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Dedicated to Dr. John J. Alpar and his perpetual curiosity.

ITFK

"The [pecan] trees act not as individuals, but somehow as a collective. Exactly how they do this, we don't yet know. But what we see is the power of unity. What happens to one happens to us all. We can starve together or feast together. All flourishing is mutual." - Robin Wall Kimmerer, *Braiding Sweetgrass*

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ABSTRACT

This thesis serves to explore engineering mechanisms by which dendritic cells and their interactions are capable of modulating immune responses. In this, I explore the generation, characterization, and use of a novel cytokine fusion, Flt3L-SA, to therapeutic ends involving both the innate and adaptive immune system. Chapter 1 defines the major players of the immune system. Herein I also explore how these cellular and soluble factors interact and play off of one another to generate beneficial immune responses (pro- and anti-inflammatory) as well as characterize when an immune response malfunctions and the issues it may cause. Finally, I review past and future protein- based technologies involved in immunomodulation to skew immune responses back in an appropriate direction in instances such as pushing inflammation forward to generate anti- tumor immunity, or skewing away from pathogenic inflammation to an inert form. In Chapter 2, I introduce my novel engineered-cytokine. The fusion of Flt3L to serum albumin (called Flt3L-SA), is then rigorously characterized for binding, activity, and pharmacokinetics and dynamics. We here explore how treatment impacts players of both the adaptive and innate systems with an obvious focus on Dendritic Cells, but also how this expansion of DCs impacts the T cell compartments, primarily that of Tregs. Following the general characterization of treatment with Flt3L-SA, Chapter 3 explores the utility of such a treatment in many settings. The first setting this is characterized in is the use of Flt3L-SA with an immunogenic protein drug as a model of enzyme replacement therapy and the prevention of antibodies to the foreign drug. Furthermore, as we note a major immune skewing in the immune system related to the gut, we also explore how oral antigen treatment in combination with Flt3L-SA changes the context in which immune education occurs. Finally, Chapter 4 discusses future works and directions involving this fusion protein in tolerance and wound healing.

CHAPTER 1

INTRODUCTION

The immune system as a surveillance mechanism is an incredible feat of evolution and natural engineering. There are specialized organs which allow for the differentiation, clustering and education of the individual components of the system, called primary and secondary lymphoid organs; however, due to the surveillance-related requirements necessary of the immune system, much of the peripheral effector processes actually occur on the cell-to-cell level rather. In this way, the immune system is a continuous and meticulous orchestration of numerous switches and decisions which cumulatively determine the response and the ultimate protection of the organism. These decisions range from those that induce tolerance to self-proteins and innocuous foreign antigens to toxicity and organized killing to clear pathogens and cull misbehaving cells. In some instances, these decisions may misfire and either induce immunity to harmless antigens (i.e.: allergy or autoimmunity) or fail to recognize and kill harmful entities such as cancerous tissue. In this chapter, I will describe: the immune system as a whole, including the major cellular players and their roles in coordination of this defense; signals which may lead the response astray and induce a shift along the anti- to pro-inflammatory spectrum; and review recent engineering feats and attempts to bring the response back to an appropriate equilibrium.

1.1 The Immune Spectrum

Acting through various cellular and soluble players, the immune system is capable of establishing and maintaining an equilibrium state. Environmental cues, such as infection, push the system towards a pro-inflammatory response. Upon clearing the offending pathogen, the immune system will then reel itself back in and establish anti-inflammatory protocols to return to a homeostatic state. Through this process, the system is able to generate a memory response which upon subsequent infection will react quicker than the initial offense and in turn require a less severe and prolonged inflammatory response to clear the pathogen. These functions occur through two major branches of the immune system: the innate system and the adaptive. The innate system, comprised of cells like Innate Lymphoid Cells (ILC), dendritic cells (DC), and granulocytes, are immunologic "first-responders" to disrupted homeostasis. These cells activate and respond after sensing conserved patterns which signal to tissue damage and/or pathogen invasion. The adaptive immune system, primarily T cells and B cells, on the other hand, is known for the ability to respond to a wide array of signals and adapt to such cues by genomic recombination of the cellular receptor (discussed later in this section). This section will first introduce the cell-based and soluble components of the immune system as well as delineate their responsibilities and then describe how each of these roles coalesce into the full pro- and anti-inflammatory cascades.

1.1.1 Cellular Mediators of the Immune System

Innate Immunity

Granulocytes

Granulocytes are immune cells defined by the many granules of effector molecules found within their cytoplasm. They can be divided into four groups based initially on the presence or absence of multi-lobed nuclei (mast cells have a rounded nucleus) and further by how

they stain using pH sensitive dyes with basophils staining in basic dyes, eosinophils staining dark red in acidic dyes, and neutrophils staining pink. All of the granulocytes play key roles in protecting against pathogens; however, neutrophils, which make up the majority of circulating white blood cells, primary role is in protection and clearance of bacterial invaders and the other granulocytes more so focus on clearance of parasites. Parasitic clearance by these cells occurs primarily via cellular degranulation and release of soluble mediators, which will be covered in more depth in later sections of this chapter. Neutrophils in addition to degranulation react in ways more suited for bacterial clearance and can also serve to phagocytose and clear the foreign pathogens. The most interesting of these responses is the release of Neutrophil Extracellular Traps (NETs).(1) Upon activation, neutrophils will decondense their nuclei and release it to the extracellular space, trapping pathogens and beginning a pro-inflammatory cascade.(2)

Innate Lymphoid Cells

Innate lymphoid cells (ILC) are a recent but ever-growing classification of primarily tissue-resident lymphocyte.(3; 4) These are cells capable of responding to innate cues but without clonally variable receptors. ILCs fall into three distinct subclasses: ILC1–which react to cues to trigger an antiviral reaction similar to $CD8^+$ and Th1 T cells and have recently been broadened to include NK cells (discussed in further depth below) and are distinguished by expression type 1 cytokines like IFN-γ, ILC2—responsible for stimulating a Th2 response in reaction to barrier disruption and alarmin release and are distinguished by expression of Gata3 and type 2 cytokines such as IL-5 and IL-13,(5–11) and ILC3—a broadened class of cell primarily active in the gut mucosa which respond to microbial signals to release Th17-related cytokines as well as encompassing cells responsible for development of peripheral lymphoid structures (lymphoid tissue inducers) and are distinguishable by expression of Rorγt, IL-17 and IL-22.(12–16)

Natural killer (NK) cells, recently recategorized as a subset of ILC1,(17) are an interesting

portion of the innate immune system, as they are able to synthesize information gathered via numerous receptors to decide whether or not to kill the potentially infected or cancerous cell. They choose dependent on the summation of various activating and suppressive signals including: the presence of opsonization cues like antibodies or components of the complement pathway (both described later), the presence of MHCI and other components of antigen presentation, cytokine signals present in the surrounding milieu, and some proteins expressed upon viral infection. The ultimate response of these cells is primarily dependent on the type of signaling domain present on the cytoplasmic tail of the triggered receptor (ITIM domains on receptors can suppress activation upon signaling, while ITAM domains on activating receptors contribute to cellular degranulation).(18–20) If the ITAM signals outweigh the ITIM signals, the NK cell will activate and is then capable of secreting enzymes and factors similar to those of cytotoxic T cells including perforins, granzymes, and immune activating cytokines. In this way, they are capable of acting in a way reminiscent of T cells, but with a timescale more in line with the innate system and without a clonally variable receptor.

Monocytes, Macrophages, and Dendritic Cells

The primary role of these cells is to serve as professional antigen presenting cells (APCs), to survey the surrounding tissues, endocytose material from these tissues (both potential pathogens as well as cells and material from the tissue itself), and show this to the adaptive immune system in a way to elicit a response according to the environment where this antigen was found. Processes such as these are where these cells get the title of "professional APC" in that they are capable of providing all three signals necessary to educate and license the T cell response.(21) These cells act as a bridge between the innate and adaptive immune systems by sensing and responding to environmental stimuli and subsequently translating that signal to naive T cells by presenting portions of the peripheral antigens on MHCII and in specific cases MHCI (known as cross presentation). This group of cells senses such environmental stimuli through receptors meant for scavenging (ie: sirpα or C-type lectins)(22)

as well as immunomodulating pattern-recognition receptors (PRR) — Toll-like receptors (TLR), NOD-like receptors (NLR), and Retinoic-acid Inducible Gene Like Receptors (RLR) to name a few — which are capable of sensing signals related to pathogen invasion, Pathogen Associated Molecular Patterns (PAMPS), as well as those related to cellular damage, called Damage Associated Molecular Patterns (DAMPS).(23) Being one of the few groups of cells capable of expressing MHCII and presenting antigen to the $CD4^+$ T_H compartment, this is critical in instigating the adaptive response to antigens, as well as priming of the $CD8^+$ T cell compartment to kill foreign infected cells.(24) This family of immune cell is also responsible for instigating pro-resolution responses. The plasticity of these cells allows them to switch from immunostimulatory processes to those related to immune inhibition, from secreting factors such as IFN-γ and TNFα to anti-inflammatory signals like IL-10, TGF-β, and glucocorticoids. In addition to this role as a sensor and translator, these cells also act to amplify the signals they receive.(25) This occurs by expression of markers and soluble signals related to the pro- and anti-inflammatory cascades, thus generating a feed-forward loop to continue the response.

