Divergent effects of a Treg-selective IL-2 mutein on Influenza specific T cell responses

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Abstract

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Enhancing regulatory T cell (Treg) function offers a compelling therapeutic strategy for autoimmune disease. Engineered IL-2 muteins more selectively expand functional Tregs with minimal impact on other immune cells compared to wild type IL-2, but their potential to compromise antiviral immunity remains largely unexplored. Here, we used a murine model of Influenza A virus (Flu) infection to determine how IL-2 mutein shapes T cell responses to respiratory virus infection. IL-2 is a pleiotropic cytokine and may have divergent effects depending on the time of administration relative to infection. IL-2 mutein administration prior to infection suppressed Flu-specific (Flu-sp) CD8 T cell responses and altered their localization and phenotype within the lungs, without affecting bystander CD8 T cells. This suppression correlated with reduced antigen presentation molecule expression on conventional dendritic cells (cDCs) early after infection but did not impact Flu-sp CD8 T cell priming. In contrast, administering IL-2 mutein during infection exacerbated disease and drove CD25-dependent expansion of Flu-sp CD8 T cells. Despite these opposing effects on effector responses, regardless of

when Fc.Mut24 was given relative to infection, Fc.Mut24-treated mice generated robust antibody responses and protective T cell memory which were maintained for at least 170 days. These findings reveal that Fc.Mut24 has temporally distinct effects on antiviral immunity, dampening early effector responses when given before infection, but enhancing effector expansion and disease severity when delivered during infection. Our results provide critical context for the therapeutic application of IL-2 muteins and highlight the importance of treatment timing in balancing immune modulation with protective immunity.

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Chapter 1: Introduction

Immune Tolerance & Breakdown

A fundamental feature of the immune system is its ability to distinguish danger from safety. The classical 'self' versus 'non-self' theory has been useful but is incomplete: immune responses to self-antigens occur even in healthy individuals (Yu et al., 2015). Normally, these responses are restrained by mechanisms of immune tolerance. When these checkpoints fail, especially in the context of danger signals, the immune system can mount destructive responses against self, leading to autoimmunity (Matzinger, 1994; Pradeu and Cooper, 2012). Autoimmunity represents a growing clinical concern and arises from a breakdown in tolerance mechanisms (Hayter and Cook, 2012). The first barrier against self-reactivity is central tolerance in the thymus. Developing thymocytes undergo positive selection for recognition of self–MHC and negative selection to eliminate clones with high affinity for self-antigens (Klein et al., 2014; Anderson and Su, 2016). Cells that survive this process largely emerge as conventional T cells, CD4 or CD8 T cells that are equipped to recognize foreign peptides and mount protective immune responses upon pathogen encounter (Goodnow et al., 2005). However, a subset of self-reactive CD4 T cells go on to form what are known as Regulatory T cells (Tregs), which serve as the breaks to the immune system.

Conventional T cells

Naïve $CD4^+$ and $CD8^+$ T cells circulate through secondary lymphoid organs via the blood and lymph, scanning antigen-presenting cells (APCs) for their cognate peptide–MHC (pMHC) complexes. Their activation requires three signals. The first signal is TCR recognition of pMHC, which triggers downstream signaling cascades including NFAT, NF-

κB, and AP-1. The second is co-stimulation, such as CD28:CD80/CD86 interactions, which reinforce survival and prevent anergy and apoptosis. The third is cytokine input from the surrounding environment or APCs, which shapes lineage commitment and effector function. Together these three signals are crucial to mount an effective and appropriate immune response to a given peptide.

While $CD4^+$ and $CD8^+$ T cells share these activation requirements, they differ in their response to and production of IL-2. $CD4^+$ T cells are the primary source of IL-2, rapidly producing it upon activation (Malek and Castro, 2010). They constitutively express the intermediate-affinity IL-2 receptor (CD122 and CD132) and, upon receiving signals 1 and 2, transiently upregulate CD25 (IL-2Rα) to form the high-affinity IL-2 receptor, enabling robust proliferation and effector differentiation (Smith, 1984). By contrast, $CD8^+$ T cells produce little IL-2 themselves but strongly upregulate CD25 in response to TCR and co-stimulatory signals, allowing them to consume IL-2 from their environment (Kalia et al., 2012). IL-2 signaling promotes $CD8^+$ proliferation and the acquisition of effector molecules. Together these cells can promote inflammation and mount immune responses to clear pathogens. Despite the rigor of central tolerance, some autoreactive T cells escape into the periphery. Here, peripheral tolerance mechanisms, particularly those mediated by regulatory T cells (Tregs), are critical to prevent autoimmune disease (Josefowicz et al., 2012).

Regulatory T cells

$CD4^+FoxP3^+$ regulatory T cells (Tregs) suppress aberrant immune responses and play an essential role in maintaining peripheral tolerance. Researchers first revealed the importance of Tregs when they transferred $CD4^+CD25^+$ T cells into thymectomized mice

and reversed fatal inflammation (Sakaguchi et al., 1995). The identification of FoxP3 as the lineage-defining transcription factor, demonstrated in the scurfy mouse and in patients with IPEX syndrome, established Tregs as a central regulator of immune homeostasis (Bennett et al., 2001; Brunkow et al., 2001). In the absence of Tregs, as seen in scurfy mice or in humans with IPEX, uncontrolled activation of self-reactive T cells leads to widespread multi-organ autoimmunity, lymphoproliferation, and early mortality, highlighting their non-redundant role in restraining immune responses (Bennett et al., 2001). It was later understood that Tregs arise primarily in the thymus, where they are selected from $CD4^+$ T cell precursors that recognize self-peptide:MHC class II complexes with intermediate-to-high affinity, a process dependent on TCR signaling (Josefowicz et al., 2012). This unique developmental pathway enables Tregs to prevent autoimmunity by restraining self-reactive T cells that escape deletion during normal development. Indeed, impaired Treg number or function is linked to a spectrum of autoimmune diseases, including type 1 diabetes and multiple sclerosis, emphasizing their essential role in immune tolerance (Wing et al., 2019).

IL-2 and Tregs

Tregs depend critically on IL-2 for survival and function and are therefore IL-2 is also implicated in many autoimmune diseases. Unlike conventional $CD4^+$ T cells, they cannot produce IL-2 but constitutively express the high-affinity IL-2 receptor (Fontenot et al., 2005; Wu et al., 2006; Setoguchi et al., 2005). This dependence ties their maintenance and suppressive capacity to the availability of IL-2 while balancing their population with conventional $CD4^+$ T cells (Setoguchi et al., 2005). When IL-2 production wanes or Tregs fail to consume it effectively, Tregs cannot prevent self-reactive T cells and autoimmune

disease can emerge, often proving fatal (Sadlack et al., 1995; Suzuki et al., 1995). Beyond survival, IL-2 signaling through STAT5 directly sustains Foxp3 expression and the transcriptional program of Tregs, ensuring their lineage stability (Burchill et al., 2007; Yao et al., 2007). Consistent with this, therapeutic augmentation of IL-2 has been shown to selectively expand and stabilize Tregs in both preclinical and clinical settings, highlighting the cytokine's potential as an immunotherapy for autoimmunity (Koreth et al., 2011; Saadoun et al., 2011).

Treg Suppressive mechanisms

Tregs suppress conventional T cells and prevent autoimmunity through multiple mechanisms that target each of the three activation signals. For signal 1 (TCR:pMHC recognition), Tregs can outcompete effector T cells for antigen-presenting cells (APCs) via their own TCRs, limiting access to pMHC complexes. For signal 2 (co-stimulation), Tregs express CTLA-4, which disrupts CD28:CD80/86 interactions through a process known as trogocytosis (Qureshi et al., 2011). For signal 3 (cytokines), Tregs consume IL-2 through high CD25 expression, depriving conventional T cells of survival and proliferative signals (Kalia et al., 2012). Beyond these canonical pathways, Tregs employ additional suppressive mechanisms: secretion of IL-10 reduces MHC expression and inflammatory cytokine production by APCs (Gondek et al., 2005; Josefowicz et al., 2012); expression of CD39 and CD73 generates extracellular adenosine, which inhibits T cell activation (Deaglio et al., 2007); and production of TGF-β directly dampens effector T cell responses and supports peripheral tolerance (Li et al., 2006). Together, these overlapping strategies restrain autoreactive T cells and maintain immune balance.

Treatment Strategies for Autoimmunity

Rebalancing the immune system has long served as the guiding principle for treating autoimmune disease. The earliest therapeutic approaches relied on broad immunosuppression. Corticosteroids and cytotoxic agents such as azathioprine or cyclophosphamide effectively ablated immune responses but carried profound toxicities, including heightened susceptibility to opportunistic infections, impaired wound healing, and systemic side effects. While these interventions could provide short-term disease control by reducing effector T cells, they did not restore immune tolerance and often led to cumulative morbidity. The advent of monoclonal antibodies represented a pivotal shift. Targeted blockade of inflammatory pathways, exemplified by anti–TNF-α (Ramos-Casals et al., 2007) and anti–IL-6 therapies (Calabrese et al., 2014), offered greater precision and markedly improved outcomes in diseases such as rheumatoid arthritis and inflammatory bowel disease (Garcillan et al., 2022; Dey et al., 2022; Salliot et al., 2009). Nonetheless, these biologics suppressed critical host defense mechanisms, leaving patients with persistent risks of infection and malignancy. Similarly, small-molecule inhibitors of signaling pathways, including JAK/STAT and other kinases (Dowty et al., 2014), provided an additional layer of disease control but shared the limitation of broad immune suppression. Although many of these drugs are effective in many patients do not restore tolerance or cure disease. Resistance develops (partly due to anti-drug antibody responses) and symptoms often return.

Recognition of these shortcomings catalyzed efforts to move beyond suppression toward the active restoration of immune tolerance. Tregs with their central role in enforcing tolerance, emerged as an attractive therapeutic target (Chinen et al., 2016;

Dashwood et al., 2025). Strategies designed to expand or enhance Tregs hold the promise of correcting the underlying immunologic imbalance rather than merely suppressing symptoms. Among these strategies, manipulation of the IL-2 pathway has been particularly compelling. Because Tregs constitutively express the high-affinity IL-2 receptor (CD25/CD122/CD132), they are uniquely poised to respond to limiting amounts of IL-2 (Chinen et al., 2016). Conventional $CD4^+$ and $CD8^+$ T cells, by contrast, typically express only the intermediate-affinity receptor and cannot compete for IL-2 in the steady state. This biology provided the rationale for early trials of low-dose IL-2 therapy in autoimmune disease and graft-versus-host disease, which demonstrated that a limiting amount of exogenous IL-2 could selectively expand Tregs *in vivo*. However, the therapeutic application of native IL-2 is constrained by several limitations. IL-2 undergoes rapid clearance through renal filtration, proteolytic degradation, and receptor-mediated endocytosis, which restricts bioavailability and necessitates frequent dosing, which can lead to off target effects including vascular leak syndrome (Klatzmann et al., 2015). Furthermore, once conventional T cells or NK cells upregulate CD25, they too can respond to IL-2, resulting in immune activation. Together these features narrow the therapeutic window and limit clinical efficacy of low dose IL-2.

