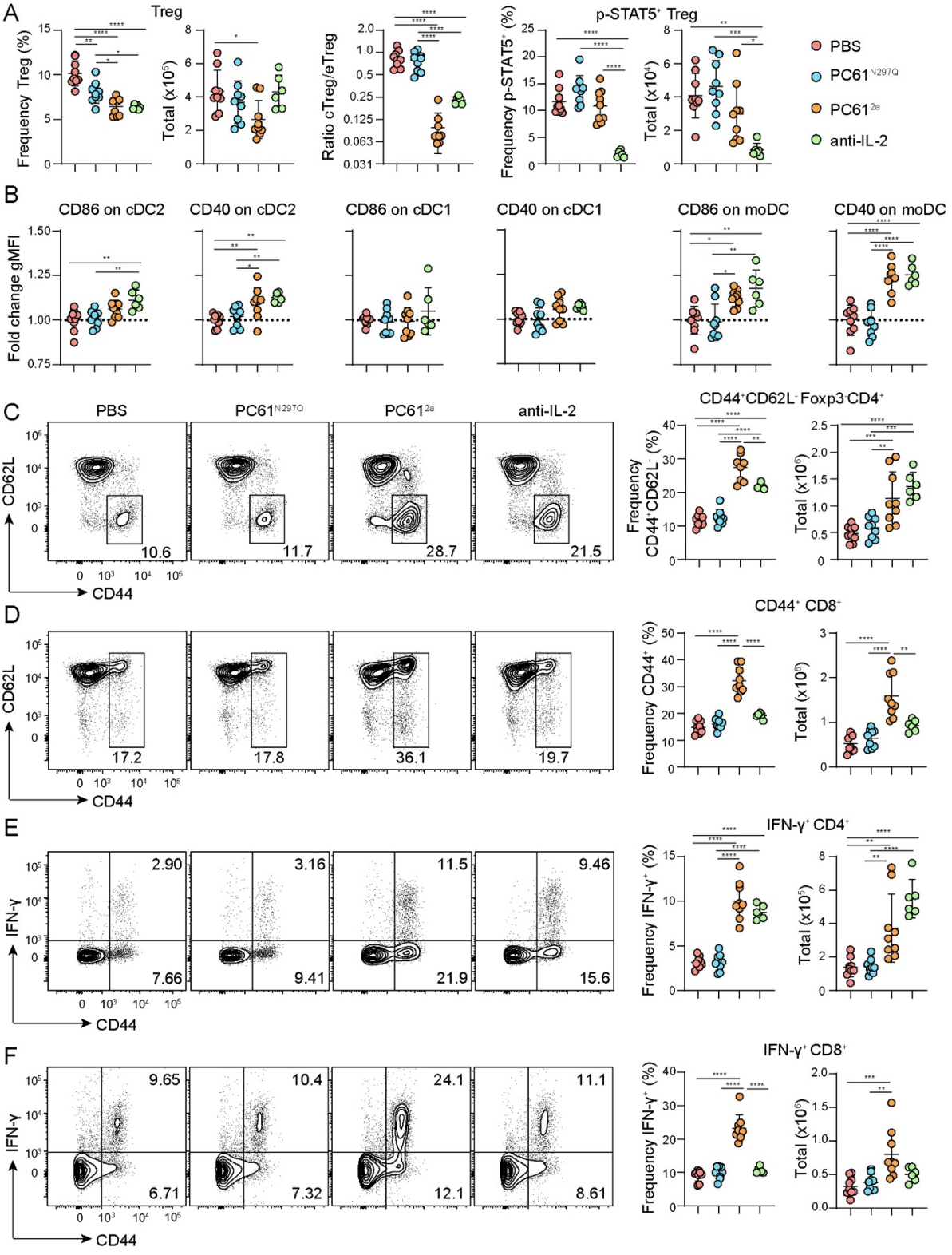

Figure 5. Impact of long-term blockade of IL-2 or CD25 on Treg cells and immune homeostasis

WT B6 mice were treated with PBS, $PC61^{N297Q}$, or $PC61^{2a}$ every 7 days or anti-IL-2 every 5 days, and sacrificed after 28 days for analysis. (A) Left, graphical analyses of frequency and total number of splenic Treg cells in each treatment group. Middle, graphical analyses of the ratio of $CD62L^+$ cTreg to $CD44^+$ eTreg in the spleens of each treatment group. Right, graphical analysis of frequency and total number of $pSTAT5^+$ splenic Treg cells in each treatment group. (B) Graphical analysis of fold change gMFI over PBS controls of CD86 and CD40 in splenic cDC2s, cDC1s, and moDCs in each treatment group. Representative analysis of CD44 and CD62L expression by gated splenic $CD4^+Foxp3^-$ T cells (C) and $CD8^+$ T cells (D) in each treatment group. Right, corresponding graphical analyses of frequency and total number of gated $CD4^+Foxp3^-CD44^+CD62L^-$ (C) and $CD8^+CD44^+$ (D) T cells. (D) Representative analysis of CD44 and IFN-γ expression in gated $CD4^+$ (E) and $CD8^+$ (F) T cells from each treatment group after stimulation *in vitro* for 4 hours with PMA, ionomycin and monensin. Right, graphical analysis of frequency and total number of IFN-γ producing $CD4^+$ and $CD8^+$ T cells in each treatment group. Data is combined from three independent experiments, 6-9 mice per group total. Significance determined by one-way ANOVA with Tukey post-test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Discussion

The importance of CD25 for Treg cell-mediated suppressive function is well established. Although genetic models using conditional ablation of CD25 on Treg cells demonstrate this [55-57], it is less clear how therapeutically inhibiting CD25 or IL-2 impacts Treg cells. As the IL-2/CD25 axis is central to mediating immune homeostasis, a precise understanding of how it could be targeted to treat human disease is critical. In this study, we define how different aspects of immune regulation are disrupted when the IL-2 signaling pathway is altered by targeting CD25 or IL-2 in various ways. Treg depletion with $PC61^{2a}$ treatment or IL-2 blockade led to enhanced DC activation. Similarly, $CD4^+$ T cell activation and expansion relies on a combination of decreased Treg cell numbers and reduced Treg cell function due to blunted or absent IL-2 signaling. By contrast, activation and proliferation of $CD8^+$ T cells does not require loss of IL-2 dependent functions in Treg cells but seems to occur when Treg cell consumption of IL-2 is impaired, as in the $PC61^{2a}$ treatment. Strikingly, although $PC61^{N297Q}$ reduces IL-2 sensitivity ~10-50-fold in Treg cells, this is not sufficient to upset their competitive advantage over Teff cells and NK cells in accessing IL-2 and does not disrupt homeostasis of any of the aforementioned immune parameters, even after prolonged treatment.

Whereas previous studies have struggled to distinguish requirements for IL-2 in earlier developmental stages versus subsequent maintenance in adult peripheral immune tissue, we demonstrate here that continued IL-2 signaling in the periphery is not necessary for maintenance of Treg cell function and immune homeostasis in the short term but does eventually lead to some immune dysregulation that is far more mild than complete loss of IL-2 or CD25. Previously, we and others have shown that maintenance of eTreg cells in non-lymphoid tissues can be IL-2

independent [64, 115], and the present study shows this can be true in the lymphoid organs as well.

The population sizes and activation states of DCs, Teff cells and Treg cells are dynamically interconnected [9, 10, 109], and work from our lab previously determined that the frequency and function of IL-2 dependent Treg cells in the spleen depends on antigen presentation to autoreactive $CD4^+$ T cells largely by CD80/86-bearing cDC2s [71]. Here, we extend these findings to show that when IL-2 is neutralized by JES6 in the short term, immune homeostasis is maintained despite a lack of pSTAT5 and reduction in suppressive molecules in Treg cells. However, S4B6 treatment blocks IL-2 signaling in Treg cells, and simultaneously redirects IL-2 to NK cells and $CD8^+$ T_{CM}, resulting in rapid upregulation of the costimulatory ligands CD86 and CD40 on DCs. This shows that acute blockade of IL-2 signaling is not sufficient to functionally inactivate Treg cells.