Adaptive Immunity

As mentioned previously, the adaptive immune system is known for the ability to generate a novel DNA sequence to create a hypervariable receptor. This is possible thanks to expression of enzymes during cellular differentiation which recombine their germ-line DNA sequence recombining variable (V), diversity (D), and joining (J) segments to generate a functional receptor. These V, D, and J segments number roughly 70 V and 61 J segments for the TCRα chain and 52 V, 2 D, and 13 J segments for TCRβ.(26) Taking this diversity as well as the chance of mutations and frameshifts caused by recombination, $\alpha\beta$T cells alone are capable of over 10^{18} receptors to be encoded within a much smaller section of the genome. Similar to T cells, the BCR is germline-modified during cellular development through a similar process.

Considering the average number of V, D, and J segments in the BCR locus for both heavy and light chains, recombination of the BCR is able to encode a less diverse repertoire than T cells (roughly $5x10^{13}$ receptors); however, this repertoire diversity is increased through a process which occurs during BCR activation, known as somatic hypermutation.(27) During somatic hypermutation, loci within the BCR become accessible to enzymes which change the genomic sequence again during a directed evolution process where B cells compete for T cell help by generating higher affinity receptors. Responsibilities of these cell types are discussed below.

B Cells

The primary role of B cells is to recognize three-dimensional surfaces of antigens through their highly variant B cell receptor. Once a naive B cell recognizes an antigen, the cell will again undergo genomic-level changes to become even more specific, called somatic hypermutation, as well as changing the class of its receptor (class-switch recombination) in accordance with the surrounding milieu.(28; 29) From here, the cell is differentiating into many phenotypes including memory B cells, short-lived plasma cells and plasmablasts, and long-lived plasma cells.(28; 29) Memory B cells serve to repopulate immune reactions upon subsequent challenges by capturing antigen and reinvigorating the adaptive response; acting as antigen presenting cells and exacerbating a T cell response, with the additional benefit of potentially undergoing further somatic hypermutation to become more specific to another serotype of pathogen, such as is the case in infections with minorly different cases of influenza.(28; 29) Plasmablasts are a rapidly dividing phenotype of B cell memory. Through rapid expansion, these cells are able to populate a large portion of lymphoid organs before becoming a plasma cell.(28; 29) Finally, the plasma cell phenotype acts to secrete copious amounts of antibodies – the non-membrane bound form of the B cell receptor (described more in-depth later) – to the blood. Short-lived plasma cells act primarily during the active infection to clear the pathogenic material and aid in healing, whereas long-lived plasma cells take up more per-

manent residence in lymphoid organs, secreting antibodies and providing a protective role in preventing the infection to begin with.(28; 29)

T Cells

T cells are one of the most powerful actors of the immune system, and as such they are also the most restrained. T cells recognize antigen shown to them as peptides on molecules known as Major Histocompatability Complexes (MHC) which come in two forms, MHCI presenting antigen to $CD8^+$ T cells and MHCII which presents antigen to $CD4^+$ T helper cells.(30) The $CD8^+$ T cell compartment, also known as Cytotoxic T Lymphocytes (CTL), functions to kill cancerous and virally infected cells as they are capable of recognizing antigen presented by any cell type and primarily secrete pro-inflammatory cytokines and enzymes related to cell lysis (granzymes, perforins, etc.). Because they are only capable of responding to antigen on MHCII as presented by APCs, $CD4^+$ T helper (T_H) cells differentiate to react to different contexts in an appropriate manner (discussed in more detail in Sections 1.1.3-1.1.4).(31) This differentiation and specialization occurs after three signals are sensed by the naive $CD4^+$ T cell. These differentiation cues are: signal 1, signaling through the T cell receptor via peptide-loaded MHC; signal 2, costimulation through receptors such as CD28 on the T cell by CD80 and CD86 on the APC; and finally signal 3, cytokine milieu in the area which signals and opens chromatin regions in the T cell genome, allowing specialization to the particular pathogen.(32) As is the case with B cells, T cells are capable of generating memory once they have been initially educated. This memory is then able to respond in a quicker manner to subsequent infection than in the case of primary infection. In addition, because T cells only recognize linear epitopes of 8-24 amino acid peptides, they may be less susceptible to antigen evolution and escape than B cells (which require full three-dimensional structures) if the epitope is found within a crucial functional domain.(33; 34)

1.1.2 Soluble Mediators of the Immune System

Complement Cascade

The complement cascade is evolutionarily one of the earliest forms of the immune system and is comprised of numerous steps and pathways with the main contributing proteins being named a subset of C with the number 1-9. The ultimate goal and final endpoint is to generate what is known as the membrane attack complex (MAC), a set of proteins which insert into the membrane of invading pathogens and create an opening between the cell and the outside world, killing the pathogen. Along the way of generating the MAC, various portions of the complement cascade – C3a, C4a, and C5a – are released as soluble anaphylatoxins. They are called such as they induce symptoms similar to those seen in an IgE-mediated anaphylactic response including vasodilation, cell recruitment, and mast cell degranulation. In small amounts, complement derived anaphylatoxins are beneficial in recruiting white blood cells and increasing blood flow to the site. The other portions of these proteins – C3b, C4b, and C5b – then remain bound to the cell surface and further the cascade towards production of the MAC as well as acting as opsonins, proteins which promote clearance and phagocytosis of the offending material.(35)

Cytokines

Cytokines are defined as a broad category of proteins released by cells to signal a response; these include factors such as growth factors, chemokines, interferons, and interleukins, to name a few broad categories and may act in autocrine, paracrine, and/or endocrine fashions. Due to the breadth of this classification, cytokines act in many ways to induce some form of cellular response, be it proliferation, recruitment, activation , or secretion of other cytokines to induce a feedback loop. The cytokines most relevant for further discussion are those related to the immune response, primarily interleukins (IL) – which are capable of distinguishing the

flavor of immune reaction occurring and will be discussed later as they relate to stimulatory and inhibitory reactions – and chemokines, which are cytokines that induce recruitment of immune cells to sites via development of a concentration gradient.

Antibodies

Antibodies, also known as immunoglobulins (Ig), are the non-membrane bound, secreted form of the B cell receptor comprised of at least 4 chains, 2 each of the heavy and light chains. They are comprised of two functional regions, the variable and crystallizable fragments. Derived from the adaptive immune system, antibodies are incredibly specific for the antigen they recognize and bind Ag through the variable fragment. This domain contains the portion of the antibody which undergoes mutations to ensure it recognizes its antigen with high affinity. Antigen specific recognition by antibody enables neutralization of various toxins, such as in antivenom, as well as preventing direct infection of cells by blocking cell binding. The crystallizable fragment of the antibody is highly conserved and serves to give the antibody function beyond binding. This region has many subclasses, as determined by the structure of the tail end of the heavy chain, which then subsequently determine how the antibody interacts with other cells of the immune system. These fall into 5 broad categories called IgD, IgM, IgA, IgG, and IgE with even further subclasses such as IgG1, IgG2, IgG3, and IgG4. Such heterogeneity allows redundancy in how cells are called to react while also providing a framework for specificity in the ultimate response.(36) An example of this would be that mast cells express the high affinity IgE receptor and cross-linking of this receptor leads to rapid degranulation of these cells and thus an anaphylactic response; however, antibodies of the IgG subclass have a range of affinities for components of the complement cascade and are thus able to tune how rapidly immune complexes fix complement and induce anaphylatoxin generation.(37) Further Fc receptor mediated antibody functions include antibody mediated cellular responses such as Antibody Dependent Cellular Cyto-

toxicity (ADCC) and phagocytosis where the presence of an antibody coating on a pathogen allows for cells (such as neutrophils, NK cells, or macrophages) to sense said offender and respond either by directly killing the cell or by phagocytosing the cell.(36)

1.1.3 The Pro-inflammatory Response

The immune system has evolved to react to many types of environmental in a specialized manner to enable clearance of these pathogens with minimal damage to the host. This section will serve to summarize how the players denoted in the previous section come together to fight off infection.