Engineering targeted IL-2

Pharmaceutical companies and researchers have increasingly focused on engineering IL-2 variants with improved pharmacokinetics and receptor selectivity to combat the limitations of low dose IL-2 therapy (Scheid et al., 2025; Silverberg et al., 2025). These next-generation IL-2 molecules aim to extend serum half-life, bias receptor binding/signaling toward Tregs, and enable more precise dosing. Protein engineering

strategies have attempted to overcome these barriers by prolonging systemic exposure. Two of the most widely used approaches include covalent attachment of polyethylene glycol (PEG) chains and fusion to the crystallizable fragment (Fc) domain of immunoglobulin G (IgG), typically with effector function disabled (Scheid et al., 2025; Silverberg et al., 2025). Both strategies increase molecular size, reduce renal clearance, and exploit FcRn recycling, thereby extending half-life and supporting more consistent dosing regimens. Parallel efforts have focused on improving receptor selectivity to favor Tregs. Because Tregs constitutively express the high-affinity IL-2 receptor (CD25/CD122/CD132), they are uniquely poised to respond when IL-2 signaling requires CD25 engagement (Scheid et al., 2025; Silverberg et al., 2025; Khoryati et al. 2020). Mutating specific amino acid residues in IL-2 to weaken its interaction with CD122 shifts signaling dependence toward CD25 (Khoryati et al. 2020). This engineering strategy enhances Treg selectivity while reducing the activation of conventional T cells and NK cells. Molecules incorporating these modifications are commonly referred to as IL-2 mutant proteins or muteins.

Our laboratory combined both approaches in a murine IL-2 mutein. We fused IL-2 to an Fc domain containing the N297G mutation to abolish effector function, and we introduced targeted mutations (N103R and V106D**)** to reduce binding affinity for CD122, thereby increasing reliance on CD25 (Khoryati et al. 2020). This dual engineering strategy produced a cytokine with enhanced potency for Tregs and limited off-target activity. Upon administration, the IL-2 mutein robustly expanded Tregs, drove upregulation of effector molecules such as CTLA-4 and IL-10, and increased proliferative activity (Jamison et al., 2024). Expanded Tregs also suppressed antigen presentation by APCs, a hallmark of

their regulatory function (Jamison et al., 2024). Notably, we observed limited impact on off-target populations, while the Fc fusion extended serum half-life to approximately 80 hours. This IL-2 mutein is known as Fc.Mut24. In line with this, in a preclinical model of type 1 diabetes, treatment with Fc.Mut24 prevented disease onset, in contrast to native IL-2, which accelerated incidence (Khoryati et al. 2020). Further, the majority of treated mice remained disease free even after Fc.Mut24 therapy withdrawal, suggesting long-term suppression of autoimmunity. These findings highlight the therapeutic promise of engineered IL-2 variants as tools to restore tolerance by directly augmenting the regulatory arm of the immune system. However, as with any immune-modulating therapy, the broad application of IL-2 muteins raises important questions regarding durability, specificity, and potential drawbacks such as decreased lung disease, secondary infection, or even death.

IL-2-expanded Tregs and Infectious Disease

Tregs play a multifaceted role in infectious disease by restraining immune activation to limit host tissue damage while simultaneously risking impaired pathogen clearance. During infections, Tregs suppress excessive effector T cell responses, curbing immunopathology that can otherwise lead to fatal outcomes. For instance, in respiratory viral infections such as influenza, Tregs reduce pulmonary inflammation and prevent immune-mediated tissue destruction, but this same suppression can blunt antiviral $CD8^+$ T cell responses and delay viral clearance (Betts et al., 2012; Brincks et al., 2013). Importantly, Tregs are not limited to general immune suppression; antigen-specific populations have been identified. For instance, influenza infection elicits HA-specific Tregs, highlighting that regulatory responses can be tailored to viral antigens, with direct

consequences for shaping immunity to respiratory viruses (Bedoya et al., 2013). IL-2–based biologics such as Fc.Mut24, which preferentially expand Tregs by targeting their constitutive high-affinity IL-2 receptor, may amplify these regulatory functions *in vivo* against pathogens (Spangler et al., 2015; Khoryati et al., 2020). While such expansion holds clear therapeutic promise for restoring tolerance in autoimmunity, it also raises the risk of strengthening virus-specific Tregs that may further restrain antiviral responses. In influenza models, Treg expansion preserves lung integrity but prolongs infection, whereas Treg depletion accelerates viral clearance but exacerbates immunopathology (Betts et al., 2012; Brincks et al., 2013).

These findings underscore the double-edged nature of Treg-targeted therapies. On one side, Fc.Mut24-mediated Treg expansion represents a promising strategy to restore tolerance in autoimmune disease; on the other, it may alter protective immunity in the setting of acute viral infections. Thus, the timing, dosing, and clinical context of such therapies will be critical to ensure that the benefits of Treg expansion outweigh the risks of impaired pathogen control.

Respiratory Viral Infection

Viral infections are among the most pervasive and consequential threats to human health, causing acute illness, chronic disease, and global pandemics. Respiratory viruses, in particular, represent a substantial burden due to their high transmissibility, frequent seasonal circulation, and capacity to trigger epidemics and pandemics**.** Seasonal influenza alone causes hundreds of thousands of hospitalizations and deaths each year worldwide, disproportionately affecting young children, the elderly, and immunocompromised individuals. Other respiratory pathogens, including respiratory

syncytial virus (RSV), parainfluenza viruses, and coronaviruses such as SARS-CoV-2, further highlight the persistent vulnerability of human populations to respiratory viral infection. Vaccines have been a cornerstone of prevention, but their efficacy is often limited by rapid viral evolution, including antigenic drift and shift, as well as the relatively short-lived durability of immune memory further necessitating understanding how these pathogens impact patients on immunosuppressive therapies (Yuzefpolskiy et al., 2022; Salliot et al., 2009; Patterson et al., 2024).

Within this context, influenza represents a well-characterized and clinically relevant model for studying antiviral immunity and the effects of immunomodulatory interventions. Influenza viruses elicit both humoral and cellular immune responses, with $CD8^+$ cytotoxic T lymphocytes (CTLs) playing a critical role in clearing infected cells and limiting viral replication. In immunocompromised or broadly immunosuppressed populations, such as patients receiving therapies for autoimmune diseases, these responses can be impaired or altered, increasing susceptibility to severe disease.

Antigen Presentation and Cytokine Cues in Shaping $CD8^+$ Immunity during Influenza

Dendritic cells (DCs) are the principal initiators of antiviral $CD8^+$ T cell responses. During influenza infection, subsets of conventional DCs capture viral antigens and present them via direct infection or cross-presentation pathways (Jenkins et al., 2021). The magnitude and quality of $CD8^+$ priming is shaped by antigen density, duration of presentation, co-stimulatory signals, and cytokine cues. Among these, IL-2 plays a pivotal role in supporting clonal expansion, survival, and differentiation of effector $CD8^+$ T cells (Williams et al., 2006; Cheng et al., 2002). While $CD4^+$ T cells are the dominant source of IL-2, activated $CD8^+$ T cells upregulate the high-affinity IL-2 receptor, allowing

them to respond to IL-2 in their microenvironment (Kalia et al., 2023). Thus, IL-2 availability functions as a key throttle of effector magnitude and sets the stage for memory formation, highlighting a potential intersection with Treg-targeted IL-2 mutein therapies. This additionally could allow CD25-biased IL-2 therapies to have further unintended consequences.

CD8⁺ T Cell Responses in Influenza

Among adaptive immune responses, $CD8^+$ cytotoxic T lymphocytes (CTLs) play a critical role in limiting viral replication and clearing infected cells in a timely manner. However, virus can be cleared, at least in murine models, without CTLs, though the disease is significantly worse, and clearance is delayed (Cao et al. 2016). Following infection, Influenza antigens are captured and presented by dendritic cells (DCs), which migrate to the draining lymph nodes and spleen to prime naïve $CD8^+$ T cells (Jenkins et al., 2020). Activated $CD8^+$ T cells undergo rapid proliferation, differentiate into effector subsets, and traffic to the lung, where they recognize and eliminate infected epithelial cells via perforin- and granzyme-mediated cytotoxicity (Ely et al., 2003). In addition to direct lysis, $CD8^+$ T cells produce antiviral cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor (TNF), which restrict viral replication and recruit additional immune effectors (Ely et al., 2003).

The magnitude and quality of the $CD8^+$ T cell response can determine the outcome of infection. Robust effector responses accelerate viral clearance, whereas delayed or impaired responses permit viral persistence and can exacerbate disease. However, excessive $CD8^+$ T cell activity can also cause collateral tissue damage in the delicate architecture of the lung, contributing to immunopathology and in some instances death

(Newton et al., 2016). Thus, protective $CD8^+$ immunity requires a balance between antiviral efficacy and immune regulation and tissue repair during influenza infection.

Development of Memory and Tissue-Resident $CD8^+$ T Cells during Influenza

Following resolution of infection, the majority of effector $CD8^+$ T cells contract, leaving behind a pool of long-lived memory T cells. Circulating central memory (Tcm) cells provide systemic surveillance, whereas tissue-resident memory Trm $CD8^+$ T cells persist at barrier sites such as the respiratory mucosa (Liang et al., 1994; Werbist et al., 2011). Trm cells are characterized by expression of CD69, which anchors them within tissue, and CD103, which facilitates retention in epithelial niches. These cells can mount rapid recall responses upon re-exposure to related viral strains, even when antibodies fail to neutralize serologically distinct viral variants. In influenza, Trm populations in the lung have been shown to mediate heterosubtypic protection, emphasizing their importance in generating protective memory (Liang et al., 1994).