It remains unclear why S4B6 can complex with endogenous IL-2 to stimulate $CD122^{hi}$ cells, but JES6 does not do the same with $CD25^{hi}$ cells. S4B6 acts as a structural mimic of CD25 when bound to IL-2 and interacting with the IL-2R [104], and we show here that it can potentiate receptor binding signaling even with limiting amounts of IL-2. In contrast, JES6 has a complicated exchange mechanism with the IL-2R that only allows binding on IL-2 with sufficient CD25 expression on the target cell [104]. We speculate that this "hand-off" is dependent on the relative concentrations of IL-2 and JES6 antibody. This could explain why JES6 blocks IL-2 signaling in Treg cells when given in vast excess *in vivo* but expands Treg cells when given as *ex vivo* generated immune complexes with recombinant IL-2 at optimal molar ratios.

In addition to robust expansion, Treg cells displayed high levels of suppressive molecules and reduced levels of costimulatory markers on DCs following treatment with IL-2/JES6 immune complexes (our work and [108]). These data mirror results from Chinen and colleagues [55], in which Treg cells with constitutively active pSTAT5 had an enhanced ability to form conjugates with DCs, resulting in their decreased expression of costimulatory molecules. Treg cells can outcompete naïve T cells to bind to DCs [116], and modulate levels of costimulatory molecules on those DCs through provision of the inhibitory receptor CTLA-4 *in vitro* [117] and *in vivo* [108, 118]. The trend toward decreased CTLA-4 expression we observed on Treg cells in the anti-IL-2-treated mice likely led to decreased inhibition of DCs with prolonged treatment. IL-2-dependent regulation of other adhesion and inhibitory molecules could also be involved in providing Treg cells' enhanced ability to interact with and even strip MHCII-peptide from DCs in order to limit priming of conventional T cells and maintain immune homeostasis [119]. However, consumption of IL-2 by Treg cells in order to sequester it away from conventional T cells seems to play as critical a role in restraining activation and expansion of Teff, especially in the $CD8^+$ compartment as suggested previously [55].

We further show that Treg cells maintain substantial CD25 function and selective access to IL-2 in a CD25-dependent manner in the presence of PC61, critically clarifying the effects of this commonly used antibody in murine models. Our data strongly support the conclusions of Huss et al. that any effects observed in mice treated with PC61 must be due to active depletion of $CD25^{hi}$ Treg cells [103]. However, conclusions made in this study and others based on the ability of PC61 to inhibit CD25 function on Treg cells that are not depleted should be re-evaluated [101, 103]. The $PC61^{N297Q}$ antibody was designed expressly for the purpose of assessing effects of IL-2 blockade in Treg cells compared to depletion with the $PC61^{2a}$ antibody, and these prior studies

assumed that the binding action of the PC61 antibody to CD25 blocks the CD25-dependent IL-2 survival signal in Treg cells. However, we show definitively this is not the case, as Treg cells in the presence of $PC61^{N297Q}$ sustained normal levels of IL-2 signaling and preserved immune homeostasis after treatment for four weeks, while Treg cells treated with anti-IL-2 lost function in the absence of IL-2 signaling and failed to prevent immune activation.

Vargas and colleagues recently demonstrated that tumor-bearing mice treated with a strong depleting CD25 antibody have a significant reduction of intra-tumoral Treg cells and subsequent improved control of tumors, while a non-depleting CD25 antibody has no effect [120]. In cancer therapy this result is desirable. In contrast, more subtle inhibition of the IL-2/CD25 axis, such as treatment with $PC61^{N297Q}$, may be more advantageous for treatment of autoimmunity. For instance, we found that continued IL-2 signaling in splenocytes treated with PC61 was skewed towards the highest CD25 expressing Treg cells compared to the untreated controls. This could be beneficial in an autoimmune setting, particularly in humans where CD25 is expressed on $CD56^{bright}$ NK cells and activated T cells, but at a lower level than on Treg cells [121]. The presence of $PC61^{N297Q}$ could therefore allow a further advantage for Treg cells to preferentially access IL-2 over other effector cells and improve tolerance in a mechanism similar to Treg selective IL-2 'muteins' [122, 123], or even be given in combination IL-2 muteins for an exceedingly selective $CD25^{hi}$ cell response.

In humans, the anti-CD25 antibodies daclizumab and basilixumab have been used as anti-inflammatory agents to treat MS and prevent graft rejection. This is somewhat counterintuitive given the central role of IL-2/CD25 in maintaining immune tolerance, and the mechanisms of action of these drugs is not well understood. Unlike PC61, these antibodies directly bind to and occlude the IL-2 binding site of CD25, and this results in a reduction in Treg cell frequency

(although these cells retain function [124, 125]), increased serum levels of IL-2, and an IL-2-dependent increase in $CD56^{bright}$ NK cells [126-129]. The increase in $CD56^{bright}$ NK cells correlates positively with therapeutic response in patients with MS. While normally thought to be regulatory and more immature, $CD56^{bright}$ NK cells expanded in daclizumab treated patients display enhanced expression of activation markers and receptors that mediate NK cell activation and cytolytic capacity [130], and *in vitro* studies indicate the ability of these NK cells to kill activated encephalitogenic T cells [127, 131]. Although NK cells appear to have a beneficial therapeutic effect in this setting, it is also possible they are contributing to adverse events in these treated patients, such as dermatitis, malignancies, infections, and encephalitis [89, 132]. Identification of antibodies that limit CD25 function but allow Treg cells to maintain their selective access to IL-2 may also be therapeutically beneficial in autoimmunity for limiting Teff cell and NK cell responses while maintaining robust Treg cell function.

Targeting of the IL-2/CD25 axis holds incredible promise for treatment of immune dysfunction. Our study emphasizes the complexity of this pathway, and that changes in the sensitivity of cells to IL-2 can produce strikingly different effects on the immune system. Total blockade of IL-2 gradually progresses to immune dysregulation, whereas residual CD25 function, even in the face of inhibitory or depleting antibodies, can maintain Treg cell preferential access to IL-2 and therefore allow preservation of immune homeostasis to varying degrees. Thus, subtle differences in CD25 antibody specificity and activity could result in a wide range of beneficial outcomes and could be selectively utilized to maximize therapeutic benefit.

Supplemental Figures

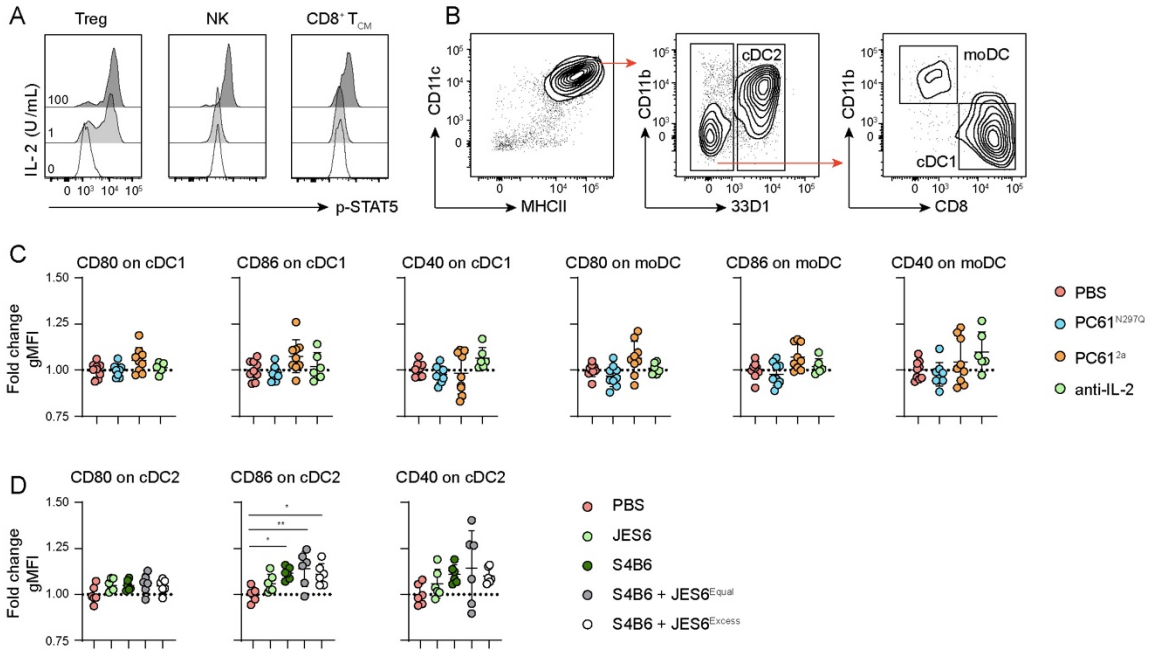

Figure S3.1.