Type 1 Immunity — Antiviral and Antibacterial Response

Upon sensing infection via pattern recognition receptors (PRR), myeloid cells such as neutrophils, monocytes, and dendritic cells will react by releasing factors such as type one interferons and endocytosing the material before traveling to the draining lymph node. The tissue itself responds to these interferons, instigating an innate type of antiviral response and upregulating MHCI expression, thus increasing the chance of presenting the pathogenic epitope to the adaptive immune system. In the lymph node, the activated APCs secrete signals like IL-12 and interferon-γ (IFN-γ) while upregulating costimulatory molecules like CD80 and CD86 as well as increasing cell surface expression of MHCII. These signals together inflame the lymph node and begin crosstalk with the adaptive immune system. Presentation of antigens in this context prime naive T cells (both $CD4^+$ and $CD8^+$) and imprints what is known as a Type 1 immune profile on these cells. T_H cells emerging from this reaction take on what is known as a type 1 phenotype stabilized by the transcription factor Tbet, in that activation of these cells causes production of cytokines like IL-2, Tumor Necrosis Factor-α (TNF-α), and IFN-γ which then propagate the reaction in a feed forward manner,(38) further eliciting cytotoxic effects from the myeloid compartment as well as directly killing

infected cells. In addition to development of T_{H1} responses, B cells in this milieu also go on to become educated here, going through germinal center reactions to generate highly specific antibodies. Class switching in this environment opens up chromatin in areas leading to a predominance of antibodies of IgG2c class,(39) as well as others, but most importantly IgE is excluded from production here. Brakes on this immune cycle take the form of expression of immunomodulatory cytokines such as IL-10, expression of co-inhibitory receptors, and development of regulatory T cells (Tregs), all of which are discussed later in subsection 1.1.4. Resolution of this immune process occurs once antigen is depleted and the infection is cleared, leading to a contraction in the adaptive system, leaving behind a small subset of memory effectors of the adaptive response and clearance of the resulting debris by the phagocytes.

Type 2 Immunity — Antiparasitic Response

As opposed to single-cell or viral pathogens (previous section), multi-cellular pathogens require a different response from the immune system to be cleared. Ultimate end goal of these responses relates more so to activation of granulocytes, whose effector molecules, such as histamine, prostaglandins, and IL-4, lead to smooth muscle contraction, mucus production, and epithelial reactions which are all related to the expulsion of parasites. For type 2 immunity, a break in barrier integrity triggers the release of signals known as alarmins, such as IL-33 or the nuclear factor high-mobility group box 1, and other damage associated molecular patterns (DAMPs) from the epithelial tissues which activate resident monocytes and dendritic cells in the tissue to migrate to the draining lymph nodes.(40) Once in the lymph node, presence of IL-4 and IL-13 from granulocyte activation instigates a feed-forward loop where APCs are matured, presenting costimulatory factors with increased MHCII, and education of naive $CD4^+$ T cells can ensue. T cells educated in this milieu, called T_{H2} cells, stabilize expression of the transcription factor Gata3, which enforces a transcription profile of cytokines like IL-4, IL-5, and IL-13 further feeding into pathways to clear parasitic

pathogens.(38) Simultaneously, B cells experiencing antigen in this environment undergo class-switching towards effector antibodies primarily of the IgE subtype as well as IgG1 in mice.(41) All of these work in concert to enable ultimate clearance of the pathogen and protection from future insult.

Other Pro-inflammatory Cascades

There are many adaptive immune reactions beyond T_{H1} and T_{H2}, one of which relates primarily to clearance of fungal and extracellular pathogens, which generates a cell type called T_{H17} due to the expression of the effector cytokine family of IL-17s. Cells of this subclass are generated from education in a milieu of Transforming Growth Factor Beta (TGF-β) and IL-6 which induces and stabilizes expression of the transcription factor Rorγt. This class of cell is especially beneficial at mucosal sites as these cells express cytokines, such as the IL-17 family and IL-22, relevant for production of antimicrobial peptides and promoting barrier integrity.(42; 43) Another T cell response of importance is the T follicular helper (T_{fh}) cell. These cells are critically important for the generation of B cell germinal centers which create high affinity antibody responses and serve to provide T cell help through signals such as IL-21, ICOS, and CD40L. They are defined primarily by their expression of the transcription factor Bcl6 and the receptor CXCR5.(42; 44) Other inflammatory pathways which involve more innate type sensing, such as the inflammasome, STING, NLR, and TLR cascades, are also tightly intermingled with these adaptive responses and also contribute to such immune activation. In fact, these innate pathways are the primary mode of keeping infections in check until the adaptive response is able to emerge for pathogen clearance.(45)

1.1.4 The Tolerizing Response

With the initiation of inflammation, anti-inflammatory cascades are also induced as a means to prevent excessive host damage while clearing the infection. In other words, if the host

dies in the process of clearing the infection, yes the infection is gone, but so is the host. This section will summarize ways in which the immune system puts out brakes to protect the host including: regulatory T cells, immune checkpoints, adaptive tolerance (such as T cell anergy and exhaustion), and finally cytokines which signal to slow the immune response.

Regulatory T cells

Tregs are a heterogeneous group of cells defined by their suppressive functions and expression of the transcription factor Foxp3. These cells are either derived in the thymus via interaction with their antigen as expressed by *AIRE*, called tTregs, or develop in the periphery by subsequent antigen exposure and engagement in a Treg inducing milieu (namely TFG-β), called pTregs. Beyond just Foxp3, functionally active Tregs also constitutively express the high-affinity IL-2 receptor, called CD25, which has brought to question how IL-2 signaling relates to the suppressive function of Tregs.(46–48) The dependence of these cells on IL-2 signaling is coming into question, however. Data from the past two decades have interpreted the presence of CD25 as a fully functional Treg with stabilized effector functions; more recently, it seems that such stabilization signals may arise from other fates as well. For example, one study found that intestinal Tregs lose CD25 and thus decrease the necessity of IL-2 signaling while maintaining suppressive functionality via CD39, CD73, CTLA-4, and IL-10 expression.(49) Other signals may also contribute to stabilization of the Treg phenotype, such as the transcription factor helios. Initially thought of as a marker of tTregs, more recent research seems to point to helios as a factor which stabilizes Treg effector functions.(50; 51) Loss of helios seems to change the suppressive potential of Tregs in that Foxp3^{+}Helios^{-} Tregs maintain ability to express pro-inflammatory cytokines such as IFN-γ and TNF-α. Overall, Tregs constitute a powerful suppressive population of cells which control inflammation in a manner termed "dominant tolerance" in that transfer of these cells are enough to halt pro-inflammatory cascades. Furthermore, there is increasing evidence coming from the transplant

tolerance field of processes known as “infectious tolerance” and “linked suppression,” wherein antigen specific Tregs are capable of converting effector T cells, which recognize either the same antigen or a different antigen presented on the same APC, respectively, into Tregs themselves.(52; 53) As such, research into induction and destruction of these cells is a critical research path for autoimmunity and cancer therapeutics.

Checkpoint Molecules

Upon activation, immune cells will upregulate immunosuppressive markers to prevent death of the host as a result of unchecked inflammation. These markers have recently been termed immune "checkpoints" as they provide balance to the immune reactions. One such example of this is that upon antigen recognition and T cell activation, one of the first markers which is upregulated is the Programmed Death receptor PD-1. Signaling through this receptor recruits inhibitory kinases to the immune synapse, thus dampening the overall response.(54) A few other inhibitory checkpoints on T cells are CTLA-4, LAG3, and TIM3 all of which act through different mechanisms to blunt the response.(54) Checkpoint receptors are also expressed on myeloid populations and can enforce a tolerogenic state on the APC. One example of this interaction would be upregulation of CD200R on myeloid populations upon activation, which upon engagement with CD200 increases the threshold required to activate and mature such myeloid cells.(55; 56) Checkpoint molecules have become increasingly important as they are overactive in malignancies and promote tumor survival; as such, these molecules will also be covered in subsection 1.2.1 and section 1.3.

Anti-inflammatory Cytokines and Enzymes

Cytokines offer pleiotropic signals in that the context in which they are present changes their function; however, 3 cytokines in particular – TGF-β, IL-10, and IL-35 – are primarily considered immunosuppressive in function and are all produced by Tregs as effector

molecules.(57) Otherwise, cytokines which are normally induced by type 1 immune reactions (IFN-γ and IL-12) tend to skew type 2 immune responses away from a pathogenic state, while cytokines involved in type 2 immunity (IL-4 or IL-13) are capable of halting aggressive type 1 and 17 immune reactions.(58) With this in mind, our lab as well as others have sought to induce or engineer these cytokines to aid in suppressing allergy and autoimmunity, as reviewed in section 1.3. Beyond these functional cytokines, cells are also capable of producing enzymes which may function in immunosuppression by depleting the local environment of key signals for immune activation. Enzymes in this category include Arginase(59) and Indoleamine 2,3-dioxygenase (IDO)(60) which metabolize the amino acids arginine and tryptophan, respectively. These amino acids are key in T cell and general immune functionality and depletion of them results in a blunted immune response.

1.2 Unproductive Shifts Along the Immune Spectrum

The immune system does not come without inherent flaws and misjudgments. In some cases, the system does not respond to signals when it should, or the offending agent is capable of evolution to avoid the host immune system. In other cases, some signal gets mixed and the immune system hyper-reacts to an otherwise innocuous agent and develops a lasting memory. These processes will be reviewed in the following section with the former related to cancer malignancies, and various ways cancers are able to subvert the immune system. The latter case is exemplified through autoimmunity and allergy responses, corresponding to overactive type 1 and type 2 immunity, respectively.