Therapeutic Considerations: IL-2 Muteins and Influenza-Specific Immunity

Expansion of Tregs with IL-2 muteins, such as Fc.Mut24, presents a double-edged scenario: while Tregs protect lung tissue from immunopathology, they may suppress influenza-specific effector and memory $CD8^+$ T cells, potentially prolonging infection or altering memory responses. Conversely, exogenous IL-2 administration in murine influenza models can enhance effector $CD8^+$ T cell expansion (Cheng et al., 2022). Therefore, Fc.Mut24 may also enhance effector functions on CD8 T cells that have upregulated CD25 leading to increased pathology and detraction from the memory compartment due to hyperproliferation and terminal differentiation. Therefore, elucidating

how IL-2 mutein driven Treg expansion interacts with the response to respiratory viral infection is critical for balancing autoimmune therapy with infection risk.

Summary and Unanswered Questions:

Targeting immune pathways remains an important strategy for treating autoimmune disease, but such interventions risk altering the ability to mount effective and durable immune responses to respiratory viral infections, like Influenza. Administration of an IL-2 mutein, for example, promotes Treg expansion, which could limit dendritic cell priming of $CD8^+$ T cells, thereby impairing viral clearance, enhancing disease severity, and leading to rapidly waning immunity. Conversely, IL-2 can also function as an immune activator by directly stimulating activated $CD8^+$ T cells. This may increase effector cell numbers but at the cost of heightened immunopathology and excessive terminal differentiation, ultimately diminishing the memory pool. In the following work, we assess how IL-2 mutein treatment shapes influenza-specific $CD8^+$ T cell responses when administered either prior to or after infection. We further evaluate its impact on the generation of protective memory and survival following lethal viral challenge.

Chapter 2: Divergent effects of a Treg-selective IL-2 mutein on Influenza specific T cell responses

Introduction:

Increasing regulatory T cell (Treg) abundance and function is a promising strategy for treating autoimmune diseases, as Tregs suppress inflammatory responses that drive disease pathology (1, 2). This can be accomplished by either expanding and re-infusing Tregs in various cell therapy approaches, or by boosting the abundance

and/or function of endogenous Tregs. In the second category, one approach involves targeting the interleukin-2 receptor (IL-2R), which is critical for Treg development and function (3). The IL-2R exists in two distinct forms - a high-affinity form (composed of CD25, CD122, and CD132) is constitutively expressed by Tregs and activated CD4 and CD8 conventional T cells (Tconv), whereas the low-affinity IL-2R (composed of CD122 and CD132), is primarily found on resting naïve and memory Tconv and is highly expressed by NK cells. This differential expression of IL-2R forms helps support Treg homeostasis and function by allowing them to effectively compete for limiting amounts of IL-2 thereby preventing excessive immune activation (4). However, when IL-2 is produced in excess it can act on Tconv to support their differentiation into effector and memory populations (5). This receptor expression pattern also provides the rationale for low-dose IL-2 therapy, which aims to preferentially expand Tregs in autoimmune disease (6).

An emerging approach to more effectively target Treg cells for treatment of autoimmunity is to use engineered IL-2 mutant proteins ('muteins'), which are IL-2 variants designed to enhance Treg selectivity (2, 7). Several Treg-biased IL-2 muteins have been developed, including the Fc-fused IL-2 mutein from Merck (MK-6194) and pegylated IL-2 from Nektar (Rezpegaldesleukin), each of which show enhanced Treg-selectivity and potent Treg expansion in pre-clinical models or early clinical trials (8, 9). Data from Rezpegaldesleukin trials have demonstrated efficacy in improving atopic dermatitis in Phase 1b (9), showing the potential of this therapeutic approach. We developed an Fc-fused murine IL-2 mutein (known as Fc.Mut24) with two amino acid substitutions that decreased CD122 binding and thus increased dependence on CD25 to

induce functional signaling (10, 11). Although Fc.Mut24 is a weaker IL-2R agonist *in vitro* than an Fc-fused version of wild-type IL-2 (Fc.WT), it is highly Treg-selective and more effectively expands Tregs *in vivo*. In addition to increasing Treg abundance, Fc.Mut24 also enhances suppressive functions of Tregs, including CTLA-4-mediated transendocytosis of the co-stimulatory ligands CD80 and CD86 (12). Importantly, unlike Fc.WT, Fc.Mut24 can be administered at high doses while maintaining Treg selectivity and effectively prevented disease progression in the non-obese diabetic (NOD) mouse model of Type 1 Diabetes (10). Thus, Fc.Mut24 provides a novel tool for pre-clinical mechanistic studies of IL-2 mutein therapy.

Given their ability to potently expand Tregs and broadly suppress the immune system, IL-2 muteins represent a promising new class of autoimmune therapies. However, immunosuppressive treatments—including corticosteroids, IL-6 inhibitors, co-stimulation inhibitors, tumor necrosis factor (TNF)-α inhibitors—also impair immune responses to infections and reduce vaccine efficacy (13-17). While these therapies target specific inflammatory pathways, Tregs use multiple immunosuppressive mechanisms to dampen immune responses (18), and therefore therapies that augment Treg abundance and function may be even more detrimental for responses to infection. Respiratory virus infections, such as RSV, Influenza and SARS-CoV-2, are particularly relevant in this context, as several immunosuppressive therapies increase the risk of severe infection (19, 20), and Tregs play a role in dampening immune responses and promoting tissue repair during respiratory virus infections (21, 22), raising the possibility that IL-2 mutein therapy could increase the risk of poor outcomes. Influenza, in particular, remains a major global health burden causing significant morbidity and mortality each year. Given these

risks, it is important to determine whether IL-2 muteins compromise antiviral immunity. In addition, IL-2-based therapies could also promote inflammation by activating T cells that upregulate CD25 and express the high-affinity IL-2R. Therefore, administering IL-2 mutein during infection, when T cells are already activated and have upregulated the high-affinity IL-2 receptor, could enhance signaling and drive excessive T cell activation, proliferation, and immunopathology. Thus how the various activities of IL-2 mutein combine to impact the outcome of respiratory virus infection is complex, and may depend on the timing of IL-2 mutein administration.

We used a murine model of Influenza A virus (Flu) infection to investigate how Fc.Mut24 influences antiviral T cell responses during respiratory infection. Administering Fc.Mut24 before infection suppressed Flu-specific (Flu-sp) CD8 T cells at 9 days post-infection (dpi) and altered their localization and phenotype within the lung. This suppression was specific to Flu-sp CD8 T cells, as bystander cells remained unaffected. Although Fc.Mut24 reduced antigen presentation molecule expression on conventional dendritic cells (cDCs) at 3dpi, the number of Flu-sp CD8 T cells was unchanged at 4dpi. In contrast, administering Fc.Mut24 during infection worsened disease severity and drove an expansion of Flu-sp CD8 T cells that was dependent on cell-intrinsic CD25 expression. Despite the divergent effects of Fc.Mut24 treatment before or during infection, Fc.Mut24-treated mice generated protective memory that persisted through at least 170dpi. These findings demonstrate that the immunologic context in which IL-2 mutein therapy is administered critically shapes antiviral T cell responses, highlighting the need to evaluate how immune-modulating treatments for autoimmunity may influence host defense.

Results:

2.1 Fc.Mut24 treatment before infection suppresses Flu-specific CD8 T cell responses

In patients receiving IL-2 muteins for autoimmunity, administration of mutein and expansion of Tregs just prior to respiratory virus infection could dampen the immune response, worsen outcomes, and compromise development of durable memory against re-infection. To model this possibility, we gave 10 μg of Fc.Mut24 to female C57BL/6 (B6) mice one day before intranasal infection with 2000 plaque-forming units (PFU) of Influenza A virus (strain A/HK-x31(H3N2)) (X31). The relative timing of Fc.Mut24 treatment and infection aimed to maximize Treg expansion during the T cell priming phase to determine how this impacted the Flu-sp immune response. We assessed T cell responses in the lungs and spleen at 9dpi, corresponding to the peak of the effector T response in our model.

Consistent with its ability to potently expand Tregs, Fc.Mut24-treated mice exhibited a higher frequency and total number of Tregs in both lungs and spleen compared to vehicle controls (Figure 2.1A). Flu-sp $CD8^+$ T cells play a critical role in viral clearance, memory formation, and cross-protection against heterosubtypic influenza strains, and we therefore focused our analyses on the impact of Fc.Mut24 treatment on this population, using an MHC class I tetramer recognizing the immunodominant nucleoprotein antigen $(NP)_{366\text{-}374}$ to identify Flu-sp cells. At 9 dpi Fc.Mut24-treated mice had a significant reduction in both the frequency and total number of Flu-sp $CD8^+$ T cells in the lungs (Figure 2.1B) and spleen (Supplemental Figure 2.1a) compared to vehicle-treated controls. The lung is a complex organ that is highly vascularized, and T cells specific for respiratory pathogens can be found in both the lung parenchyma and

patrolling the lung vasculature (23). Therefore, we used intravenous (IV) CD45 antibody labeling to distinguish Flu-sp T cells found in the lung vasculature (IV^+) and lung parenchymal (IV^-) Flu-sp $CD8^+$ T cells. Fc.Mut24-treated mice had a significant enrichment of Flu-sp $CD8^+$ T cells within the lung parenchyma compared to vehicle-treated controls (Figure 2.1C). However, despite this skewed distribution in the lung parenchyma, the total number of Flu-sp $CD8^+$ T cells remained lower in both the lung vasculature (~7-fold reduction) and parenchyma (~3-fold reduction) compared to control animals (Figure 2.1C).

To further investigate the altered differentiation and migration of Flu-sp $CD8^+$ T cells, we analyzed markers associated with tissue residency and vascular retention. Consistent with IV labeling results, the frequency of Flu-sp $CD8^+$ T cells expressing KLRG1 and CX3CR1 (molecules linked to retention in the lung vasculature (24) was reduced in Fc.Mut24-treated mice, whereas expression of the tissue-residency markers CD103 and CD69 was increased (Figure 2.1D). This did not simply reflect their altered distribution, as Fc.Mut24-treated mice had higher expression of CD69 and CD103 compared with control mice even within the lung parenchyma fraction (Supplemental Figure 2.1c), and similarly showed significantly reduced expression of CX3CR1 and KLRG1 in the blood-exposed fraction (Supplemental Figure 1d) compared to the parenchymal compartment. To further determine how Fc.Mut24 treatment altered the functional differentiation of Flu-sp $CD8^+$ T cells, we examined expression of transcription factors and cytokine receptors that promote effector and memory cell differentiation (Supplemental Figure 2.2a). Flu-sp $CD8^+$ T cells from Fc.Mut24-treated mice showed a slight reduction in expression of the transcription factor EOMES, but no difference in

expression of TCF1 or T-bet. However, consistent with increased memory potential, Fc.Mut24 treatment increased expression of the IL-7R component CD127 (25).