(A) Representative flow cytometric analysis of p-STAT5 in gated splenic Foxp3+ Treg cells, NK cells, and CD44+CD62L+ CD8+ cells from WT B6 mice after stimulation *in vitro* with rIL-2. (B) Gating strategy to identify DCs in the spleen. (C) WT B6 mice were treated with PBS, $PC61^{N297Q}$, $PC61^{2a}$, or anti-IL-2 (JES6), and sacrificed for analysis after seven days. Graphical analysis of fold change gMFI over PBS controls of CD80, CD86 and CD40 in splenic cDC1 and moDC from all treatment groups. (D) WT B6 mice were treated IP with PBS, 150µg JES6 alone (no S4B6), 150µg S4B6 alone (no JES6), 150µg S4B6 and 150µg JES6, or 500µg JES6 and 150µg S4B6 on day 0 and day 5, and sacrificed on day 7 for analysis. Graphical analysis of fold change gMFI over PBS controls of CD80, CD86 and CD40 in gated splenic cDC2 cells from all treatment groups. Data is combined from two or three independent experiments, 6-9 mice per group total. Significance determined by one-way ANOVA with Tukey post-test for multiple comparisons. *$p<0.05$, **$p<0.01$.

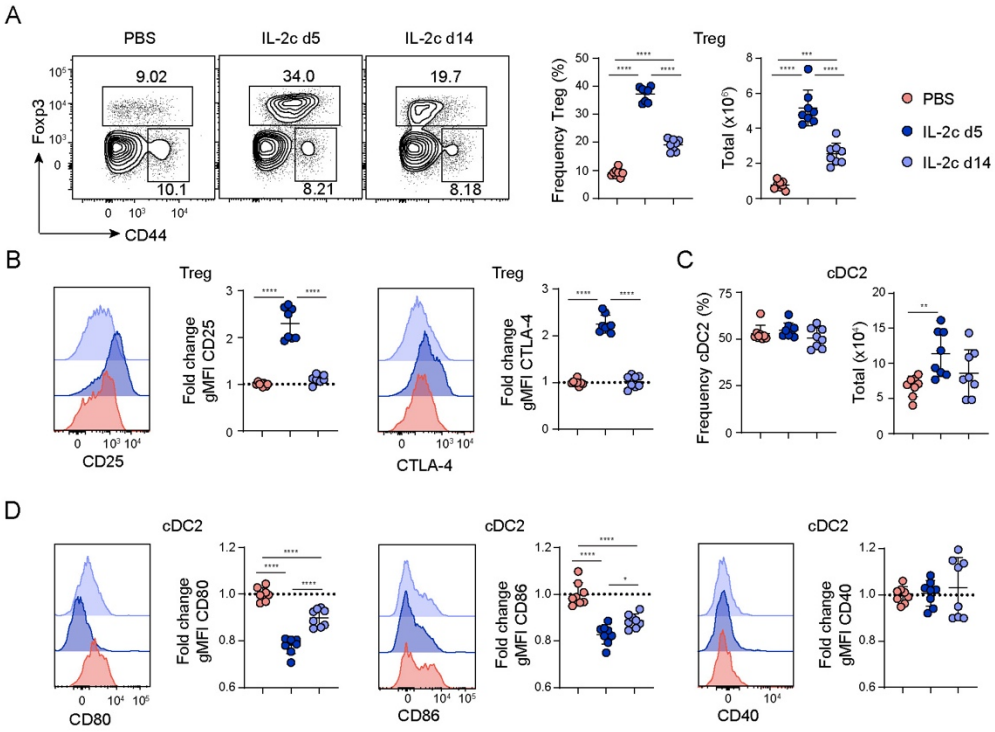

Figure S3.2.

WT B6 mice were treated IP with PBS or IL-2 complex (IL-2 and JES6) on day 0 and day 2, and sacrificed on day 5 or day 14 for analysis. (A) Representative flow cytometric analyses of Foxp3 and CD44 expression by gated splenic CD4+ T cells. Foxp3+ Treg cells are gated as indicated. Right, Graphical analysis of frequency and total number of splenic Treg cells in each treatment group. (B) Representative flow cytometry histograms of CD25 PC61 and CTLA-4 staining in Treg cells. Corresponding graphical analysis of fold change in gMFI over controls of CD25 PC61 and CTLA-4 staining by Treg cells in each treatment group. (C) Graphical analysis of frequency and total number of cDC2 in the spleen of each treatment group. (D) Representative flow cytometry histograms of CD80, CD86 and CD40 staining in cDC2. Corresponding graphical analysis of fold change in gMFI over controls of CD80, CD86 and CD40 staining by cDC2 in each treatment group. Data is combined from two independent experiments, 8 mice per group total. Significance determined by one-way ANOVA with Tukey post-test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

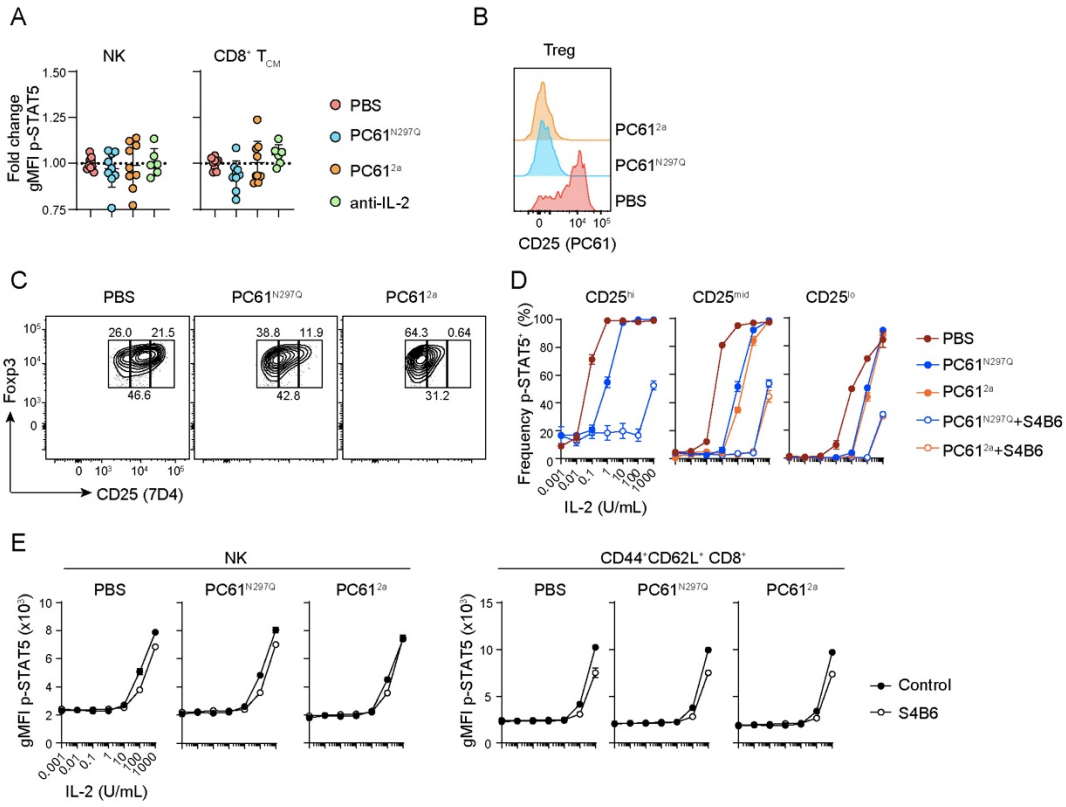

Figure S3.3.