1.2.1 Cancer and Immune Evasion

Cancer constitutes a situation where the immune response has been blunted and prevents clearance of the offending tissue. This blunting usually occurs through directed evolution of the pathogenic cell following pressures by the immune system, a process known

as immunoediting.(61) One mechanism by which cancer avoids immune recognition is by down-regulation of MHCI.(62; 63) This lessens the ability of CTLs to recognize and kill the cancerous tissue. With this down-regulation, however, the cancer must also upregulate inhibitory markers to avoid recognition by NK cells and the innate immune system, such as CD47.(64) known as a "don't eat me" signal, CD47 expression prevents phagocytosis and clearance of healthy cells; upregulation of CD47 on cancer cells however inhibits uptake and presentation of possible neoantigens to the adaptive system.(65) Furthermore, cancer cells upregulate checkpoint molecules like PD-L1 as well as enforce immunosuppressive environments to blunt the response even more. Expression of factors like TGF-β may lead to excessive extracellular matrix (ECM) deposition, providing a physical barrier for immune cell infiltration as well as continuing the feed-forward loop of immunosuppression.(66) These various factors, taken together, have led to a system of scoring immune infiltration within the tumor and predicting responses to immune related therapeutics. Tumors with high immune infiltration are likely to respond well to therapy and are known as immunologically "hot," whereas tumors with little to know immune infiltration are known as "cold."(67; 68) The last subset are tumors in which the growth and ECM prevents immune interaction within the tumor boundaries and immune cells are secluded to the outskirts of the tissue, known as immune "excluded."(69) Hot tumors have likely enforced phenotypes on the local immune environment leading to T cell exhaustion.(70) Characterized by high expression of checkpoint molecules and loss of expression of TNF-α, T cell exhaustion is an interesting immune phenomena wherein chronic exposure and signaling through the TCR leads to an ineffective response as well as a type of repopulating memory state.(71; 72) Overall, the tumor microenvironment constitutes a heterogeneous space, the study of which has expanded our immunologic knowledge of how regulatory networks come together. The search for how best to address these immunologic shortcomings has revolutionized immunology and will be addressed further in section 1.3.

1.2.2 Autoimmunity and Allergy

Autoimmunity and allergy present as conditions in which tolerance to the "self" or innocuous "non-self" is broken and improper immune responses occur and have been termed "hypersensitivites." One method to classify drug related hypersensitivities is the Gell-Coombs system.(73) While used to explain reactions to drug treatments, these hypersensitivity descriptions are useful in describing both autoimmunity as well as reactions to foreign entities.(74) Hypersensitivities in this system fall under four primary distinctions: type I–traditional IgE mediated anaphylaxis-like hypersensitivities; type II–non-IgE antibody mediated reactions involving complement fixation; type III–IgG mediated immune complex formation leading to local damage as well as kidney damage; and type IV–delayed, T cell mediated reactions leading to direct cell death or local release of inflammatory cytokines. A few examples of autoimmunity (primarily type IV hypersensitivity in this system, but some bleed over with antibody mediated effects) are: type 1 diabetes (immunity developed against pancreatic β cells), multiple sclerosis (immunity development against myelin related proteins and other components of the central nervous system), rheumatoid arthritis (immune reactions against components of the joint), and inflammatory bowel diseases (immune reactions against commensal microbes in the gut and eventually against the tissues of the gut itself). Treatments for these types of reactions have traditionally been supplementation of the lost signals (ie: insulin injections for diabetics) or broad immunosuppression to prevent further tissue destruction. Recent advancements have allowed for a more tailored approach by blocking immune signaling cascades overactive in the patients case, such as TNF-α blocking treatments for many indications or IL-17 blocking treatments for IBD and psoriasis (reviewed in more depth in section 1.3).

Food and environmental allergies are the best examples of type I hypersensitivities. Upon exposure to the allergen, IgE crosslinking on granulocytes (primarily mast cells) induces degranulation and the release of soluble mediators such as histamine and chemokines related to

recruitment of eosinophils and basophils. Histamine signaling leads to vasodilation and mucus production causing local swelling around the airways and a drop in total blood pressure, termed anaphylactic shock. Chronic inflammation of this type to environmental allergens – house dust mite, pollen, mold – leads to asthma development and chronic airway constriction. Treatments for type I hypersensitivities primarily consists of blocking histamine reactions, using anti-histamines, vasoconstriction using epinephrine to prevent anaphylactic shock, and local steroid use to bring down inflammation in asthmatic attacks.

1.3 Therapeutics and Immunomodulation

Due to the important context of cell-to-cell interactions and communications in determining the overall response, the immune system is a prime space for bioengineering. By changing one small signal between immune cells, say blocking a single receptor or adding/removing a cytokine signal, the entire response can be modified to a therapeutic end. As we have learned more and more about the immune system over the past six decades, these therapeutics have finally begun to come to fruition and provide long-lasting benefit in conditions which may have been otherwise untreatable.

1.3.1 Antigen Specific Immunotherapies

Since scientific recognition of the specificity of the immune system, a long-standing goal was the ability to induce antigen specific responses to both noxious and harmless proteins. This section will cover methods tested to induce antigen specific immunosuppression, herein termed tolerogenesis, in cases of autoimmunity and allergy or inflammation as well as antigen-specific immune activation in cases of cancer.

The primary, and lofty, goal of antigen-based therapies in autoimmunity is the generation of functional and lasting Tregs. As discussed in subsection 1.1.4, Tregs are a powerful suppressor cell type capable of exerting "dominant tolerance," where generation or transfer

of these cells is sufficient for reduction of autoimmune pathologies. With this in mind, research into the generation of technologies related to modifying antigen location and signals around the T cell at time of education to induce Treg phenotypes has been fruitful. These involve targeting antigens to: tolerogenic niches like the liver(75) or skin,(76; 77) recycling pathways of cells in general as well as that of red blood cells (called efferocytosis),(78; 79) and targeting subsets of cells capable of presenting antigen in the steady state, such as DC and liver resident APCs like sinusoidal-endothelial cells (LSECs).(80–83) In addition to direct generation of Tregs, another goal of these immunotherapies is to induce antigenic spreading away from just the epitope delivered, in this case called bystander or infectious tolerance. Herein, the presence and proliferation of Tregs induces a milieu of cytokines and signals – such as IL-10, TGF-β, and IL-35 – capable of promoting the generation of novel Treg lineages.(84–88) Another goal of antigen specific tolerance, while less powerful than Treg induction but important nonetheless, is to educate the relevant T cells in a context without excessive inflammatory signals to potentially induce immunologic dysfunction, called anergy and exhaustion.(89) These fates arise from chronic stimulation through the TCR ultimately leading to a loss of reactivity, which is beneficial in cases to reduce inflammation. One simple but useful methodology to induce such a fate can be seen in the use of both allergy shots and oral immunotherapy, the latter of which overcame a major hurdle in the FDA approval of the first oral immunotherapy, Palforzia™, in 2020. However, while the exact mechanism by which Palforzia™works is unknown, recent work in oral immunotherapies suggest chronic antigen exposure leads to an initial burst of allergic responsiveness followed by either a crash of those populations or a shift to non-allergic type responses (IgA, IgG4, and potentially Tr1 expansion).(90) These methods are capable of inducing a transient loss of reactivity, but not all patients experience lasting tolerance.

Instigating immune reactions against cancers provides a unique challenge as opposed to initiating immunity against foreign proteins, due to the cancer having evolved in the pres-

ence of the immune system.(91; 92) Considering this, therapies to induce novel reactions against tumors tend to focus on initiating responses to over expressed proteins or to develop reactions against novel epitopes generated by the high mutational burden on tumors, termed "neoantigens". The straightforward approach to this problem would be to sequence the tumor genome and match against that of healthy tissue before determining potential epitopes that the immune system may react to, and generating a vaccine against such an epitope.(93–98) However, the cost and level of personalization that this procedure requires makes the effort moot.(99; 100) A more reasonable approach to this is to directly adjuvant the tumor and generate an "in situ" vaccine, which allows the immune system to perform the selection process on its own.(101–103) Alternatively, recent research has looked to exploit tumor associated antigens (such as CD20, Mucin-1, epithelial growth factor receptor, or carcinoembryonic antigen to name a few)(104–107) to engineer and transduce artificial receptors, made of an antibody-based recognition domain fused to T cell signaling machinery, specific to these markers to generate chimeric antigen receptor T (CAR-T) cells. Expression of these receptors on the patient's own T cells allows for a more specific method of tumor cell deletion; however, while these work exceedingly well for blood-borne malignancies,(108) solid tumors pose unique challenges related to the ability of the cells to infiltrate the tumor and the suppressive tumor microenvironment.(109; 110) Engineering strategies to enhance the efficacy of these cells are underway, but these technologies fall outside of the scope of this thesis.