Despite the reduced magnitude and altered distribution and phenotype of the Flu-sp $CD8^+$ T cell response, Fc.Mut24 treatment did not affect disease severity, as measured by weight loss (Figure 2.1E), viral RNA load (Figure 2.1F), or NP-specific IgG antibody titers (Supplemental Figure 2.1a) compared to vehicle controls. Notably, while Fc.Mut24 treatment did not alter the magnitude of the Flu-sp $CD4^+$ conventional T cell response (Supplemental Figure 2.1f), there was a decrease in $FoxP3^-$ $CD4^+$ T cells in the lungs, suggesting a broader immunomodulatory effect (Supplemental Figure 2.1g). Thus, Fc.Mut24 treatment resulted in sustained Treg expansion, and decreased Flu-sp $CD8^+$ T cell abundance associated with altered localization and phenotype, with a larger fraction of cells localized to the tissue parenchyma, potentially positioning them for effective viral clearance. This is supported by the lack of change in disease severity or viral loads despite the overall reduction in Flu-sp $CD8^+$ T cells.

Figure 2.1: Fc.Mut24 administration before X31 infection limits Flu-sp CD8⁺ T cell responses. *Mice were treated one day before infection with 2000PFU of X31 with either vehicle (blue) or Fc.Mut24 (red). All data represent takedown at 9dpi.* ***A)*** *Representative flow cytometry analysis of CD25 and Foxp3 expression by gated CD4⁺ T cells in the lungs of vehicle- and Fc.Mut24-treated mice (left), and quantification of the number and frequency of Tregs in the lungs (right).* ***B)*** *Representative flow cytometry analysis of CD44 expression and $NP_{366-374}$ tetramer staining by gated CD8⁺ T cells in the lungs of vehicle- and Fc.Mut24-tretted mice (left), and quantification of the number and frequency of Flu-sp T cells in the lungs (right).* ***C)*** *Representative histogram of IV label staining on gated Flu-sp T cells in lungs of vehicle- and Fc.Mut24-treted mice (left), and quantification of the frequency and number Flu-sp CD8 T cells in parenchymal vs blood exposed compartments (right).* ***D)*** *Representative flow cytometry staining and quantitative analyses showing expression of the selected markers by gated $CD44^{lo}$ naïve CD8⁺ T cells in the lungs of vehicle-treated mice, or by Flu-sp T cells in the lungs of vehicle- or Fc.Mut24-treated mice as indicated.* ***E)*** *Weight loss as a percent of baseline in vehicle- or Fc.Mut24-treated mice during the course of X31 infection.* ***F)*** *Quantification of viral genes M1/M2 relative to HPRT by PCR in vehicle- or Fc.Mut24-treated mice as indicated. Each point represents analysis of an individual animal. All data are representative of two separate experiments *p < 0.05, **p < 0.01, ****p < 0.0001, by unpaired T test for A-C and one way ANOVA with multiple comparison for D and F and with mixed effects for E.*

2.2 Fc.Mut24 Treatment Selectively Limits Flu-Specific Responses Without Suppressing Bystander CD8 T Cells

In addition to reducing the abundance and localization of NP-specific CD8⁺ T cells, Fc.Mut24 treatment also reduced the number of tetramer-negative $CD44^{hi}$ ($CD44^{hi}$) CD8⁺ T cells in the lungs at 9 dpi (Figure 2.2A), and these tetramer-negative cells also tended to localize more toward the lung parenchyma, although this trend did not reach statistical significance (Supplemental Figure 2.3a). These cells also showed a similar shift in expression of markers of T cell migration and functional differentiation as the NP-specific CD8⁺ T cell population (Supplemental Figure 2.3b). Ki-67 expression was also reduced in $CD44^{hi}$ cells from Fc.Mut24-treated mice, suggesting decreased proliferation of these antigen-experienced cells (Supplemental Figure 2.3b). These tetramer-negative cells could represent Flu-sp cells recognizing epitopes other than the $(NP)_{366-374}$ epitope, or

alternatively could be bystander activated $CD8^+$ T cells that also infiltrate the lungs during influenza infection (26, 27). To determine if Fc.Mut24 altered this bystander response, we infected mice intraperitoneally with 2000 PFU of Vesicular Stomatitis virus that expresses the SIINFEKL epitope from ovalbumin (VSV-OVA) and allowed 28 days for resolution. This generated SIIN-specific $CD8^+$ T cells, providing a bystander population that could be identified and tracked via staining with a K^b/SIINFEKL MHCI tetramer. We then treated the mice with Fc.Mut24 or vehicle and, one day later, challenged half the mice intranasally with X31 or mock infection, creating four experimental groups for tracking the SIIN-sp $CD8^+$ T cell response. In contrast with the response of Flu-sp $CD8^+$ T cells, we observed no significant differences in the number of SIIN-sp $CD8^+$ T cells in

the lungs across all groups (Figure 2.2B). Although the frequency of SIIN-sp was reduced in the VSV + Flu + vehicle group,

Figure 2.2: Fc.*Mut24 treatment does not impact bystander CD8 T Cells.* ***A)*** *Mice were treated one day before infection with 2000PFU of X31 with either vehicle (blue) or Fc.Mut24 (red). Representative flow cytometry analysis of CD44 expression and* $NP_{366-374}$ *tetramer staining by gated* $CD8^+$ *T cells in the lungs of vehicle- and Fc.Mut24-tretted mice (left), and quantification of the number and frequency of* $CD44^{hi}Tetramer^-$ *T cells in the lungs (right).* ***B)*** *Mice were infected with VSV-OVA 30d before treatment with vehicle or Fc.Mut24 and X31 infection. All data represent takedown at 9dpi with X31. Representative flow cytometry analysis of CD44 expression and SIINFEKL tetramer staining by gated CD8+ T cells in the lungs of mice treated as indicated (left), and quantitative analysis of the number and frequency of SIIN-sp CD8 T cells across groups (right). N = 5 per group. Each point represents analysis of an individual animal. *p < 0.05, **p < 0.01, ****p < 0.0001, by by unpaired T test for A and one way ANOVA with multiple comparison for B.*

this likely reflects the influx of Flu-sp $CD8^+$ T cells (Figure 2.2B). Moreover, SIIN-sp $CD8^+$ T cells showed no differences

in localization between the parenchyma and blood-exposed compartments across all groups (Supplemental Figure 2.3c). We also found no changes in the expression of selected markers that were altered on $CD44^{hi}$ $CD8^+$ T cells (TCF, CD127, CD69, Ki-67, KLRG1, or CX3CR1) on SIIN-sp $CD8^+$ T cells (Supplemental Figure 3d). Together, these results indicate that

Fc.Mut24 does not suppress the SIIN-sp response, suggesting that the observed suppression in the $CD44^{hi}$ population likely reflects effects on Flu-sp cells that were not directly tracked, rather than non-specific bystander responses.

2.3 Fc.Mut24 treatment prior to infection reduces cDC antigen presentation capacity with minimal impact on CD8 T cell proliferation at 4 days post-Influenza infection

Given the significant reduction in Flu-sp $CD8^+$ T cells during the effector phase of the response, we wanted to determine if this was associated with changes in antigen presentation capacity of conventional dendritic cells (cDCs), which prime Flu-sp $CD8^+$ T cell responses in the draining mediastinal lymph nodes (medLN) and spleen following Flu infection in mice (28). Indeed, by 3dpi, both cDC1 and cDC2 subsets strongly upregulated key antigen presentation markers, including CD80, CD86, and MHC I (Supplemental Figure 2.4a&b). We have previously shown that Tregs expanded through Fc.Mut24 upregulate CTLA-4 which targets co-stimulatory molecules CD80 and CD86 on cDCs, and that IL-10 production from expanded Tregs can modulate surface MHC expression (12). Therefore, to further assess how Fc.Mut24 affects Treg expansion and cDC

activation in the context of Flu infection, we analyzed lung, medLN and spleen at 3 dpi. Fc.Mut24-treated mice showed a marked increase in FoxP3^{+} Tregs in both tissue sites (Figure 2.3A), which exhibited an effector phenotype defined by high expression of CTLA-4 and CD25 (Supplemental Figure 2.4c). Consistent with our previous results in LPS-treated mice and in NOD mice, the fraction of activated CD80^{hi}CD86^{hi} cDC1 and cDC2 subsets in the lungs and spleen were significantly reduced in Fc.Mut24-treated mice at 3 dpi, whereas only cDC2 cells were significantly impacted in the medLN, (Figure 2.3B).

Decreased antigen presentation capacity by cDCs could impair Flu-sp CD8^{+} T cell priming, resulting in the decreased expansion and infiltration of Flu-sp CD8^{+} T cells in the lungs we observed in Fc.Mut24-treated mice. To test this, we adoptively transferred congenically marked OT-I CD8^{+} T cells into Fc.Mut24- or vehicle-treated mice, then infected them with a recombinant X31 expressing the SIINFEKL peptide (X31-SIIN). At 4 dpi, we measured OT-I expansion based on cell counts in the lung, medLN, and spleen. Surprisingly, although the number of OT-1 cells trended lower in both the lungs (Figure 2.3C), spleen (Supplemental Figure 2.4d), Fc.Mut24-treated mice showed no significant reduction in OT-I expansion in any tissue, including the medLN compared to controls at

4dpi, and there were no significant differences in expression of the activation markers CD69, CD25,

Figure 2.3: Fc.Mut24 treatment before X31 infection reduces antigen presentation capacity of DCs in the lungs and spleen. *Mice were treated one day before infection with 2000PFU of X31 with either vehicle (blue) or Fc.Mut24 (red).* ***A)*** *Representative flow cytometry analysis of CD25 and Foxp3 expression by gated $CD4^+$ T cells in the lungs of vehicle- and Fc.Mut24-treated mice (left), and quantification of the number and frequency of Tregs in the lungs (right) at 3dpi.* ***B)*** *Representative flow cytometry analysis of CD80 and CD86 expression by gated cDC1 ($CD3/B220^-Ly6G^-F4/80^-MHCII^{high}CD11c^{high}XCR1^+SIRPalpha^-$) and cDC2 ($CD3/B220^-Ly6G^-F4/80^-MHCII^{high}CD11c^{high}XCR1^-SIRPalpha^+$) cells from lungs at 3dpi with X31 (top). Quantification of activated $CD80^+CD86^+$ cDC1 or cDC2 in vehicle- or Fc.Mut24-treated mice as indicated.* ***C)*** *$CD8^+$ T cells from $CD45.1^+CD45.2^+$ OT-1 mice were enriched and transferred to $CD45.2^+$ C57BL/6 recipients concurrent with vehicle- or Fc.Mut24-treatment one day prior to infection with X31-SIINFEKL. Representative flow cytometry analysis of CD8 and CD45.1 expression by gated live lymphocytes (left), and quantification of the frequency and number of transferred OT-1 T cells (right) from the lungs of recipient mice at 4dpi (right).* ***D)*** *$CD8^+$ T cells from $CD45.1^+CD45.2^+$ OT-1 mice were activated for 48hrs and then transferred into transferred to $CD45.2^+$ C57BL/6 recipients at 3dpi with X31-SIINFEKL. Representative flow cytometry analysis of CD8 and CD45.1 expression by gated live lymphocytes (left), and quantification of the frequency and number of transferred OT-1 T cells (right) from the lungs of recipient mice (right) at 9dpi (6 days after cell transfer). Panels A, C and D are representative of one experiment n = 5 mice per group. Panel B is representative of two separate experiments n = 10 per group. Each point represents analysis of an individual animal. *p < 0.05, **p < 0.01, ****p < 0.0001, by unpaired T test for all panels.*

and CD44 in the lung or spleen (Supplemental Figure 2.4e). Thus, despite reducing co-stimulatory ligand expression by cDC2, pre-treatment with Fc.Mut24 had only minimal effects on the initial activation and tissue localization of virus-specific $CD8^+$ T cells.