(A) WT B6 mice were treated IP with PBS, PC61^{N297Q}, PC61^{2a}, or anti-IL-2 (JES6), and sacrificed after seven days for analysis. (A) Graphical analysis of fold change in gMFI over controls of p-STAT5 in NK and CD44+CD62L+ CD8+ cells in each treatment group. Data is combined from three independent experiments, 6-9 mice per group total. Significance determined by one-way ANOVA with Tukey post-test for multiple comparisons. (B-E) WT B6 mice were treated IP with PBS, PC61^{N297Q} or PC61^{2a} and sacrificed after 24 hours. (B) Representative flow cytometry histograms of CD25 (PC61) staining on Treg cells. (C) Representative flow cytometry analysis of CD25 (7D4) staining on gated Treg cells, and gates defining CD25^{hi}, CD25^{mid} and CD25^{lo} cells are shown. (D) Graphical analysis of frequency of p-STAT5+ Treg cells in response to *in vitro* IL-2 +/- S4B6 in each *in vivo* treatment group (PBS, PC61^{N297Q} or PC61^{2a}) as indicated. (E) Graphical analysis of gMFI p-STAT5 NK or CD44+CD62L+ CD8+ cells in response to *in vitro* rIL-2 +/- S4B6 in each *in vivo* treatment group (PBS, PC61^{N297Q} or PC61^{2a}). Data is from one representative experiment, with three technical replicates per condition. Experiments were repeated independently at least three times.

Chapter 4: IL-2 signaling focuses the immune response to pathogen

Introduction

IL-2 is a cytokine with pleiotropic effects on a variety of T cell populations, making it a likely player in T cell-dependent immune responses. Surprisingly, mice deficient in IL-2 or IL-2R components, but within experimental settings where autoimmunity is controlled such as bone marrow chimeras, mount effective immune responses against a variety of pathogens [133]. Notably, many of these studies demonstrate that $CD4^+$ and $CD8^+$ T cell proliferation associated with an acute immune response occurs in the absence of IL-2 signaling [134-140]. Whereas *in vitro* experiments indicate that IL-2 is important for activation induced cell death (AICD) and effector cell differentiation [141, 142], *in vivo* both maturation and contraction appear to happen relatively normally [137, 138, 143-146]. Specific functional defects in cytotoxicity or inflammatory cytokine production have been reported in antigen-specific T cells activated in the absence of IL-2 [145]. Although initiation of immune responses does not require IL-2, in some models the primary response is attenuated, and subsequent memory responses are impaired [143, 144]. Collectively, these studies show that IL-2 independent T cell proliferation and effector cell development do occur and lead to generally effective primary immune responses.

Total deficiency in IL-2 or components of its receptor is rare in humans, but functional SNPs are much more prevalent. Genome wide association studies (GWAS) have linked genetic variation at *IL2* and *IL2RA* with susceptibility to multiple inflammatory disorders [147], but the vast majority of disease-associated SNPs are non-coding, and their impacts on IL2/IL2RA expression and function are not clear. Subtle changes in the bioavailability and activity of IL-2 can have powerful immune effects, as evidenced by therapeutic modulation of the IL-2 signaling pathway in various disease settings. Since viral infection is considered a primary environmental

factor in initiation or exacerbation of autoimmune diseases [148], understanding how subtle changes in IL-2 during acute infection disrupt immune regulation may shed light on how these contribute to autoimmunity, and how they could be therapeutically modulated.

In this preliminary study, we addressed this question by infecting mice with vesicular stomatitis virus (VSV) and analyzing responses in animals where IL-2 was blocked with an antibody (JES6), as well as mice with impaired IL-2 production due to deletion of an enhancer element that augments IL-2 expression. Virus-specific $CD8^+$ T cells expanded normally in mice with altered or absent IL-2 signaling compared to controls. Treg cells were reduced in mice with abnormal IL-2 signaling to a similar extent whether or not the mice were infected with virus. In contrast, $CD44^+CD62L^-$ CD4 Teff cells showed increased proliferation in infected mice with impaired or blocked IL-2 signaling compared to controls, but not uninfected mice. $CD44^+CD62L^-$ CD4 Teff cell responses to viral peptide stimulation were similar regardless of the level of IL-2 signaling. Our data suggest that viral infection under suboptimal IL-2 conditions allows expansion of bystander $CD4^+$ T cells that could be the early stages of an autoinflammatory or autoimmune state.

Results

Disrupted IL-2 signaling does not impair acute $CD8^+$ T cell response to VSV

To investigate the role of IL-2 signaling during acute infection, WT mice were infected intranasally (i.n.) with VSV or given PBS as a control. Within both the infected and uninfected groups, mice were then treated with JES6 i.p. to block IL-2 signaling, or PBS as a control. Mice were sacrificed on day 7 post infection, and the spleen, lung, and lung-draining mediastinal lymph node (medLN) were assessed for antigen-specific $CD8^+$ T cell responses using a VSV nucleoprotein (N) MHC-I [H-$2K^b$/RGYVYQGL] tetramer. As expected, all mice infected with

VSV displayed a robust population of tetramer^{+}CD44^{hi} antigen-specific CD8^{+} T cells in all organs analyzed, and treatment with JES6 did not hinder priming or expansion of these cells (Figure 4.1 A-B). At this timepoint after infection, CD8^{+} T cells can be divided into terminal effector cells (TECs) which express KLRG1, and memory-precursor effector cells (MPECs) which express CD127. Frequencies of TECS and MPECs were not significantly different between VSV and VSV + JES6 treated mice (Figure 4.1 C-D). Thus, blockade of IL-2 during VSV infection does not alter the activation or differentiation of antigen-specific CD8^{+} T cells at day 7.