Overall, methodologies to induce tolerance and immunity to specific antigens remain the elusive holy grail of immunomodulation. With recent advancements and expanding knowledge of the immune system, the interactions of the system with the rest of the body, and how the microbiome is involved in these reactions, antigen specific therapeutics are becoming more and more realizable.

1.3.2 Blockades – Cytokine and Checkpoint

Due to the interconnections between so many immunologic processes, decreasing a single signal can completely change the context and downstream cascades of the immune system. This concept is a driving force for the generation of novel therapeutics which block pro- and anti-inflammatory signals for autoimmunity and cancer. These therapeutics are reviewed below.

Research in the early 1990s demonstrated the importance of the cytokine TNF-α in rheumatoid arthritis pathogenesis.(111; 112) This knowledge in hand, researchers recombinantly fused either the extracellular portion of the TNF-α receptor or the antigen recognition site of a mouse anti-TNF-α antibody to the Fc domain of a human antibody, effectively blocking cytokine signaling and generating an improved half-life, initiating the start of a field of novel human therapeutics.(113; 114) Etanercept and infliximab have since been approved for numerous autoimmune conditions, including rheumatoid arthritis, ankylosing spondylitis, and IBD to name a few. Following etanercept and infliximab, novel cytokine blocking immunotherapeutics have hit the market with great promise to provide alternatives to patients who develop resistances to initial treatments — at least five TNF-α blockers on the market, acting as next in line treatments — as well as providing a potentially more specific avenue of protection with lessened side effects and decreased risk of becoming totally immunocompromised, in the case of therapeutics blocking IL-17A or IL-23 for psoriasis or IBD.(115–117) However, as previously alluded to, these cytokines are not only functional in autoimmune cascades but are also responsible for signaling related to infection and cancer control. Recent meta-analyses seem to sate fears related to cancer occurrence or recurrence in patients on blockade therapies(118); however, susceptibility to novel infections as well as worsening chronic infections like tuberculosis has been documented.(119–121) With this being said, these effects are also recapitulated with chronic use of immunosuppressive drugs like methotrexate, rapamycin, or corticosteroids. This broad immunosuppression and the

risks associated with it is one reason for the interest in antigen-specific immunotherapies. In addition to blocking signals related to type 1 and type 17 immunopathologies, recent approval has also been granted to therapies meant to interfere with type 2 pathologies; these therapeutics focus on blocking such systems as IgE binding to mast cells with regular infusion of αIgE-Fc monoclonal antibodes (Xolair™) or more recently the approval of a monoclonal antibody which blocks the α chain of the IL-4 and IL-13 signaling receptor (Dupixent™).(122–125) This form of therapy may also come with a similar issue, in that by blocking type 2 responses the other arms of the immune system could become dysregulated and hyperactive.

Beyond blocking pro-inflammatory signals, antibody therapeutics have shown incredible clinical success as cancer treatments. Focusing instead on blocking immune "checkpoints," these therapeutics are known by their ability to release the breaks which cancer implements on the immune system. The importance of these breakthroughs is not only emphasized by the eruption of the checkpoint blockade field, but also in the awarding of the 2018 Nobel Prize in Medicine going to James Allison, for the discovery and blockade of CTLA-4, and Tasuku Honjo, for the discovery of PD-1.(126; 127) These immunotherapeutics offer the opportunity for a subset of cancer patients to experience lasting remission, even in cases of advanced metastatic cancer. Releasing the immune system in these cases allows for the dissemination of tumor reactive immune cells throughout the body to reinvigorate the antitumor response. Since the discovery and approval of checkpoint blockades, research into other targetable moieties of immunosuppression have exploded. From therapies acting along similar axes to reinvigorate the immune system, like those against LAG3 or TIGIT,(128–130) to antibodies enhancing immunologic uptake of tumor cells and boosting the potential of presentation of neoepitopes, like those blocking the "don't eat me" signal CD47,(64; 131; 132) checkpoint blockade and immunotherapeutics have made a lasting impression on the cancer research field and will continue to do so.

1.3.3 Cytokine Immunotherapies

Cytokine therapy research hit a boom in the 20th century with FDA approvals coming in the 1990s.(133) After the first wave of approval in the early 1990s, with very strict guidelines on usage, engineering strategies to address issues of toxicity and pleiotropy of these molecules have emerged, but no new cytokines have reached FDA approval since. In addition, strategies focused on other cytokines and indications have arisen.(83; 134–136) This section will briefly review attempts to engineer these cytokines.

A primary issue encountered by cytokine therapeutics is the relative short half-life of these proteins due in part to their small size (usually <30 kilodaltons). Engineering strategies for this tend to focus on either increasing the relative size of the protein or by exploiting natural methods of protein recycling to bypass the clearance by the kidneys. Clinically successful examples of the former include the chemical fusion of the cytokine to polyethylene-glycol (PEG);(137; 138) however, with recent research related to PEG exposure and immunogenicity thereof, this methodology has become contentious.(139–141) Other methods designed to increase cytokine recycling involve recombinant fusion of the protein to the Fc fragment of antibodies(142–144) or, of particular relevance to the project contained in this thesis, to serum albumin.(58; 138; 145–147) These protein domains have an innate capability to bind recycling receptors, such as the neonatal Fc receptor, which enables recirculation of the protein out of the kidney tissue and back into blood. Although successful, with the increased systemic exposure to the cytokine, methods related to solely increasing circulation may come at the cost of increased risk of toxicities.

As previously stated, the power and pleiotropy of cytokines poses a double-edged sword to the therapeutic potential of these protein drugs. Alternative strategies to engineering these proteins may take the form of changing affinity for portions of a receptor complex. One such example of this could be the novel forms of pegylated IL-2 from the biotech company NEKTAR.(23; 148; 149) Another alternative of this would be the *de novo* generation

of cytokines through learning algorithms trained to emphasize some faces of the receptor complex and avoid others, as the case in recent work on "Neoleukin-2" and Neo-4.(150–152) In addition to this, other attempts have also come about to modify the binding capabilities of IL-2 to the various portions of the receptor complex (IL-2Rα, IL-2Rβ, γc) to promote or dissuade Treg binding and proliferation.(153–159) Alternatively, previous work in our lab and others has sought to change the toxicity profile of cytokines by recombinantly fusing the protein to a masking domain.(160–163) Changing the affinity to the receptor complex helps to avoid toxicities encountered through cytokine pleiotropy by avoiding unintended signaling or by requiring a higher local concentration to achieve signaling. To this end, cytokine engineering has also taken the form of adding other domains to the cytokine to adapt cell-specific or tissue-specific tropisms to the molecules.(164–167) One branch of this, termed immunocytokines, fuses the cytokine to the variable fragment of an antibody with research in this domain focused on targeting broad signals found primarily in tumor tissue, such as necrosis and dysregulated vasculature.(168–174) Alternatively, our lab and others have also focused on adding domains of other proteins to imbue a targeting capability without the use of antibody regions – such as binding exposed collagen to localize or retain signaling to both tumors and sites of inflammation.(165; 167; 175–177) In addition to tissue targeting approaches, research to preferentially target cytokines to cellular subsets via fusion to an antibody fragment or the addition of a natural binding moiety for receptors on the particular cell type is also coming into focus.(178–180) These approaches seem to maintain benefit in pre-clinical models, but further research is necessary to understand if toxicity measures are reduced in people.

CHAPTER 2

ENGINEERED FLT3L-SA PRODUCTION AND CHARACTERIZATION IN HEALTHY ANIMALS

2.1 Abstract

Dendritic cells have come to be a focus of immunotherapeutic targeting, due in part to their impressive capability as a professional antigen-presenting cell. Operating as the decision matrix of the adaptive immune system – sampling the environment, bridging the gap between innate and adaptive systems, and determining the ultimate flavor of the immune response – dendritic cells demonstrate a particular strength considering their relative scarcity as a portion of immune cells. Herein, we develop a novel engineered fusion of Flt3L to serum albumin (Flt3L-SA) and characterize the biochemical and pharmacological properties of the drug. We demonstrate profound effects on the DC compartment in the overall immune system as well as that of the gut and note a relative increase of TGF-β producing DCs. Furthermore, we characterize the effects on the adaptive immune system in expansion of regulatory T cells. We finally attempt to determine how the expansion of DCs leads to concurrent Treg proliferation and serendipitously note a potential feedback mechanism of PD-1/PD-L1 signaling in restraining Treg proliferation.