In addition to potentially impacting their initial priming, Fc.Mut24-mediated Treg expansion may suppress the migration and activation of Flu-sp $CD8^+$ T cells within the lungs themselves. To test this, we activated OT-I $CD8^+$ T cells *in vitro* with anti-CD3/CD28 Dynabeads for 48 hours to mimic T cell priming and then transferred them into recipient mice that had been pre-treated with vehicle or Fc.Mut24 and infected with X31-SIIN three days earlier and sacrificed the mice for analysis 6 days later (9dpi). Although the

frequency and number of transferred cells were reduced in the spleens of Fc.Mut24-treated mice, we observed no reduction in the number of OT-I cells in the lungs (Figure 2.3D, Supplemental Figure 2.4f). Additionally, rather than being suppressed the transferred OT-1 cells showed increased expression of activation markers in the lungs but not the spleen (Supplemental Figure 2.4g). Together, these results indicate that while Fc.Mut24-driven Treg expansion dampens cDC activation and reduces the expansion and differentiation of Flu-sp cells, it has limited impact on their initial priming, and may even potentiate reactivation of virus-specific $CD8^+$ T cells in the lungs.

2.4 Fc.Mut24 administration during infection enhances Flu-specific CD8 T cell responses

A second important clinical scenario to model for how IL-2 mutein therapy may impact anti-viral immune responses is a patient receiving IL-2 mutein after initiation of infection, which would likely impact the Flu-sp T cell response differently than pre-infection treatment. These effects could include 'off-target' stimulation of $CD25^+$ responding T cells leading to increased proliferation and effector cell differentiation that could enhance viral clearance but exacerbate detrimental immunopathology. However, Tregs also help coordinate tissue repair responses in the lungs during Flu infection, and this may help offset these inflammatory effects. Therefore, to model the effects of post-infection treatment, we administered Fc.Mut24 or vehicle to mice at 4dpi with X31 and analyzed immune responses at 9 dpi (five days after mutein treatment). Fc.Mut24 treatment at 4dpi robustly increased both the number and frequency of Tregs in the lung and spleen (Supplemental Figure 2.5a), confirming that it retained its ability to expand Treg even in highly inflammatory conditions as previously reported (10). However, rather than dampening the Flu-sp $CD8^+$ T cell response, Fc.Mut24 given at 4dpi did not

significantly change the frequency of Flu-sp $CD8^+$ T cells, and increased their absolute number in the lung (Figure 2.4A) and spleen (Supplemental Figure 2.5b) at 9dpi. In

contrast to results from mice

Figure 2. 4: Fc.Mut24 treatment during infection enhances the Flu-specific CD8 T cell response. Mice were treated at 4dpi with X31 with either vehicle (blue) or Fc.Mut24 (green)**.** All data represent takedown at 9dpi. **A)** Representative flow cytometry analysis of CD44 expression and $NP_{366-374}$ tetramer staining by gated $CD8^+$ T cells in the lungs of vehicle- and Fc.Mut24-tretted mice (left), and quantification of the number and frequency of Flu-sp T cells in the lungs (right) **C)** Representative histogram of IV label staining on gated Flu-sp T cells in lungs of vehicle- and Fc.Mut24-treted mice (left), and quantification of the frequency and number Flu-sp CD8 T cells in parenchymal vs blood exposed compartments (right). **C)** Weight loss as a percent of baseline in vehicle- or Fc.Mut24-treated mice during the course of X31 infection. Dotted line indicates time of vehicle or Fc.Mut24 treatment. **D)** Quantification of viral genes *M1/M2* relative to *HPRT* by PCR in vehicle- or Fc.Mut24-treated mice as indicated. **E)** Representative H&E images from mice at 12 days post infection. All data are representative of two separate experiments with n = 10. Each point represents analysis of an individual animal. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$, unpaired T test for panels A and B and one way ANOVA with multiple comparison for D and Mixed Effects for panel C.

pre-treated with Fc.Mut24, we observed a trend toward increased Flu-sp CD8 T cell frequency in the lung vasculature in mice given Fc.Mut24-after infection, and the absolute number of cells in this compartment was significantly increased whereas cell number in the lung parenchyma was not changed (Figure 2.4B). Fc.Mut24-treatment after infection did not alter expression of KLRG1, EOMES T-bet or TCF1, however similar to mice receiving Fc.Mut24 before infection, expression of CX3CR1 was reduced and CD127 expression was dramatically increased in Flu-sp CD8 T cells from the lungs of mice treated with Fc.Mut24 at 4dpi (Supplemental Figure 2.6a).

Unlike mice given Fc.Mut24 before Flu infection, mice treated at 4dpi lost more weight and recovered more slowly than vehicle-treated controls (Figure 2.4C). This was not associated with increased viral RNA in the lungs (Figure 2.4D) and thus is likely due to exacerbated immunopathology (Figure 2.4E). As we observed with Flu-sp CD8+ T cells, Flu-sp CD4+ T cell responses were also increased in mice given Fc.Mut24 at 4dpi (Supplemental Figure 2.5c). However, despite the ability of IL-2 to inhibit the

differentiation of Tfh cells and subsequent antibody responses during infection (29, 30), NP-specific IgG titers were not altered by Fc.Mut24 treatment (Supplemental Figure 2.5d).

2.5 Expansion of Flu-specific CD8 T cells by Fc.Mut24 requires CD25 expression

Fc.Mut24 requires CD25 to initiate IL-2R signaling, and activated STAT5 downstream of this pathway can further amplify CD25 expression through a positive feedback loop (31). We therefore hypothesized that in mice given Fc.Mut24 at 4dpi, CD25 expression on primed Flu-sp $CD8^+$ T cells enables direct stimulation by Fc.Mut24, leading to enhanced expansion and altered differentiation and localization. Indeed, Flu-sp $CD8^+$ T cells expressed higher levels of CD25 in 4dpi Fc.Mut24-treated mice, and thus Fc.Mut24 may directly stimulate responding $CD8^+$ T cells and promote their excessive proliferation (Figure 2.5A). To directly test this hypothesis, we used CRISPR/CAS9 RNPs to delete the *Il2ra* gene (which encodes CD25) in naïve OT-1 T cells (32), and successful targeting was confirmed by assessing CD25 upregulation in targeted cells after *in vitro* stimulation with anti-CD3/anti-CD28 (Supplemental Figure 2.7a). We transferred 5×10^5 cells to recipient CD45.2+ animals prior to infection with X31-SIIN, and infected animals were treated with either vehicle or Fc.Mut24 at 4dpi. Indeed, loss of CD25 significantly impaired the Fc.Mut24-mediated hyper-expansion of OT-1 cells following X31-SIIN infection, whereas there was no significant difference in expansion of control and *Il2ra*-targeted cells in vehicle-treated mice (Figure 2.5B). We also analyzed the response of endogenous host-derived SIIN-sp T cells using K^b/SIINFEKL tetramers. Similar to what we observed for $CD8^+$ T cells specific for the $NP_{366\text{-}374}$ Flu epitope, Fc.Mut24 treatment led to an increased expansion of the endogenous SIIN-sp cells compared to mice that

received vehicle (Figure 2.5C), and thus the changes in expansion we observed are not epitope-specific, but rather reflect direct stimulation of CD25-expressing CD8 T cells by

Figure 2. 5: Fc.Mut24 directly expands Flu-specific CD8 T cells. *$5x10^5$ congenic OT-1 CD8 T cells were transferred to mice, that were a day later infected with X31-SIIN and treated with 10ug of Fc.Mut24 at 4dpi. Data represents 9dpi takedown of lungs of indicated mice.* ***A)*** *Mice were treated at 4dpi with X31 with either vehicle (blue) or Fc.Mut24 (green)**.** Data represent takedown at 9dpi. Representative flow cytometry staining and quantitative analyses showing CD25 expression by gated $CD44^{lo}$ naïve $CD8^+$ T cells in the lungs of vehicle-treated mice, or by Flu-sp T cells in the lungs of vehicle- or Fc.Mut24-treated mice as indicated.* ***B)*** *$CD8^+$ T cells from $CD45.1^+$ OT-1 mice were enriched transfected with CAS9 RNPs containing a guide (g)RNA targeting the Il2ra locus or a control non-targeting gRNA. Cells were transferred to $CD45.2^+$ C57BL/6 recipients that were infected with X31-SIINFEKL and treated with vehicle of Fc.Mut24 4 days later as indicated. Representative flow cytometry analysis of CD45.1 and CD45.2 expression by gated CD8+ T cells from the lungs of mice at 9dpi showing gate for donor OT-1 T cells (left), and quantitative analysis of the abundance and frequency of donor OT-1 cells (right).* ***C)*** *Representative flow cytometry analysis of CD44 expression and SIINFEKL tetramer binding by CD45.2+CD45.1- recipient cells (left) and quantitative analysis of endogenous SIIN-sp $CD8^+$ T cells from lungs of mice treated as in B. Data are from one experiment with n=5 mice/group, representative of two separate experiments. *p < 0.05, **p < 0.01, ****p < 0.0001, by one way ANOVA with multiple comparisons.*

Fc.Mut24 during infection, which likely contributes to the increased presence Flu-sp $CD8^+$ T cells and the elevated immune-pathology associated with Fc.Mut24 administration post-infection.