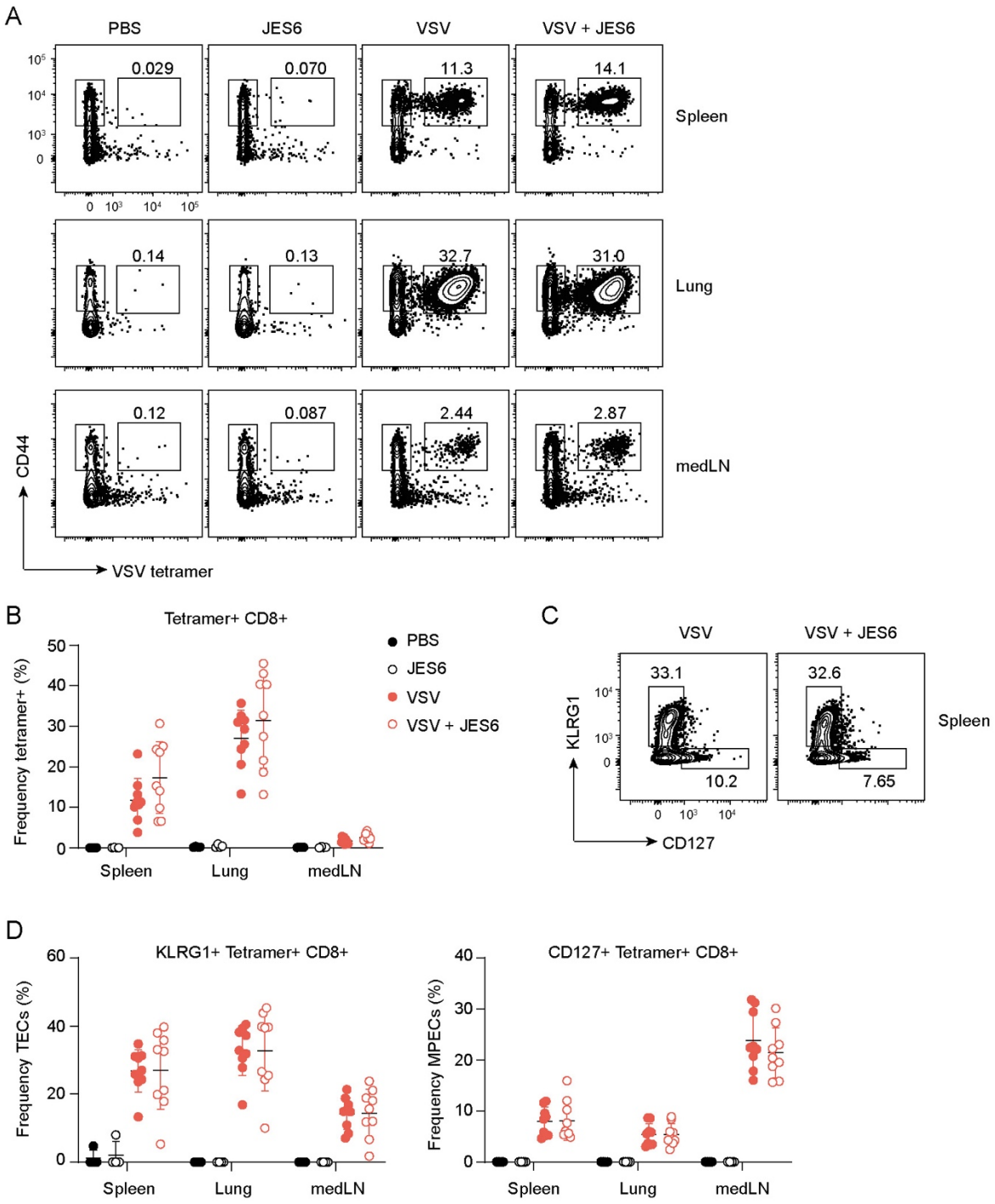

Figure 4.1. VSV-specific CD8$^+$ T cell expansion and differentiation occurs in the absence of IL-2 signaling

WT mice were infected i.n. with VSV-Ova or PBS as a control on day 0, and treated i.p. with the IL-2 blocking antibody JES6 or PBS as a control on days 1 and 5 before sacrifice at day 7 for analysis. (A) Representative flow cytometric analyses of VSV tetramer and CD44 expression by gated CD8$^+$ T cells from the spleen, lung, and medLN. Tetramer$^+$ CD8$^+$ T cells are gated as

indicated. (B) Graphical analysis of frequency of tetramer$^+$ CD8$^+$ T cells in each treatment group and each organ analyzed. (C) Representative flow cytometric analyses of CD127 and KLRG1 expression by gated tetramer$^+$ cells from the spleen. (D) Graphical analysis of frequency of KLRG1$^+$CD127$^-$ and KLRG1$^-$CD127$^+$ tetramer$^+$ cells in each organ analyzed. Data is combined from two independent experiments, 4-9 mice per group total. Significance determined by two-way ANOVA with Tukey post-test for multiple comparisons.

IL-2 blockade during VSV infection alters the balance of Treg and CD4$^+$ Teff cells

Next, we examined both the regulatory and conventional bulk (not antigen-specific) CD4$^+$ T cell compartments in mice infected with VSV compared to controls. We previously showed that blocking IL-2 with JES6 reduced Treg cells after 7 days, and this reduction in Treg cells was similar in mice whether they were infected with VSV or uninfected across all organs analyzed (Figure 4.2A). IL-2 signaling was completely blocked in both uninfected and VSV infected mice that received JES6, as shown by an absence of p-STAT5 in splenic Treg cells directly ex vivo (Figure 4.2B). CD44$^+$CD62L$^-$ Foxp3$^-$ CD4$^+$ Teff cells did not increase in uninfected mice treated with JES6 compared to controls (Figure 4.2C). However, in the spleen and lung, all VSV infected mice had expanded CD4$^+$ Teff cells compared to uninfected, and VSV + JES6 mice had significantly higher frequencies over VSV infected mice that received i.p. PBS. Thus, IL-2 blockade during acute infection rapidly disrupts the balance of Treg and CD4$^+$ Teff cells.

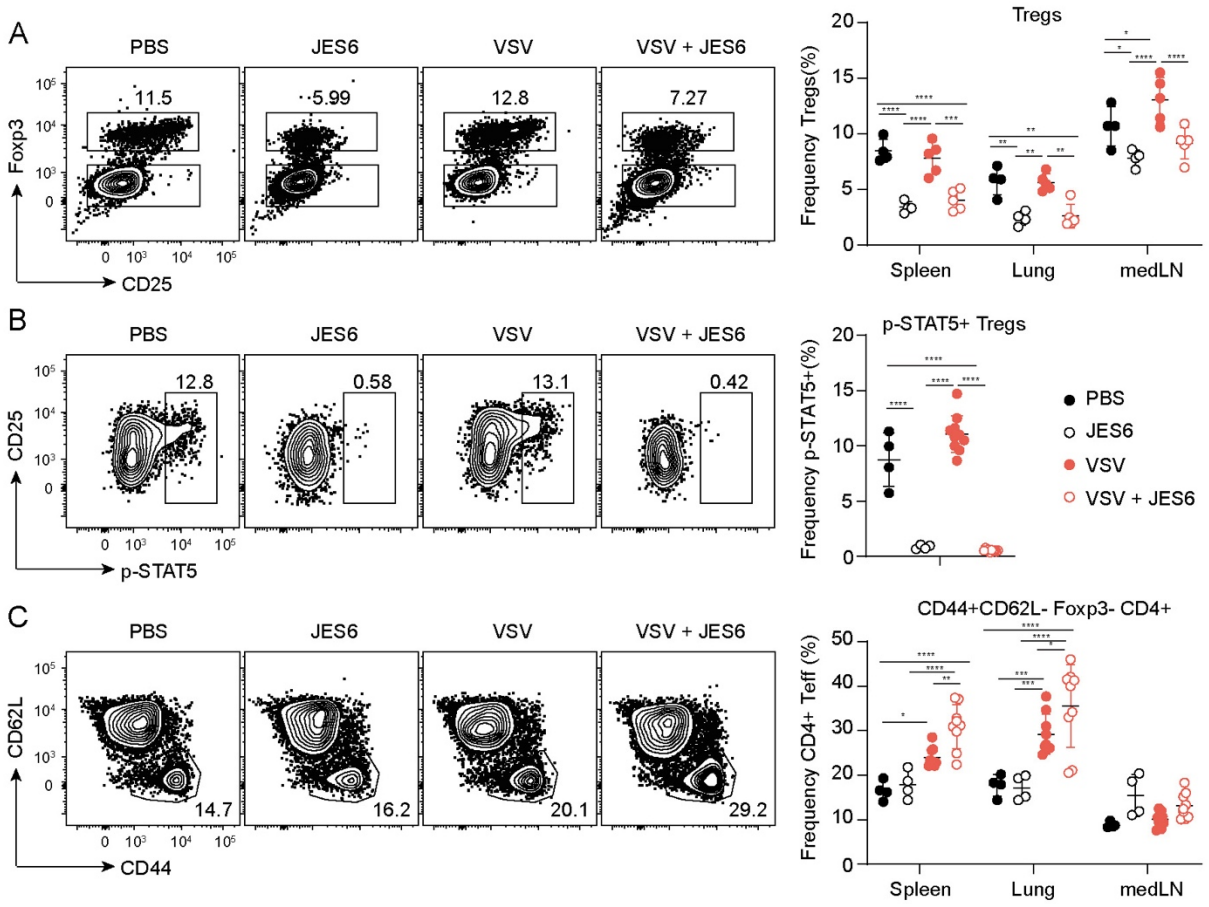

Figure 4.2. Differential effects on Treg and CD4$^+$ Teff cells with IL-2 blockade during infection

WT mice were infected i.n. with VSV-Ova or PBS as a control on day 0, and treated i.p. with the IL-2 blocking antibody JES6 or PBS as a control on days 1 and 5 before sacrifice at day 7 for analysis. (A) Representative flow cytometric analyses of CD25 and Foxp3 expression by gated CD4$^+$ T cells from the medLN. Foxp3$^+$ Treg cells are gated as indicated. Right, graphical analysis of frequency of Treg cells in each treatment group and each organ analyzed. Data are from one representative experiment, 4-5 mice per group. (B) Representative flow cytometric analysis of p-STAT5 and CD25 expression by gated Treg cells from the spleen. pSTAT5$^+$ Treg cells are gated as indicated. Right, graphical analysis of frequency of pSTAT5$^+$ Treg cells in the spleen in each treatment group. (C) Representative flow cytometric analysis of CD44 and CD62L expression by gated Foxp3$^-$ CD4$^+$ T cells in the spleen. CD44$^+$CD62L$^-$ CD4$^+$ Teff cells are gated as indicated. Right, graphical analysis of frequency of CD4$^+$ Teff cells in each treatment group and organ analyzed. Data in B and C are combined from two independent experiments, 4-9 mice per group total. Significance determined by two-way ANOVA with Tukey post-test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Impact of IL-2 signaling on T cell IFN-γ production in VSV infection