2.2 Introduction

Dendritic cells (DC) are becoming increasingly relevant as their functions beyond antigen presentation alone are being elucidated. Acting as the kill switch between pro- and anti-inflammatory cascades, DCs have the ability to dictate the continuation or halting of immune reactions, both in the production of cytokines and surface receptors as well as in crosstalk to the adaptive immune response. As such, recent research has sought to exploit these functions of DCs to each end.(81; 181–187) Primary research has focused on the importance of type 1 conventional DCs (cDC1) in the anti-tumor response; by cross-presenting exogenous, cell-bound antigens to cytotoxic $CD8^+$ T cells, cDC1s play a critical role in education and advancement of the immune reaction against tumors and infections.(165; 188–193) Enhancing

cDC1 presence in the tumor environment via chemokine localization or through expansion of these cells has shown therapeutic benefit in combination with other immunostimulatory agents, such as checkpoint blockades, irradiation, or addition of therapeutic adjuvants.(194–196) Expansion of DCs has occurred through use of irradiated tumor cells acting as factories of GM-CSF (GVax) or Flt3L (FVax) or the exogenous addition via regular injections of the purified recombinant protein.(194; 195; 197–203)

As previously stated, however, DCs act in both immunoinhibitory capacities as well as stimulatory.(204; 205) The ability to use DCs to restrain the immune reaction is an understudied field, in comparison to research involving the stimulatory ability of the cells; however, relevant research to the inhibitory capacity of DCs is also coming from cancer research. One such model is the research into a subset of DCs coined mregDCs (mature DCs enriched in immunoregulatory molecules) by the Merad group as well as others.(206) While these cells maintain antigen presentation capacity, co-expression of regulatory markers such as CD200 and PD-L1 inhibits activation of corresponding adaptive cells. In addition to this type of DC, others have described methodologies in which DCs are directly able to generate signals to amplify tolerogenesis and Treg formation including production of IL-10, indoleamine 2,3-dioxygenase (IDO), TGF-β, and retinoic acid. Overall, however, there are as many ways of defining and making a "tolerogenic DC" as there are number of people studying them, making classification and distinction of direct fate difficult.

Herein this chapter, we describe and characterize a novel cytokine therapeutic for the expansion of DCs, FMS-like Tyrosine kinase 3 Ligand (Flt3L) recombinantly fused to serum albumin (Flt3L-SA). Flt3L signaling through CD135 (Flt3) has been determined essential for DC generation, especially for cDC1 differentiation, from hematopoietic progenitors, but due to its small size therapies require repeated injections.(194; 195; 207–209) We address this limitation through the recombinant fusion of albumin, which prolongs systemic circulation and speculate that the fusion may also change the tissue tropism of the cytokine. We further

characterize phenotypic differences of DCs from Flt3L-SA treated mice to that of untreated and mice treated with a native (WT) Flt3L variant. We also explore the generation of a TGF-β expressing subset of DCs and the effect of PD-1:PD-L1 signaling on the resting adaptive immune system. Finally, we define and characterize how treatment with the novel cytokine fusion affects the resting composition of Tregs in healthy mice. We use this to provide a framework for future studies and immunotherapeutic applications of this protein, covered in more depth in Chapter 3.

2.3 Results

2.3.1 Production and In Vitro *Characterization of Flt3L Variants*

Production and Purification

Murine Flt3L was first cloned into the pcDNA3.1 plasmid with an N-terminal IgGk leader sequence and a C-terminal 6his tag (**Fig. 2.1a**). This provided both a framework for future cloning as well as an expression vector for a non-engineered, WT Flt3L. Following this, Murine Serum Albumin (SA) with an N-terminal $(G_4S)_2$ spacer sequence was subcloned C-terminally to Flt3L to produce our Flt3L-SA construct (**Fig. 2.1b**). Production of these proteins was carried out in the HEK293F system using Opti-pro transfection media and linear Polyethylenemine (see Materials and Methods for full transfection protocol). Following a week of transfection, supernatant was harvested and sterile filtered before flowing over an AKTA fplc using a HisTRAP and Size exclusion chromatography. Purity of the samples at the end was assessed via SDS-PAGE prior to aliquoting and freezing (**Fig. 2.2**).

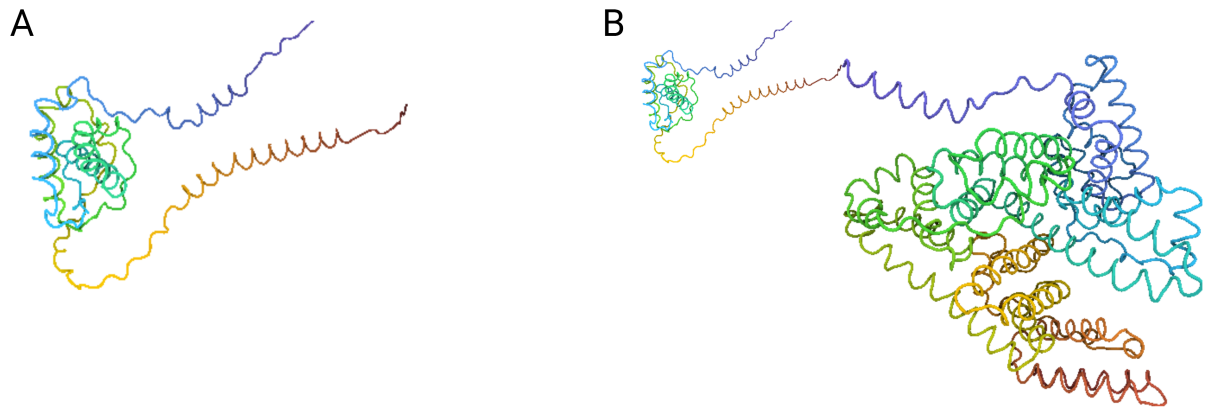

Figure 2.1: **Representative Schematic of Flt3L Constructs | a,** Representative image of native Murine Flt3L. **b,** Representative schematic of Flt3L-SA Fusion protein with the Serum Albumin C-terminal to the Flt3L. Image generated in Biorender using AlfaFold prediction structures.(210; 211)

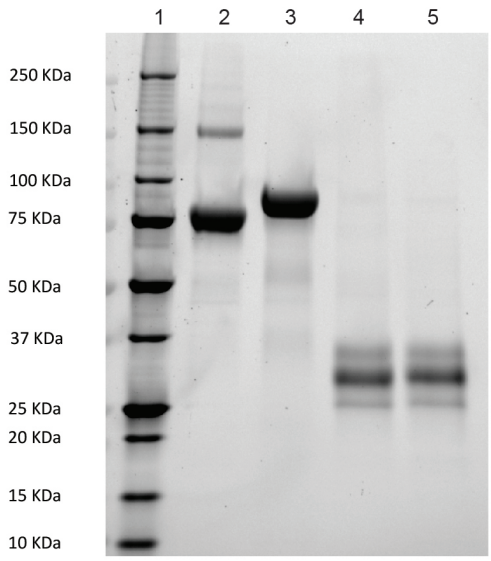

Figure 2.2: **SDS-PAGE Demonstrating Purity of Flt3L and Flt3L-SA Following Purification |** Lanes represent the following as 1-5: Ladder, Flt3L-SA under non-reducing conditions, Flt3L-SA under reducing conditions, WT Flt3L under non-reducing conditions, and WT Flt3L under reducing conditions.

Binding and *In Vitro* Bioactivity of Flt3L Variants

To then confirm functionality of both variants produced (WT Flt3L and Flt3L-SA), we first sought to determine whether binding to the cognate receptor for Flt3L, CD135 or Flt3, had been impacted by production or fusion. This was performed via an ELISA plate coated with CD135 and detection with a biotinylated anti-mFlt3L antibody followed by streptavidin-HRP based conversion of TMB. From this, we see very similar binding with affinity for CD135 measured as 160pM for both variants (**Fig. 2.3**). We also note decreased saturation of the measurement for the Flt3L-SA fusion, which we attribute to the increased size and steric inaccessibility of the detection antibody for the protein.

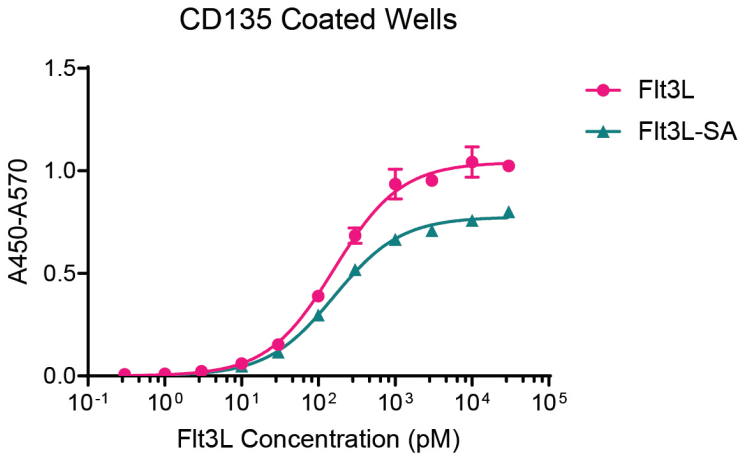

Figure 2.3: **ELISA Demonstrating Affinity of Flt3L Constructs Against Flt3 (CD135)** | ELISA coated with the receptor CD135 used to demonstrate the relative affinity of the Flt3L constructs for their receptor. Binding affinities for Flt3L and Flt3L-SA are 160.4pM and 160pM, respectively. Each sample was performed in triplicate with error bars representing SD.