2.6 Protective Memory formation and durability are maintained with Fc.Mut24 treatment

Mice treated with Fc.Mut24 before Flu infection showed reduced $CD8^+$ T cell responses, which could impair memory formation and durability. Conversely, mice treated at 4dpi exhibited enhanced proliferation and effector phenotypes, which might also compromise memory development. However, regardless of treatment time, Flu-sp $CD8^+$ T cells from Fc.Mut24-treated mice at 9dpi expressed elevated levels of the memory precursor marker CD127 which allows cells to respond to IL-7 and promotes memory cell formation. Therefore, we evaluated protective memory at 30 dpi. For this, we challenged previously infected mice with a lethal dose (5000 PFU) of the A/PR/8 (PR8, H1N1) (PR8)

influenza strain. Importantly, PR8 (H1N1) and X31 (H3N2) are serologically distinct strains, and protection against this heterosubtypic infection is partially T cell dependent (33). All mice treated with Fc.Mut24 (either before or after initial X31 infection) were fully protected from PR8 infection at this timepoint, showing weight loss and recovery comparable to vehicle-treated X31 immune controls and surviving the challenge, whereas naïve mice all succumbed to PR8 (Figure 2.6A).

To assess long-term durability of protective memory, we treated mice with Fc.Mut24 either one day before or 4 days after X31 infection, and evaluated memory T cell abundance and protective responses at 170 dpi. Flu-sp $CD8^+$ T cells remained detectable in the lungs of all animals, and both their frequency and number were comparable between Fc.Mut24- and vehicle-treated groups (Figure 2.6B and C). We also examined localization of memory cells in the lung parenchyma vs. vasculature, and expression of compartment-specific residency markers (CD69, CX3CR1, KLRG1), but found no significant differences at 170 dpi in any of our experimental groups (Supplemental Figure 2.8a&b). Upon lethal PR8 challenge infection, both vehicle- and Fc.Mut24-treated X31 immune were protected from lethal disease, and mice treated with Fc.Mut24 at 4dpi during initial X31 infection trended toward milder disease and less weight loss compared to vehicle controls, although this difference did not reach statistical significance (Figure 2.6 D and E). Moreover, the Flu-sp $CD8^+$ T cell response showed no differences in magnitude, phenotype, or tissue localization between any of the X31 immune groups (Figure 2.6E; Supplemental Figure 2.8 c&d). Thus, although Fc.Mut24 alters the acute CD8 T cell response—either suppressing or enhancing expansion of Flu-sp $CD8^+$ T cells depending on timing—it does not compromise the generation or

maintenance of protective T cell memory. Similarly, there were no differences in the maintenance of IgG responses to NP at 170dpi (Supplemental Figure 2.8e), and thus our

findings show that regardless of timing relative to initial infection, Fc.Mut24 treatment does not impact the integrity and durability of memory Flu-sp $CD8^+$ T cell responses.

Figure 2.6: Fc.Mut24 does not alter generation or durability of protective T cell memory to influenza. *Mice were infected with X31 and treated with vehicle (blue), or with Fc.Mut24 either a day before (red) or 4 days after (green) infection..* ***A)*** *Mice were lethally challenged with 5000 FU of PR8 at 30 days post X31 infection. Disease severity based on weight loss from start of lethal challenge in mice treated with Fc.Mut24 one day before (left) or 4 days after (right) initial X31 infection (left) compared with vehicle-treated and X31-naïve mice.* ***B), C)*** *Representative flow cytometry analysis of CD44 expression and $NP_{366-374}$ tetramer staining by gated $CD8^+$ T cells in the lungs of vehicle- and Fc.Mut24-tretted mice, and quantification of the number and frequency of $CD44^{hi}Tetramer^-$ T cells in the lungs at 170dpi in mice treated one day prior to* ***B)*** *or 4 days after* ***C)*** *initial X31infection.* ***D), E)*** *X31-immune mice treated with vehicle or Fc.Mut24 were lethally challenged with 5000 FU of PR8 at 170dpi. Disease severity based on weight loss from start of lethal challenge (left). Quantification of Flu-sp CD8 T cells in lung (right) in mice treated with Fc.Mut24 one day prior to* ***D)*** *or 4 days after* ***E)*** *initial X31 infection. *p < 0.05, **p < 0.01, ****p < 0.0001, by unpaired T test and two way ANOVA with mixed effects for weight loss curves in panels A, C, D and E.*

Supplemental Figure 2. 1. *Mice were treated with vehicle (blue) or Fc.Mut24 (red) one day before infection with 2000PFU of X31. All data represent takedown at 9dpi.* ***A), B)*** *Quantification of $CD4^+Foxp3^+$ Tregs* ***A)*** *and Flu-sp CD8+ T cells* ***B)*** *in spleens of infected mice.* ***C), D)*** *Representative flow cytometric analysis and quantification of expression of the indicated markers by lung parenchymal* ***D)*** *or blood exposed Flu-sp CD8 T cells.* ***E)*** *Total NP-specific IgG antibody measured by ELISA at the indicated serial dilutions of serum collected at necropsy in naïve and vehicle- or Fc.Mut24-treated mice as indicated.* ***F)*** *Representative flow cytometry analysis of CD4 MHCII tetramer against $NP_{311-325}$ staining and quantitative analysis of flu-sp $CD4^+$ T cells in the lung (left) and spleen (right) of vehicle- and Fc.Mut24-treated mice.* ***G)*** *Quantification of FoxP3 negative CD4 T cells in lung and spleen. Representative of two separate experiments n = 10, except F which n = 5. *p < 0.05, **p < 0.01, ****p < 0.0001, by unpaired T test for all panels except E where a one way ANOVA with multiple comparison was used.*

Supplemental Figure 2.2. *Mice were with vehicle or Fc.Mut24 one day before infection with 2000PFU of X31* ***A)*** *Representative marker staining and quantitative analyses of expression of the indicated makers by gated CD44loTetramer- naïve T cells from vehicle-treated mice, and from Flu-sp T cells from vehicle- (blue) or Fc.Mut24-treated (red) mice in the lungs (top) and spleen (bottom). Representative of two separate experiments n = 10 *p < 0.05, **p < 0.01, ****p < 0.0001, by one way ANOVA with multiple comparison.*

Supplemental Figure 2.3. A) Quantification of CD44hi Tetramer- CD8 T cells based on IV fractions in the lungs of mice. **B)** Quantification of selected markers on CD44hi Tetramer- CD8+ T cells. **C)** Quantification of bystander SIIN-sp CD8 T cells in lung based on IV labeling **D)** Quantification of indicated markers on SIIN-sp CD8 bystanders. Panels A and B represent two separate experiments n = 10. C and D are representative of one experiment n = 5 *p < 0.05, **p < 0.01, ****p < 0.0001, by unpaired T test for panels A and B and by one way ANOVA with multiple comparison for panel C and D.

Supplemental Figure 2.4 Mice were treated with vehicle (blue) or Fc.Mut24 (red) one day before infection with 2000PFU of X31. **A), B)** An analysis of CD80 and CD86 expression by cDC1 **A)** and cDC2 **B)** at 0, 2 or 3dpi as indicated. **C)** Representative flow cytometry and quantitative analysis of CD25 and CTLA4 expression by gated CD4+Focp3+ Treg in vehicle- or Fc.Mut24-treated mice at 3dpi (right). **D), E)** Analysis of donor OT-1 T cells given to mice before X31-SIINFEKL infection. Quantification of transferred OT-1 cells in the spleen and medLN of vehicle- and Fc.Mut24-treated mice at 4dpi D), expression of indicated activation markers by donor OT-1 cells isolated from lungs (top) and spleen (bottom) at 4dpi. **E). F), G)** Analysis of donor OT-1 T cells given to mice at 3dpi with X31-SIINFEKL infection. Quantification of donor OT-1 cells that were transferred at 3dpi and from the spleen at takedown (9dpi) **F)**, expression of indicated activation markers by donor OT-1 cells isolated from lungs (top) and spleen (bottom) at 9dpi. Experiments representative of an experiment n = 5. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, by unpaired T test for all panels.

Supplemental Figure 2.5 Mice were treated with vehicle (blue) or Fc.Mut24 (green) at 4dpi with X31. All data represent takedown at 9dpi. **A)** Representative flow cytometry analysis of CD25 and FoxP3 expression by gated CD4+ T cells in the lungs of vehicle and Fc.Mut24 treated mice, and quantitative analysis of the frequency and number of Treg cells in the lung and spleen. **B)** Number and frequencies of Flu-sp CD8 T cells spleen of mice treated as indicated. **C) F)** Representative flow cytometry analysis of CD4 MHCII tetramer staining against NP311-325 and quantitative analysis of flu-sp CD4+ T cells in the lung (left) and spleen (right) of vehicle- and Fc.Mut24-treated mice. **D)** Total NP-specific IgG antibody measured by ELISA at the indicated serial dilutions of serum collected at necropsy in naïve and vehicle- or Fc.Mut24-treated mice as indicated All data represent two separate experiments n = 10, except for C where n = 5. *p < 0.05, **p < 0.01, ****p < 0.0001, unpaired T test for all panels except for D where a one way ANOVA with multiple comparison was used.

Supplemental Figure 2.6. Mice were treated with vehicle or Fc.Mut24 at 4dpi with X31. All data represent takedown at 9dpi. **A)** Representative flow cytometry analysis of the indicated marker expression by gated CD44loTetramet- naïve T cells from vehicle-treated mice (purple), or by Flu-sp CD8+ T cells from lungs and spleens of vehicle-(blue) or Fc.Mut24-treated (red) mice, and quantification of marker expression by cells from the lungs and spleen as indicated. Representative of two separate experiments n = 10 *p < 0.05, **p < 0.01, ****p < 0.0001, by one way ANOVA with multiple comparison.

Supplemental Figure 2.7. ***A****)* Representative flow cytometry analysis of CD25 and CD69 expression by OT-1 T cells following transduction of CAS9 RNPs containing control or Il2ra-targeting guide RNA following 48h activation with plate bound anti-CD3/CD28.

Supplemental Figure 2.8. Mice were all treated a day prior to (**A and B**) or 4 days after infection with X31 with Fc.Mut24 (**C and D**). **A or C)** Markers on Flu-sp CD8 t cells in the lung of mice treated with Fc.Mut24 before or after (respectively) X31 at 170dpi. **B or D)** Markers on Flu-sp CD8 t cells in the lung of mice treated with Fc.Mut24 before or after (respectively) X31 at 170dpi and then lethally challenged with 5000 PFU of PR8. **E)** total IgG titers against the Flu NP from mice prior to 170dpi. Representative of one experiment where n = 5 *p < 0.05, **p < 0.01, ****p < 0.0001, by unpaired T test.