The expanded population of $CD44^+CD62L^-$ $CD4^+$ Teff in VSV-infected mice treated with JES6 mice could be specific to VSV, specific to self, commensal, or other antigens, or a combination. In order to test the magnitude of the $CD4^+$ T cell VSV specific response, we cultured splenocytes from all four groups of mice with or without an MHCII-restricted VSV peptide for 6 hours in the presence of monensin and measured IFN-γ production by flow cytometry. Both VSV and VSV + JES6 mice had a small frequency (1% or less) of $CD44^+CD62L^-$ $CD4^+$ Teff cells that made IFN-γ (Figure 4.3A-B). As there was no significant difference between the frequency of responding cells in VSV + JES6 compared to VSV, we hypothesize that the majority of the expanded $CD44^+CD62L^-$ $CD4^+$ Teff population found in VSV + JES6 mice is not virus specific. We also stimulated splenocytes from all groups of mice with PMA and ionomycin in the presence of monensin, in order to measure their overall potential to produce IFN-γ. In both the $CD4^+$ and $CD8^+$ Teff compartments, there was a trend towards higher frequencies of cells from VSV + JES6 mice that made IFN-γ compared to VSV (Figure 4.3A, C).

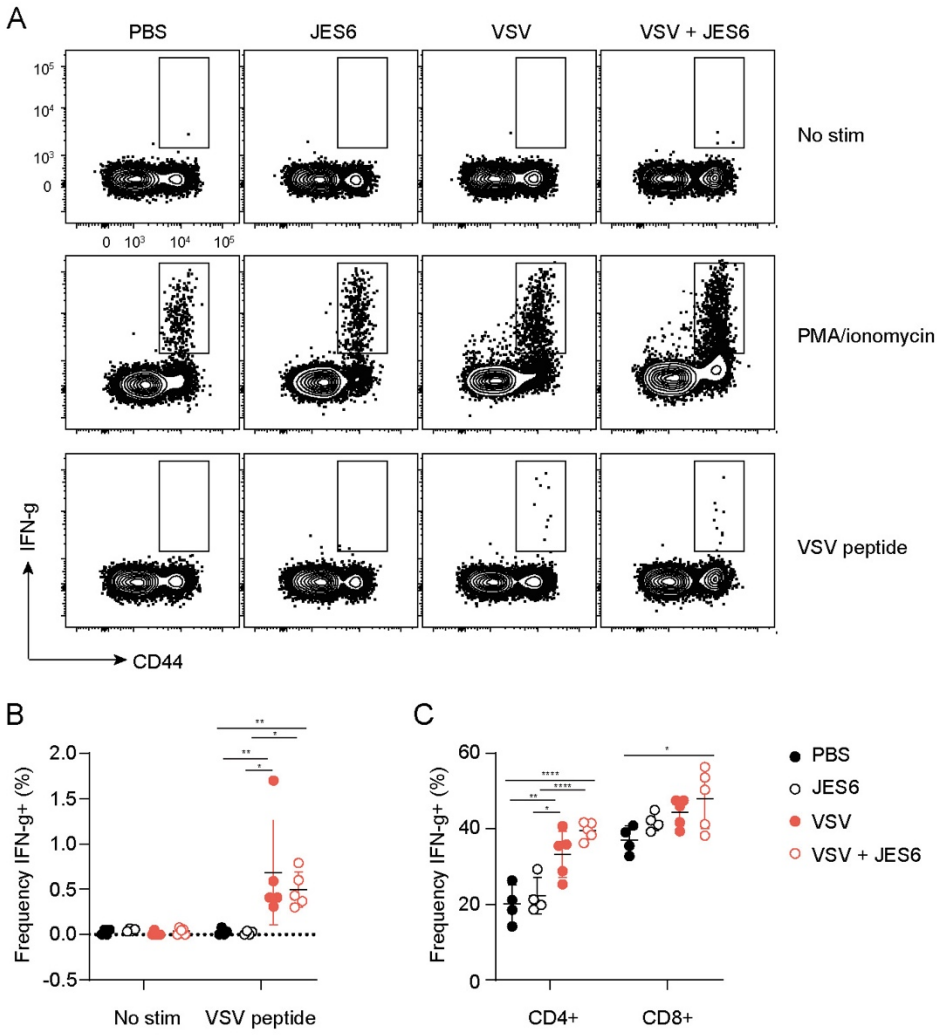

Figure 4.3. CD4$^+$ T cell IFN-γ production in response to VSV infection is IL-2 independent

WT mice were infected i.n. with VSV-Ova or PBS as a control on day 0, and treated i.p. with the IL-2 blocking antibody JES6 or PBS as a control on days 1 and 5 before sacrifice at day 7 for analysis. Splenocytes from each mouse were restimulated for 6 hours at 37°C in the presence of monensin with media (no stim), PMA and ionomycin, or VSV peptide. (A) Representative flow cytometric analyses of CD44 and IFN-γ expression by gated CD4$^+$ T cells from the spleen under different stimulation conditions. CD44$^+$IFN-γ$^+$ CD4+ T cells are gated as indicated. (B) Graphical analysis of IFN-γ$^+$ cells as a frequency of CD44$^+$ CD4$^+$ T cells in each treatment group after no stim or VSV peptide stimulation. (C) Graphical analysis of IFN-γ$^+$ cells as a frequency of CD44$^+$ CD4$^+$ or CD8$^+$ T cells in each treatment group after PMA and ionomycin stimulation. Data is from one representative experiment, 4-5 mice per group. Significance determined by two-way ANOVA with Tukey post-test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

IL-2-83E mice display normal T cell populations in the thymus and periphery

To further explore the T cell response to VSV infection under altered IL-2 conditions, we performed similar experiments in mice with impaired IL-2 production. These mice, called IL-2-83E, have a CRISPR/CAS9 mediated deletion of a response element 83kb upstream of the IL-2 promotor, which normally undergoes chromatin remodeling and loops to interact with the IL-2 promotor in a CD28-dependent manner [149]. $CD4^+$ T cells from these mice produce about half the amount of IL-2 when stimulated *in vitro* compared to WT controls (data not shown). At baseline, IL-2-83E mice did not display any overt immune phenotypic differences in the T cell compartment compared to WT mice. T cell development in the thymus appeared normal, as no differences were detected in frequencies of CD4 single positive (SP), CD8SP, or $CD4^-CD8^-$ double negative (DN) T cells (Figure 4.4A). Treg cells can develop in the thymus through two different pathways. $CD25^+Foxp3^-$ Treg precursors upregulate CD25 first upon strong TCR stimulation, and only express Foxp3 after IL-2 and STAT5 signaling to become mature Treg cells [150, 151]. In contrast, $CD25^-Foxp3^+$ Treg precursors first express Foxp3, but still require IL-2 signaling to survive apoptosis and further differentiate [152]. These developmental pathways lead to different suppressive functions [153]. IL-2-83E mice displayed normal frequencies of the various Treg cell precursors compared to WT mice (Figure 4.4B), indicating that thymic Treg cell development is not altered in these mice. In the periphery, frequencies of $Foxp3^+$ Treg cells, $CD44^+CD62L^-$ $CD4^+$ and $CD8^+$ Teff cells were largely unaffected by impaired IL-2 production in IL-2-83E mice, with the exception of a significantly reduced frequency of Treg cells in the peripheral LN (pLN) in IL-2-83E mice compared to WT controls. (Figure 4.4C-E).