Once binding was confirmed, we then sought to check bioactivity of the molecules. Based on literature related to the signaling cascade of Flt3L, CD135 autophosphorylates itself before recruiting signaling components of the ERK (Extracellular signal-Regulated Kinase) pathway system.(212) As such, we first checked signaling cascade following Flt3L treatment on starved BMDCs differentiated in the presence of Flt3L blotting for phosphorylated ERK1/2 as well as

total ERK1/2 following different times of stimulation. Herein, we can see effective stimulation of the ERK pathway using both Flt3L variants with a peak in signaling at five minutes of exposure (**Fig. 2.4**), which aligns with published reports of Flt3 signaling.(212; 213) With the same cells used in the western blots, we then sought to check signaling related to concentration in order to ascertain the EC_{50} of each of the drugs. Knowing that the proteins eventually signal through ERK1/2, we were able to stimulate the cells and then check bioactivity via flow cytometry by staining for pERK1/2. As seen in **Fig. 2.5**, EC_{50} of the WT Flt3L and Flt3L-SA were determined to be about 1.3 and 3.6 nM, respectively. Thus we see a slight decrease in total bioactivity of the SA fused protein, potentially due to steric prevention of dimerization of either the Flt3L variant or of the receptor and thus prevention of the autophosphorylation cascade. However, this effect seems minimal and provided us with evidence to move forward with *in vivo* studies.

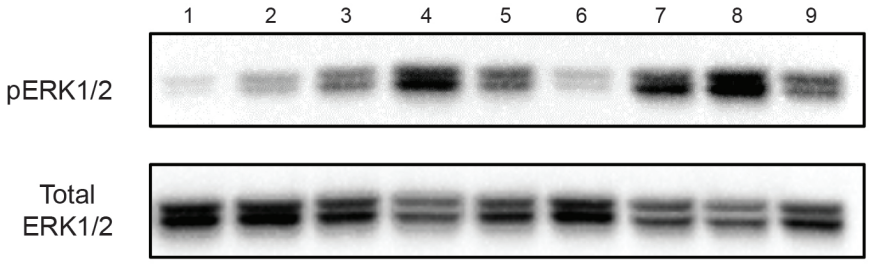

Figure 2.4: **Time-dependent Signaling through ERK Cascade Confirms Flt3L Functionality |** BMDC's generated in the presence of Flt3L were starved before stimulation with 2μM Flt3L or Flt3L-SA for times indicated as follows for lanes 1-9: media control, WT Flt3L for 1', 2.5', 5', 10', Flt3L-SA for 1', 2.5', 5', 10'. Post stimulation, cells were lysed and equivalent protein amounts were loaded per well before probing for phospho-ERK1/2 and total ERK1/2.

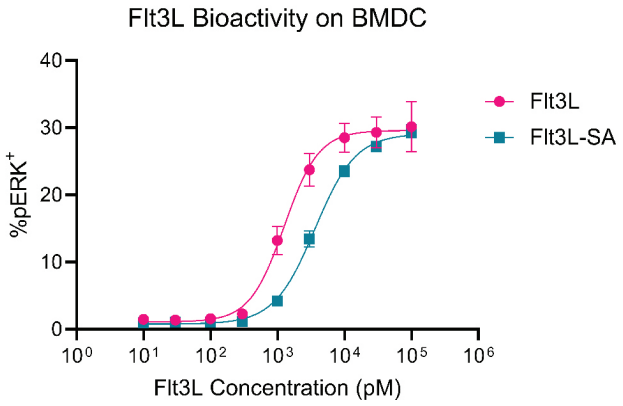

Figure 2.5: **Dose-dependent ERK Signaling Demonstrates On-cell Bioactivity** | starved BMDC's generated in the presence of Flt3L were stimulated with indicated concentrations of Flt3L or Flt3L-SA for exactly five minutes before fixation and permeabilization and staining for pERK1/2. Best fit lines gives EC_{50} values of 1.267nM and 3.641nM for WT and Flt3L-SA, respectively. Data points represent average and SD of quadruplicate samples.

2.3.2 In Vivo *Pharmacokinetics, Bioactivity, and Pharmacodynamics*

Pharmacokinetics

As it is known that serum albumin has a long half-life, due to a number of factors including its size, which prevents clearance via the renal system, and ability to bind the neonatal Fc receptor (lessening the effect of lysosomal degradation)(58; 214–217), we next sought out to determine the pharmacokinetics and *in vivo* half-lives of the Flt3L variants. To do so, C57BL/6 mice were subcutaneously (SC) injected with 10μg or the molar equivalent of Flt3L or Flt3L-SA, respectively, at time 0. Flt3L content was then measured in the plasma via Flt3L ELISA at times noted in **Fig. 2.6A**. We note a dramatic increase in the amount of Flt3L in the plasma of Flt3L-SA treated mice at all times measured when compared to those treated with WT Flt3L, which then leads to a 30 fold overall increase in Flt3L exposure over time (**Fig. 2.6B-C**). When values are normalized to the maximum detected

value, it becomes apparent that the increase in Flt3L exposure is due to both an increase in time to peak due to a depot effect, $t_{1/2rise}$ of 1.3 and 4.1 hours for Flt3L and Flt3L-SA, respectively, but also an extension of the half-life of the SA fusion once it actually reaches the bloodstream, $t_{1/2fall}$ increased from 10.6 hours for the WT variant to 55.8 hours for the SA fusion (**Fig. 2.6D**).

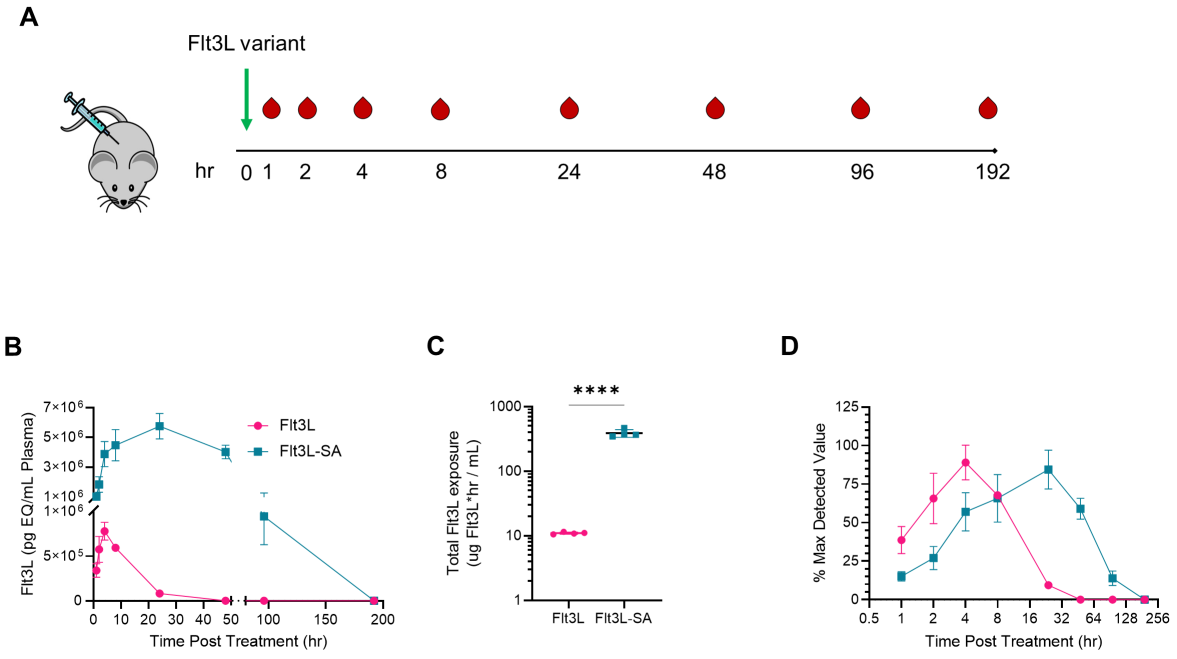

Figure 2.6: **Pharmacokinetics of Flt3L and Flt3L-SA after single subcutaneous injection | a,** representation of treatment and the bleed times following. **b,** Total Flt3L measured in the plasma via Flt3L sandwich ELISA using Flt3L or Flt3L-SA ladders using molar equivalent amounts for the Flt3L-SA variant. **c,** Total Flt3L exposure per mouse as calculated as the area under the curve reported in part b. **d,** Normalized Flt3L exposure taken by dividing each value by the maximum detected in each group as used to determine the $t_{1/2rise}$ (1.3 and 4.1 hours post-injection) and $t_{1/2fall}$ (10.6 hours and 55.8 hours post-injection) for Flt3L and Flt3L-SA, respectively. Data in B and D represent average of 4 points with SD; each point represents one mouse in C with SD. Significance calculated via one-sided t-test.