Discussion

Our study demonstrates that the timing of IL-2-based immunotherapy critically shapes its impact on anti-viral $CD8^+$ T cell responses. Using Fc.Mut24, a Treg-selective IL-2 mutein, we show that treatment before respiratory virus infection suppressed the

expansion and function of Flu-specific $CD8^+$ T cells and alters their localization within the lungs, whereas treatment after infection expands virus-specific $CD8^+$ T cells through CD25-dependent signaling and worsens symptomatic disease likely through enhanced immunopathology. However, regardless of timing of treatment, mice recovered from infection and developed durable protective immunity against lethal heterosubtypic infections. These divergent and context-dependent effects appear to reflect a balance between suppressive Treg activity, and direct IL-2 signaling that promotes differentiation of $CD25^+$ effector $CD8^+$ T cells.

Administering Fc.Mut24 before infection significantly reduced the abundance of Flu-specific $CD8^+$ T cells. Interestingly, despite this overall reduction, these cells showed a skewed localization toward the lung parenchyma. Viral load and disease severity remained unchanged despite the reduced number and altered distribution of $CD8^+$ T cells, and this indicates that even in the face of massive Treg expansion, it is possible to generate protective Flu-specific $CD8^+$ T cell responses to efficiently clear the infection. Though expansion of Treg cells expressing high levels of CTLA-4 Fc.Mut24 treatment before infection likely limits the availability of the CD80 and CD86 co-stimulatory ligands. Similarly, the large reservoir of $CD25^{hi}$ Treg cells may reduce IL-2 availability for Flu-sp $CD8^+$ T cells during the expansion phase, and together these factors may favor the priming and expansion of $CD8^+$ T cells that receive strong TCR signals and effectively clear virus and form protective memory despite the immunosuppressive environment present during priming. Indeed, their function as an IL-2 sink is a key mechanism by which Treg can suppress $CD8^+$ T cell responses (34, 35). Despite limiting expansion of Flu-sp $CD8^+$ T cells, Fc.Mut24 had minimal effects on bystander T cells during X31

infection. As the recruitment and activation of bystander $CD8^+$ T cells is driven largely by chemokines and cytokines produced by cells of the innate immune system (36), this supports a mechanism whereby Treg cells interrupt key pathways central to elaboration of the adaptive immune response.

Interestingly, we also observed no differences in total IgG specific for the viral NP protein at any time point examined. This is surprising given that IL-2 is a key inhibitor of Tfh differentiation and function (29, 30), and therefore restricting IL-2 availability by expanding Treg prior to infection would be expected to promote better antibody responses. Indeed, despite the reduced $CD8^+$ T cell response we observed in Fc.Mut24 pre-treated mice, these animals successfully cleared the virus and established durable protective memory that was quantitatively indistinguishable from that observed in vehicle-treated mice, indicating that long-term memory formation and maintenance was not strictly dependent on the magnitude of the initial clonal expansion.

When administered after infection had begun, Fc.Mut24 drove hyper-expansion of Flu-sp $CD8^+$ T cells. Under these conditions, Fc.Mut24 can signal through CD25 on responding Flu-sp $CD8^+$ T cells and promote their expansion. We hypothesize that this results in enhanced immune-mediated pathology in the lungs (37), consistent with the increase weight loss observed in animals given Fc.Mut24 4 days after influenza infection. However, as we observed in with mice given Fc.Mut24 before infection, protective T cell memory is still generated and does not wane more rapidly in the context of a single Fc.Mut24 treatment before or after infection. Indeed, it may be that Fc.Mut24 treatment during initial infection actually boosts lasting protective memory formation, as mice treated

as such trended towards less weight loss than vehicle controls upon PR/8 rechallenge. Others have also described IL-2 administration as boosting $CD8^+$ memory T cell generation and function in the context of active inflammation (38, 39). Enhanced memory formation and function in mice treated with Fc.Mut24 (either prior to or during infection) may be attributable to the upregulation of the IL-7 receptor component CD127 on Flu-sp $CD8^+$ T cells in Fc.Mut24-treated mice. CD127 expression is associated with memory cell differentiation and survival (25, 40), and therefore may as a mechanism to generate durable protective memory and compensate for the altered phenotypes we observed during primary infection. Further the lack of waning antibody responses may be explained by the enhanced proliferation of Tregs which have been shown to favor Tfh generation during influenza infection (41), thus enabling sufficient antibody responses and explain why we did not see reduced total IgG against the viral NP.

These findings demonstrate that although treatment with Fc.Mut24 exerts immunosuppressive effects through Treg expansion, respiratory viral infection is still effectively controlled and durable memory responses are established, even when peak Treg expansion coincides with the initial priming phase. This observation has important implications for use of IL-2 muteins and other Treg-expanding immunotherapies, suggesting that the presence of Treg-mediated suppression does not necessarily preclude effective anti-viral T cell responses, viral clearance and development of protective immunity. However, these results also highlight a critical caveat in that IL-2-based therapies may exacerbate immunopathology if administered during active infection. This is especially relevant in the context of respiratory viral infections, where immune-driven tissue damage significantly contributes to morbidity and mortality (42).

These results underscore the need for careful consideration of the timing and context of Treg-targeted interventions in infectious disease settings, particularly when balancing immune regulation with the necessity of effective pathogen clearance.

Chapter 3: Materials and Methods

Mice

C57BL/6 (B6) (JAX Strain# 000664) mice were purchased from The Jackson Laboratory. B6.SJL-Ptprca Pepcb/BoyJ (CD45.1^{+}, JAX Strain# 002014) and C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-1, JAX Strain # 003831) mice were bred and maintained at Benaroya Research Institute (BRI). Female Mice used in experiments between the ages of 6-10 weeks at start of experiment. Female mice are used due to male B6 mice being more resistant to infection (43). For histological analyses, euthanasia was performed with tribromoethanol overdose. For all other experiments mice were euthanized by CO_2 inhalation. All experiments were performed in accordance with the guidelines and with the approval of the Institutional Animal Care and Use Committee of the BRI.

Viruses and infections

Influenza viruses A/HK-x31(x31, H3N2), A/PR/8 (PR8, H1N1) (44), and the X31-ova expressing the CD8 H2-K^{b} restricted SIINFEKL epitope (45), were all generously provided by Dr. Kimberly D. Klonowski (University of Georgia). Animals were infected with 2000 PFU of either X31 strain or 5000PFU of PR8 strain intranasally in 50uL of PBS. All mice infected with Influenza were monitored at least daily for weight loss and twice a day when mice reached 80% of baseline weight. If mice reached 70% of baseline weight, mice were promptly euthanized using aforementioned methods. For longer term studies, once mice recovered they were monitored twice weekly. VSV-OVA (46) was generously provided

from Dr. Pamela Fink (University of Washington). Mice were infected with 2000PFU of VSV-OVA, intraperitoneally in 100 uL of PBS.

IL-2 mutein treatment

The development of the murine Fc.IL-2 mutein (Fc.Mut24) was previously described (10). Fc.Mut24 was produced and purified by Olympic Protein Technologies (Seattle, WA), and contained less than 15 endotoxin units (EU)/mL. For all experiments, 10 µg of Fc.Mut24 or PBS vehicle was administered via intraperitoneal injection.

Cell isolation and flow cytometry

Where applicable, mice were anesthetized using 4% isoflurane and intravenously injected with 3µg of anti-CD45 or anti-CD45.2 antibody in 100uL for intravascular labeling. Mice were allowed to recover for two minutes before euthanasia, performed as described above. For flow cytometry, lungs were minced and digested in RPMI supplemented with Liberase (50 µg/mL) and DNase I (10 U/mL) for 20 minutes at 37 °C with agitation. Cell suspensions were filtered through 70 µm strainers into RPMI containing 10% FBS (RPMI-10), followed by red blood cell lysis with ACK lysis buffer and washing in RPMI-10. The same protocol was used for lymphoid tissues (mediastinal lymph node and spleen) when analyzing conventional dendritic cells (cDCs). Otherwise, spleens and medLNs were mechanically dissociated through a 70 µm strainer, followed by red blood cell lysis and washing as above. For dendritic cell analysis, $CD11c^+$ cells were enriched using anti-CD11c microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Enriched cells were stained with selected antibodies targeting cDC surface markers (see reagent table).

For detection of antigen-specific $CD4^+$ T cells, MHC class II tetramer staining was performed using the I-A^b/$NP_{311-325}$ (QVYSLIRPNENPAHK) tetramer (47), kindly provided by Marion Pepper (University of Washington). Samples were incubated with tetramer at room temperature for 1 hour, washed, and enriched using anti-APC microbeads (Miltenyi Biotec). Enriched cells were then stained with the CD4 flow cytometry panel, while the flow-through was used for additional staining and analysis. For $CD8^+$ T cell analysis, H-2D^b/$NP_{366-374}$ (ASNENMETM) or H-2K^b/SIINFEKL tetramers (generated by the NIH Tetramer Facility, Emory University) were used. Cells were stained with tetramer for 1 hour at room temperature prior to surface staining.

Cell surface staining for flow cytometry was performed in FACS buffer (PBS-2% BCS) using a selected antibody cocktail (See reagent table). Cells were incubated in the antibody cocktail for 20 min at room temperature and then washed in FACS buffer before collecting events on an BD Symphony flow cytometer. For intracellular staining, surface antigens were stained before fixation and permeabilization with FixPerm buffer (eBioscience) (See reagent table). Cells were washed and stained with selected antibodies. Flow cytometry data was analyzed using FlowJo software.