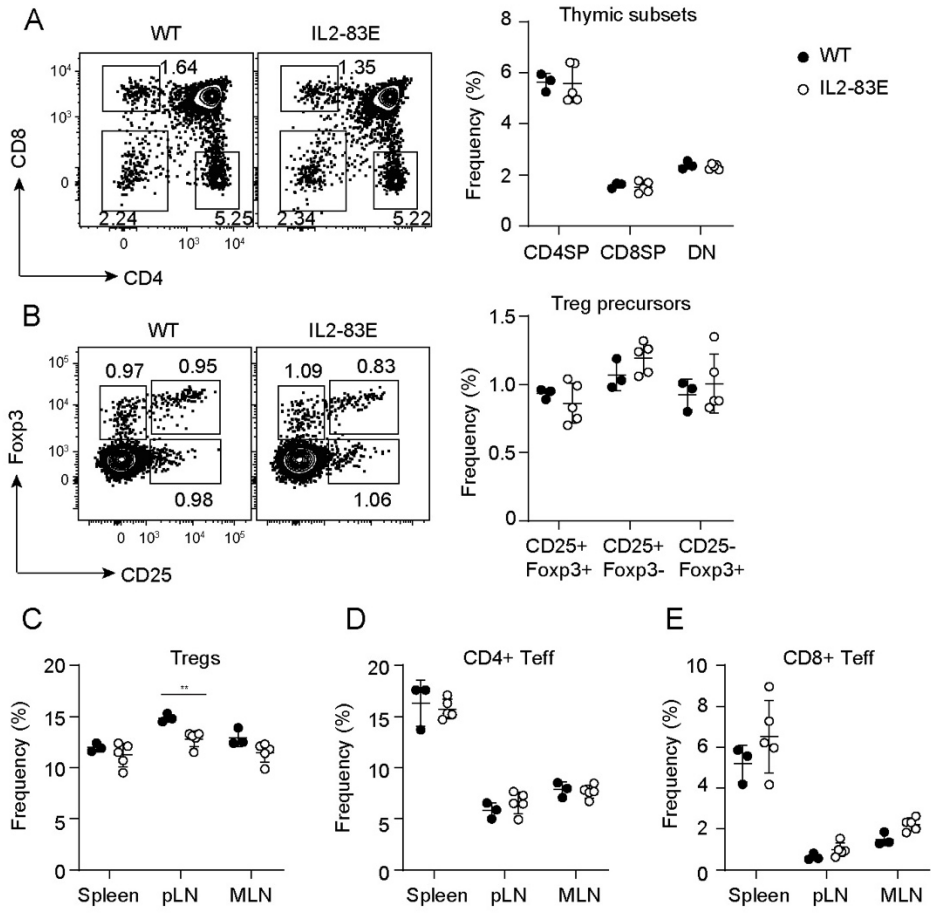

Figure 4.4. T cell populations in IL-2-83E mice at homeostasis

(A) Representative flow cytometric analyses of CD4 and CD8 expression by gated TCRβ$^+$ thymocytes in WT and IL-2-83E mice. Right, graphical analysis of frequencies of CD4SP, CD8SP and DN thymocytes. (B) Representative flow cytometric analysis of CD25 and Foxp3 expression by gated CD73$^-$ CD4SP thymocytes in WT and IL-2-83E mice. Right, graphical analysis of frequencies of CD25$^+$Foxp3$^+$, CD25$^+$Foxp3$^-$, and CD25$^-$Foxp3$^+$ thymocytes. Graphical analysis of frequencies of Treg (C), CD44$^+$CD62L$^-$ CD4$^+$ Teff (D), and CD44$^+$CD62L$^-$ CD8$^+$ Teff (E) cells in spleen, pLN and MLN from WT and IL-2-83E mice. Data are from one representative experiment, 3-5 mice per group. Significance determined by multiple t-tests with a Holm-Sidak correction. **p<0.01

Impaired IL-2 production by IL-2-83E mice leads to similar response to VSV infection as IL-2 blockade

Upon VSV infection, no significant differences in the frequency of antigen-specific CD8$^+$ cells (Figure 4.5A), or TECS and MPECs (Figure 4.5B-C) within that population, were observed

between WT and IL-2-83E mice. We did detect significant decreases in the frequency of Foxp3$^+$ Treg cells in the medLN and lung, with a trending decrease in the spleen of IL-2-83E mice compared to controls (Figure 4.5D). At this day 7 timepoint, Treg cells from both WT and IL-2-83E mice had normal levels of p-STAT5 (Figure 4.5E). Intriguingly, we also observed an increase in the frequency of CD44$^+$CD62L$^-$ CD4$^+$ Teff in IL-2-83E mice compared to WT in the spleen and lung (Figure 4.5F), mirroring what we found when we blocked IL-2 with JES6 during infection.

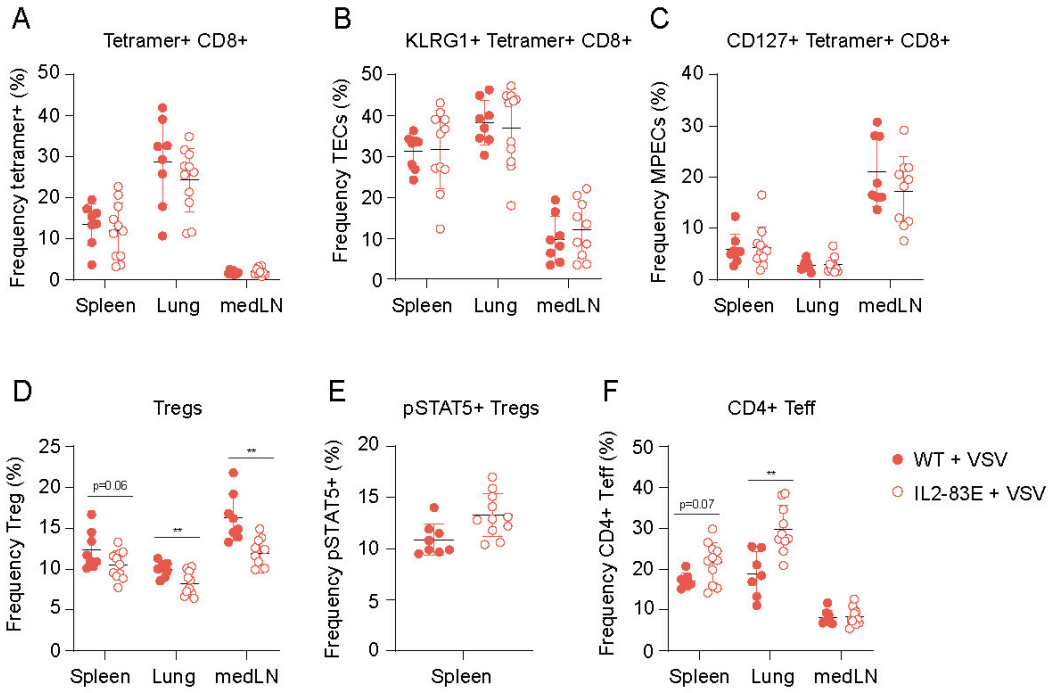

Figure 4.5. IL-2-83E mice have normal CD8$^+$ responses but impaired CD4$^+$ responses to VSV infection

WT and IL-2-83E mice were infected i.n. with VSV-Ova on day 0 and sacrificed at day 7 for analysis. Graphical analysis of frequencies of tetramer$^+$ CD8$^+$ T cells (A), KLRG1$^+$CD127$^-$ tetramer$^+$ cells (B), KLRG1$^-$CD127$^+$ tetramer$^+$ cells (C), Foxp3$^+$ Treg cells (D), pSTAT5$^+$ Treg cells (E), and CD44$^+$CD62L$^-$ CD4$^+$ Teff cells (F) in the spleen, lung and medLN in each genotype. Data is combined from two independent experiments, 8-11 mice per group total. Significance determined by two-way ANOVA with Tukey post-test for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Discussion

The role of IL-2 during T cell-dependent responses *in vivo* remains somewhat unclear. T cells deficient in IL-2 or IL-2R components appear to be largely able to activate, proliferate, and differentiate in response to antigenic challenge [36]. However, expression of IL-2 and its receptor is complex and dynamic, especially during an acute immune response. The fine regulation of this pathway, many aspects of which we still do not understand, is critical to achieving the balanced IL-2 bioactivity needed for controlled immune responses and maintenance of immunologic tolerance. Our data suggest that while IL-2 is not required to mount a response to pathogen, if concentrations are suboptimal, the balance of $CD4^+$ Treg and Teff cells needed to maintain immune homeostasis may not be restored.