Quantification of viral RNA

Total RNA was extracted from lung tissue using the Qiagen RNeasy Plus Mini Kit (cat. 74134) following the manufacturer's protocol. Tissue homogenization was performed in RLT buffer containing β-mercaptoethanol (BME), with buffer volume adjusted according to tissue weight as specified by the kit. Approximately 15 mg of tissue was used per sample to avoid overloading the RNA-binding column. Samples were lysed using 5 mm stainless steel beads (Qiagen, cat. 69989) in 2.0 mL Safe-lock Eppendorf tubes on a

TissueLyser II (Qiagen) at 30 Hz for 2 minutes. RNA yield was quantified using a NanoDrop spectrophotometer. Complementary DNA (cDNA) was synthesized from purified RNA using SuperScript II Reverse Transcriptase (Invitrogen, cat. 18064-014) and IDT ReadyMade Random Hexamer primers (cat. 51-01-18-26), including a no-RNA negative control in each batch. Reactions were assembled using a two-step thermal protocol. First, Master Mix 1 was added and samples were incubated at 65 °C for 5 minutes, then cooled to 4 °C. After addition of Master Mix 2, samples were incubated at 30 °C for 10 minutes followed by 42 °C for 50 minutes. The resulting cDNA was quantified using a NanoDrop spectrophotometer. Quantitative PCR (qPCR) was used to quantify Influenza A virus matrix (M1/M2) gene expression from PR8 and X31 strains. Reactions were prepared using IDT PrimeTime Std qPCR Assay containing a fluorescent probe and primers specific to the M segment of IAV with the following primer and probe design: Probe: (6-FAM/ZEN/IBFQ) 5′-CCTCTGCTGCTTGCTCACTCGATC-3′; Forward Primer: 5′-CAGCACTACAGCTAAGGCTATG-3′; Reverse Primer: 5′-CTCATCGCTTGCACCATTTG-3′. The probe/primer mix was resuspended at a 20x concentration. Each qPCR reaction (20 µL total volume) contained: 10 µL 2x IDT PrimeTime Gene Expression Master Mix (cat. 1055770),1 µL 20x PrimeTime qPCR Assay Mix, 4 µL diluted cDNA (~75 ng), 5 µL nuclease-free water. An internal control targeting hypoxanthine-guanine phosphoribosyltransferase (HPRT) was run in parallel to normalize viral gene expression. The Viia7 Real-Time PCR System (Thermo Fisher Scientific) was used for amplification and detection.

Adoptive transfers

Spleen and lymph nodes were harvested from OT-1 mice, passed through a 70 μm strainer and ACK lysed. $CD8^+$ cells were enriched by negative selection using a CD8 T cell isolation kit according to the manufacturer's instructions (Miltenyi). Cells were then enumerated, washed in PBS before transfer by retroorbital injection into recipient mice (0.005-0.5×10^6 cells per mouse depending on the experiment). For transfer of activated OT-1 CD8 T cells, spleens were collected from CD45.1/.2 mice and processed as described above. Once in single cell suspension, 1x10^6 cells were plated in a 12-well plate (see reagent table) in 1mL of cRPMI supplemented with 50UI/mL of rIL-2 (see reagent table). Cells were plated at a ratio 1:1 ratio with washed Dynabeads and allowed to culture for 48hrs. Following 48hrs, Dynabeads (see reagent table) were extracted using a magnet and cells washed before being transferred retroorbitally to mice at 0.5×10^6 cells per mouse.)

CRISPR deletion of Il2ra

For deletion of the *Il2ra* gene in primary naive OT-1 TCR T cells, we utilized the CRISPR/CAS9 system as previously described (32). Purified CAS9 protein, *Il2ra* crRNA (AGAUGAAGUGUGGGAAAACGGUUUUAGAGCUAUGCU), and trans-activator RNA (tracrRNA) were purchased from Integrated DNA Technologies. $CD8^+$ T cells were isolated from OT-1 mouse spleen using $CD8^+$ T cell negative selection MojoSort Kit (Biolegend). T cells were cultured in IL-7 (10ng/ml) for 24 hrs prior to transfection. CRISPR/CAS9 reagents were prepared as per the manufacturer's instructions for IDT Alt-R CRISPR-CAS9 system. The pre-cultured $CD8^+$ cells were were resuspended in T buffer (Invitrogen MPK1096) and electroporated using 6 2200V, 10ms, 3 pulses, with the Neon electroporation system. Following transfection, cells were transferred into warm RPMI

with 10% FCS and were incubated at 37°C, 5% CO2 for 4- 5 hours. Prior to adoptive transfer, the electroporation efficiency was evaluated by flowcytometry measuring ATTO uptake. Control cells were transfected with ATTO and CAS9 alone without *Il2ra* crRNA. To validate *Il2ra* deletion efficiency, the neon-transfected cells were plated on αCD3/αCD28 coated plates for activation for 48 hours followed by flow cytometry analysis of cell surface CD25 expression.

Enzyme-Linked Immunosorbent Assay (ELISA)

To quantify serum antibody responses against influenza PR8 nuclear protein, ELISA plates were coated overnight at 4°C with anti-His antibody (1:1000 dilution in PBS, 50 µL/well). Plates were then washed three times with 100 µL PBS containing 0.1% Tween-20 (PBS-T), followed by incubation with His-tagged PR8 nuclear protein (SinoBiological, Cat# 11675-V08B) at 1:100 dilution in PBS-T (50 µL/well) for 1 hour at 37°C. After three additional PBS-T washes, wells were blocked with 100 µL of blocking buffer (PBS-T supplemented with 5% BSA) for 1 hour at 37°C. Diluted serum samples were added to the plates (50 µL/well) and incubated for 1 hour at 37°C. Plates were then washed three times with PBS-T and incubated with 50 µL of biotin-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch, Cat# 115-035-062) diluted 1:1000 in blocking buffer for 1 hour at 37°C. After five washes with PBS-T, 50 µL of 1× TMB substrate was added to each well and color development was monitored. The reaction was stopped with 50 µL of H_2SO_4, and absorbance was measured at 450 nm using a microplate reader.

Histological analysis

Lungs were excised and immediately fixed in 10% formalin for at least 24hrs and then paraffin embedded. H&E staining was performed on a 5µm tissue sections by Benaroya

Research Institute Histology Core. Resulting slides were imaged using a Molecular Devices ImageXpress Confocal with the 10x objective by Benaroya Research Institute Histology Core.

Statistics

All data are presented as mean values ± SD, and graphs were created and analyzed using Prism Software (GraphPad). Comparisons between treatment groups were analyzed using two-tailed unpaired student T tests, one-, or two-way ANOVA where appropriate, adjusted for multiple comparisons using Tukey's post-test. To compare weight loss between experimental groups over time, a two-way mixed-effects ANOVA was performed. This mixed-effects approach allows for modeling the correlation structure of repeated observations and provides more accurate estimates by adjusting for inter-subject variability.

Writing disclosure

Portions of the manuscript text were edited for grammar and clarity using OpenAI's ChatGPT (version GPT-4). The tool was used solely for language refinement; all scientific content and interpretations are the original work of the authors.

Chapter 4: Concluding remarks

Our study demonstrates that the timing of Fc.Mut24 administration critically shapes its impact on antiviral $CD8^+$ T cell responses. The therapy exerts divergent effects depending on whether it is given before or during infection: pre-infection treatment suppresses Flu-specific $CD8^+$ T cell expansion and alters their localization within the lung, whereas post-infection treatment drives hyper-expansion of effector $CD8^+$ T cells, worsening symptomatic disease through immunopathology. Despite these context-dependent effects, mice consistently recovered from infection and developed durable, heterosubtypic protective immunity. These findings illustrate the balance between Treg-mediated suppression and direct IL-2 signaling on effector $CD8^+$ T cells, and highlight the immune system's capacity to generate protective memory even under pronounced immunoregulation.

More broadly, our results carry important implications for the future of IL-2 muteins and other Treg-boosting therapies. Current standard-of-care treatments for many autoimmune diseases increase susceptibility to infection and worsen outcomes during therapy. By contrast, we demonstrate that boosting Tregs did not compromise infection control at any time of administration. Although disease severity increased when Fc.Mut24 was administered during peak infection, this effect reflected direct IL-2–driven effector expansion rather than Treg targeting. Importantly, this transient pathology did not increase mortality and could be mitigated through dosing strategies, molecular refinements, or clinical safeguards such as infectious disease screening. This finding is highly significant, as many patients with autoimmune diseases are predisposed to infection-related complications; demonstrating that Treg-boosting therapies need not exacerbate this risk supports their broader clinical potential.

We further show that boosting Tregs does not diminish pre-existing T cell populations. This is encouraging for clinical translation, as vaccines remain the most effective strategy for preventing infection and limiting disease severity. Fc.Mut24 selectively modulated active immune responses without ablating pre-existing bystander cells. Thus, vaccine-induced T cell repertoires remain intact under IL-2 mutein treatment, suggesting that vaccine efficacy will be preserved. Relatedly, even during active immune responses, Treg boosting neither impaired the generation of protective memory nor accelerated its waning. Together, these findings underscore an important advantage of Treg-boosting therapies: they can restrain pathological immunity without erasing protective immune memory.

While IL-2 muteins retain some classical off-target effects by activating effector T cells, this represents a challenge of molecular design rather than a fundamental limitation of the strategy. Rational engineering to reduce effector engagement through further residue modifications, incorporation of bi-specificity, or enhanced affinity for Treg-restricted markers, will likely define the next generation of these molecules. Advances in bispecific technologies and improved understanding of Treg versus effector biology make this increasingly feasible. Coupled with the favorable safety profile and durable responses observed in our study and phase I clinical trials, these improvements point to increasing Treg selectivity as the key frontier for IL-2–based therapies.

Despite this progress, several outstanding questions remain. A key advantage of Treg-boosting therapies over conventional immunosuppressants is that they expand endogenous Tregs, which may then restrain autoimmunity in a physiologic and potentially self-sustaining manner. This is a highly attractive feature but one that remains poorly understood. In principle, such durability could allow therapeutic benefit to persist even after treatment withdrawal. Indeed, we previously showed that boosting Tregs with an IL-2 mutein led to durable arrest of diabetes progression in mice, outperforming both vehicle and low-dose IL-2. However, it remains unclear how stable this balance would be in the context of infection, or how durable Treg-mediated control would remain over years or decades in humans.

Dosing also represents an important unresolved issue. Repeated administration of IL-2 muteins is likely required, whether as an initial loading dose or for long-term maintenance. Yet, sustained elevation of Treg numbers or chronic IL-2 exposure could carry unintended consequences. Similar to other cytokine therapies, IL-2–based treatments may risk impairing the formation of new protective memory, eroding pre-existing memory populations, or increasing infection susceptibility over time. Addressing these questions will be critical to fully realize the therapeutic promise of Treg boosting. While Treg-based therapies offer the potential to recalibrate autoimmunity in a durable and physiologic way, their long-term effects on host defense, immune memory, and infection risk remain pressing challenges that will require longitudinal clinical trials.

At a broader level, our findings refine the therapeutic logic of IL-2 biology. High-dose IL-2 in cancer historically exploited effector expansion, while low-dose IL-2 in autoimmunity sought to preferentially expand Tregs despite off-target risks. Fc.Mut24 provides a case study of how molecular engineering and treatment context interact to shape outcomes across this spectrum. By showing that Treg-boosting strategies can preserve infection control, maintain vaccine efficacy, and generate durable memory even under immune suppression, our work helps resolve a central tension in cytokine therapy: how to attenuate autoimmunity without compromising host defense. As IL-2–based therapies advance in autoimmunity, transplantation, and beyond, these principles will guide the next generation of rationally designed, Treg-selective cytokines.

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