In this preliminary study, we found that $CD8^+$ Teff cells do not seem to be impaired in the primary immune response to VSV infection, but $CD4^+$ Teff cell responses are altered when IL-2 signaling is attenuated. Treg cells decrease with IL-2 blockade via JES6 whether infection is present or not, but only in presence of infection, or with treatment for longer period of time as in Chapter 3, do we see outgrowth of $CD4^+$ Teff cells. Similarly, mice harboring a mutation that reduces IL-2 production from $CD4^+$ T cells upon activation show a decrease in Treg cells and increase in $CD4^+$ Teff cells after infection. It remains to be seen what the recall responses would be like in an animal where IL-2 is blocked in primary response, or production is impaired.

We speculate that perhaps IL-2 serves an important role in the peripheral adult immune system to focus the immune response toward pathogen and away from self or commensal antigens. The fact that we observed similar frequencies of IFN-γ producing $CD4^+$ Teff cells in response to restimulation with VSV peptide, regardless of IL-2 signaling during priming, suggests that the expanded $CD4^+$ Teff cells in animals with blocked or reduced IL-2 signaling

are not virus-specific. The specificity of these T cells is the subject of ongoing work, and whether the expansion of these $CD4^+$ Teff is dependent on Treg cell reduction, lack of IL-2 signaling, or a combination remains to be determined.

We show that Treg cells maintain immune homeostasis in the short term absence of IL-2 (JES6 treatment) or at suboptimal IL-2 levels (IL-2-83E mice), but this control is disrupted by viral infection. Our findings in Chapter 3 detailed the loss of immune homeostasis with longer term IL-2 blockade, but it appears to be accelerated with infection. More work is required to determine if this 'trigger' needs to be a replicative antigen, or whether just an adjuvant would be sufficient. These studies could be highly informative in our understanding of how interactions between differences in IL-2 bioavailability and environmental or pathogenic insults may lead to immune dysregulation. Furthermore, using tools to manipulate the IL-2 signaling pathway, such as antibodies or muteins, at different points relative to immune responses could inform new therapeutic interventions to help with delaying or preventing autoimmunity.

Chapter 5: Concluding remarks

The balance of Treg and Teff cells is a central element in determining the outcome of immune responses, and the IL-2/CD25 signaling axis is a critical mediator of this balance. Despite extensive research devoted to uncovering the precise role of IL-2 signaling in Treg cell regulation in the periphery, our understanding is still incomplete. We demonstrate here that at homeostasis, residual CD25 function, even in the face of inhibitory or depleting antibodies, can maintain Treg cell preferential access to IL-2 and therefore allow preservation of immune homeostasis to varying degrees. Total blockade of IL-2 is initially well tolerated but gradually progresses to immune dysregulation, mostly due to defects in Treg cell suppressive function (Figure 5.1). DCs relieved of Treg cell-mediated suppression upregulate costimulatory molecules and contribute to activation and expansion of $CD4^+$ Teff cells. Reduced Treg cell numbers due to treatment with the CD25-depleting antibody also leads to expansion of $CD8^+$ Teff cells, likely due to increased IL-2 availability. Finally, we show definitively that PC61 does not block IL-2 signaling, as Treg cells in the presence of $PC61^{N297Q}$ sustained normal levels of IL-2 signaling and preserved immune homeostasis after treatment for four weeks. Our work clarifies the functionality of commonly used IL-2 and CD25 antibodies in mouse models, which is critical for correct interpretation of the role of IL-2 signaling in the periphery in healthy adult mice.

In the context of acute infection, we found that IL-2 blockade leads to an imbalance in Treg and Teff cells similar to what we observed at homeostasis but occurring much more rapidly (Figure 5.2). Attenuated IL-2 signaling, as in the IL-2-83E mice, is not sufficient to prevent this immune dysregulation during acute infection. Expansion of $CD4^+$ Teff cells is often required to control the invading pathogen, but upon resolution of infection the balance of Treg and Teff cells is restored. Future work is needed to determine the consequences of this exacerbated imbalance

in $CD4^+$ T cell populations under absent or suboptimal IL-2 concentrations. We speculate this may indicate a novel role for IL-2 signaling in the periphery in helping to focus the immune response to pathogen.

Attempts to manipulate the IL-2 pathway to prevent or treat autoimmunity have been successful in specific pathogen free (SPF) laboratory mice, but these have translated with limited success into human patients. Indeed, the meticulous husbandry of SPF animals does not accurately reflect the immune environment in which most humans live. The substantial impact of microbial exposure on shaping immune repertoire and response to stimuli has been well documented and more recently appreciated by the immunology field. Our speculation that IL-2 may serve to focus the immune response toward pathogen and away from self and innocuous environmental antigens in the peripheral immune system points to the value of future research using models with increased microbial exposure, which may provide better predictive power for clinical outcomes of manipulation of the IL-2 signaling pathway.

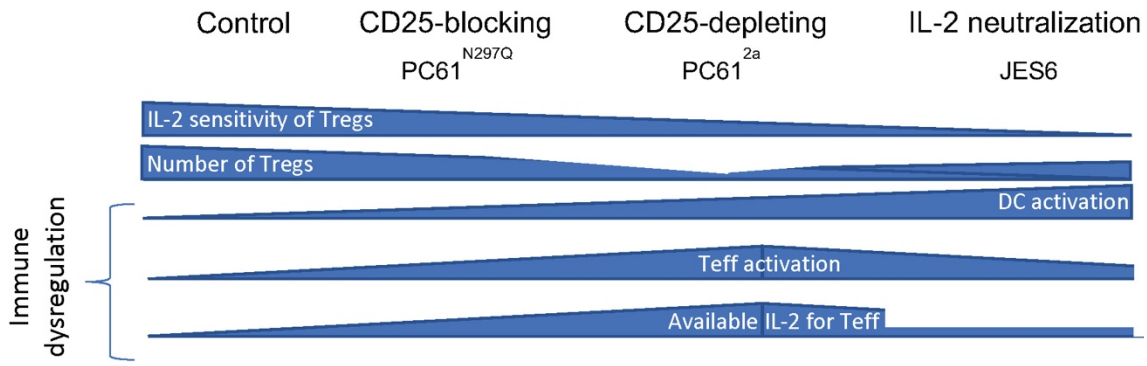

Figure 5.1. Effects of CD25 and IL-2 antibodies at homeostasis.

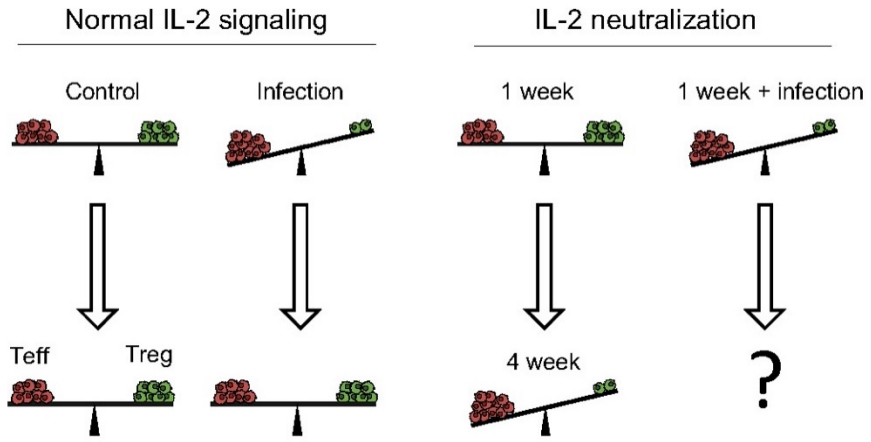

Figure 5.2. IL-2 focuses the immune response to pathogen.

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