Bioactivity

Once we knew the pharmacokinetics of the two Flt3L variants, we then tested the bioactivity of the 2 molecules *in vivo* as well as testing if administration route affected said activity. To do so, we administered 30μg or molar equivalent of either protein every other day for a total of 6 doses, beginning on day 0 and euthanizing on day 12. The proteins were administered either systemically via intraperitoneal (IP) injection or locally to one hock via SC injection, as shown in **Fig. 2.7**. Saline was injected in the opposite draining hock from the protein to compare local inflammation due to the injection. Upon euthanasia, 3 sets of organs were harvested: spleens to analyze the systemic response, injection draining auxiliary and brachial lymph nodes (LN), and the contralateral lymph nodes on the non-injection draining side of the animal. See representative gating for this experiment in **Fig. 2.8**.

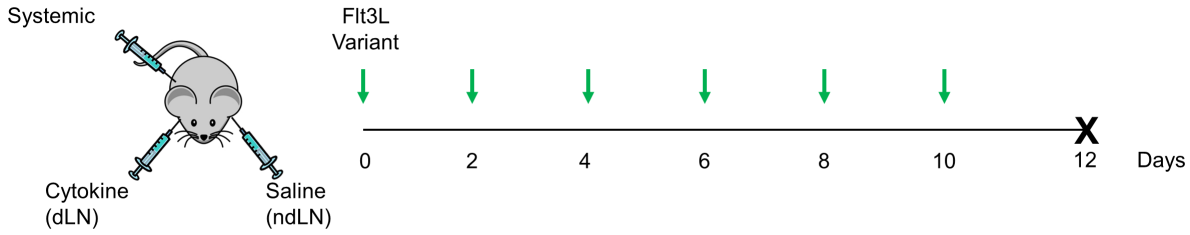

Figure 2.7: ***In Vivo* Bioactivity and Dosing Location Timeline |** Mice were treated either subcutaneously in the hock or intraperitoneal to test local and systemic responses to 30μg Flt3L given every other day for six doses. In mice treated locally, contralateral hocks were injected with saline on the same schedule to control for inflammation due to the injection itself. Mice were then euthanized two days after the final injection, and draining and non-draining lymph nodes as well as spleens were processed for flow cytometry.

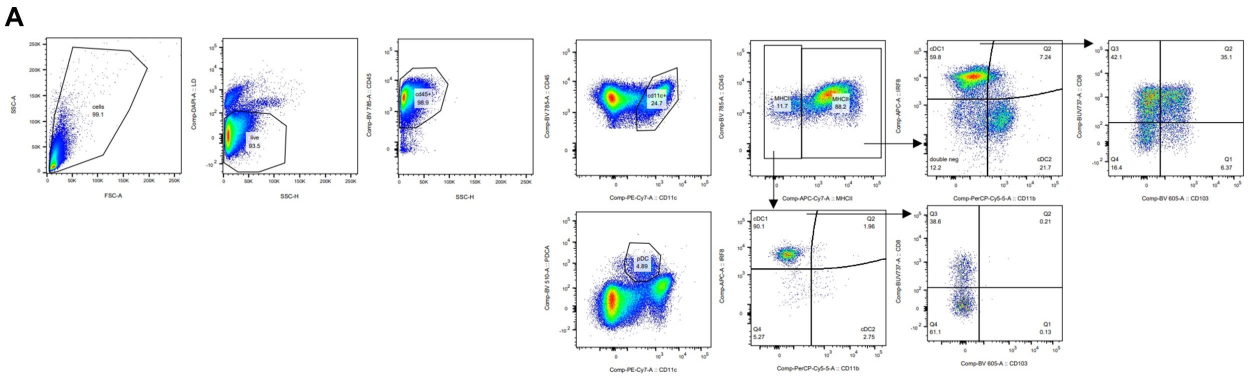

Figure 2.8: **Representative gating for Figure 2.9-Figure 2.10**

Focusing first on the overall response to the injections by looking at the splenic DC populations, we see significant increases in the overall DC composition in mice treated with either Flt3L variant, with no differences noted between the two injection routes. Furthermore, we also see a significant increase in the bulk DC population in mice treated with our Flt3L-SA construct as compared to the WT variant (**Fig. 2.9A**), likely due to the increased half-life of the construct. Diving deeper into the DC subsets in the spleen, we see similar significant effects as the bulk DC changes in the IRF8$^+$ cDC1 and a near significant trend in the CD11b$^+$ cDC2 populations when looking as a percent of the total CD45$^+$ population (**Fig. 2.9B,C**). When normalized as percent of the bulk DC populations, we see significant increases in cDC1 in all Flt3L treated groups, again with a stronger effect in mice treated with the SA variant (**Fig. 2.9D**); however, we note no differences in the percent of DCs which have a cDC2 phenotype across any treatment group (**Fig. 2.9E**). This suggests that Flt3L in general is able to generate increased numbers of all DCs, but has a preferential skewing towards generation of cDC1s, as previously described.(218–221)

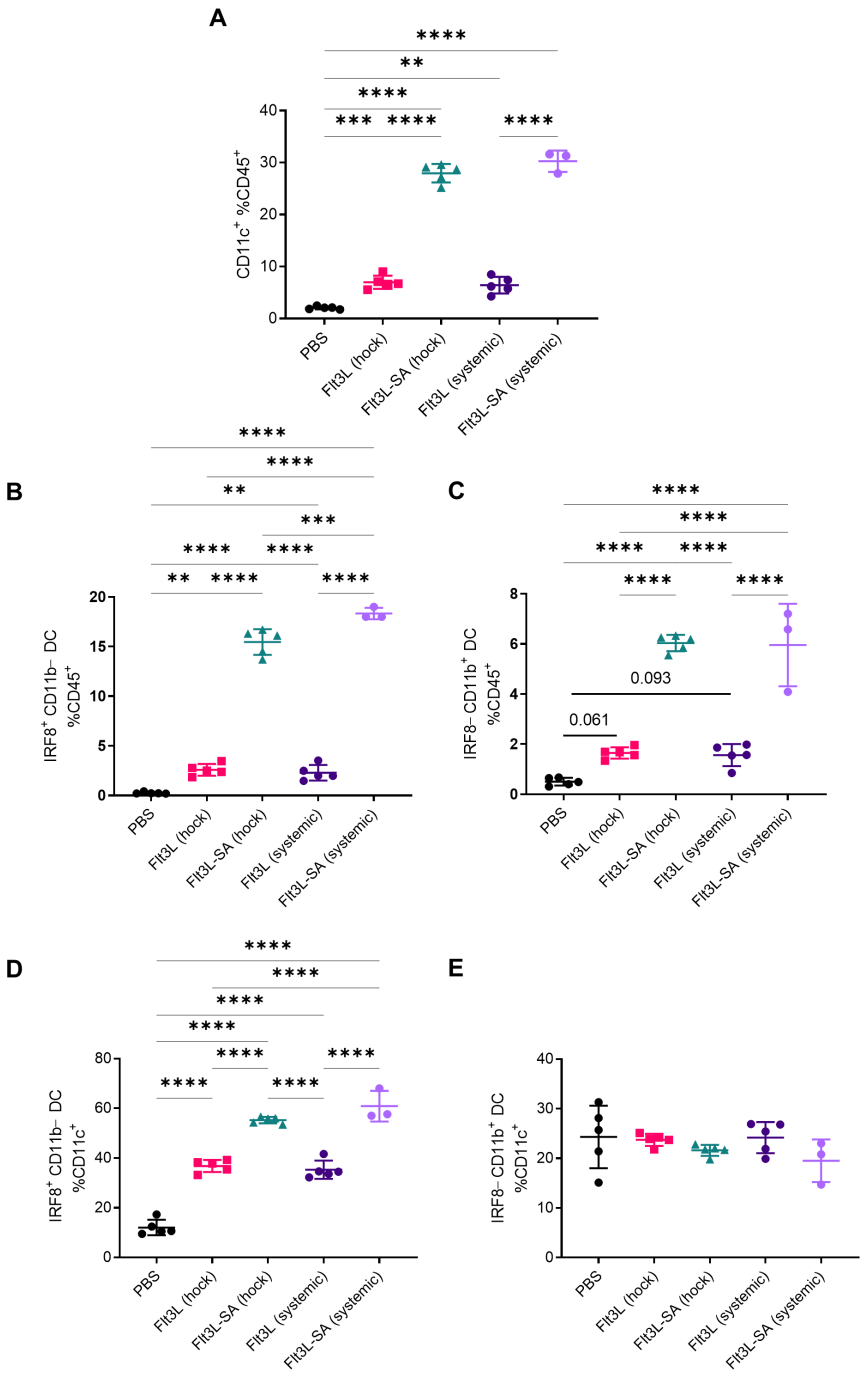

Figure 2.9: ***In Vivo* Bioactivity and Dosing Location, Splenic Dendritic Cell Characterization | a,** Total DC's as proportion of the hematopoietic ($CD45^+$) compartment for each treatment method. **b-c,** Analysis of cDC1 ($IRF8^+$) and cDC2 ($CD11b^+$) DCs as proportion of total hematopoietic compartment and **d-e,** proportion of total $CD11c^+$ cells. Each data point represents one mouse, with error bars for SD. Significance calculated via one-way ANOVA with Tukey's multiple comparison.

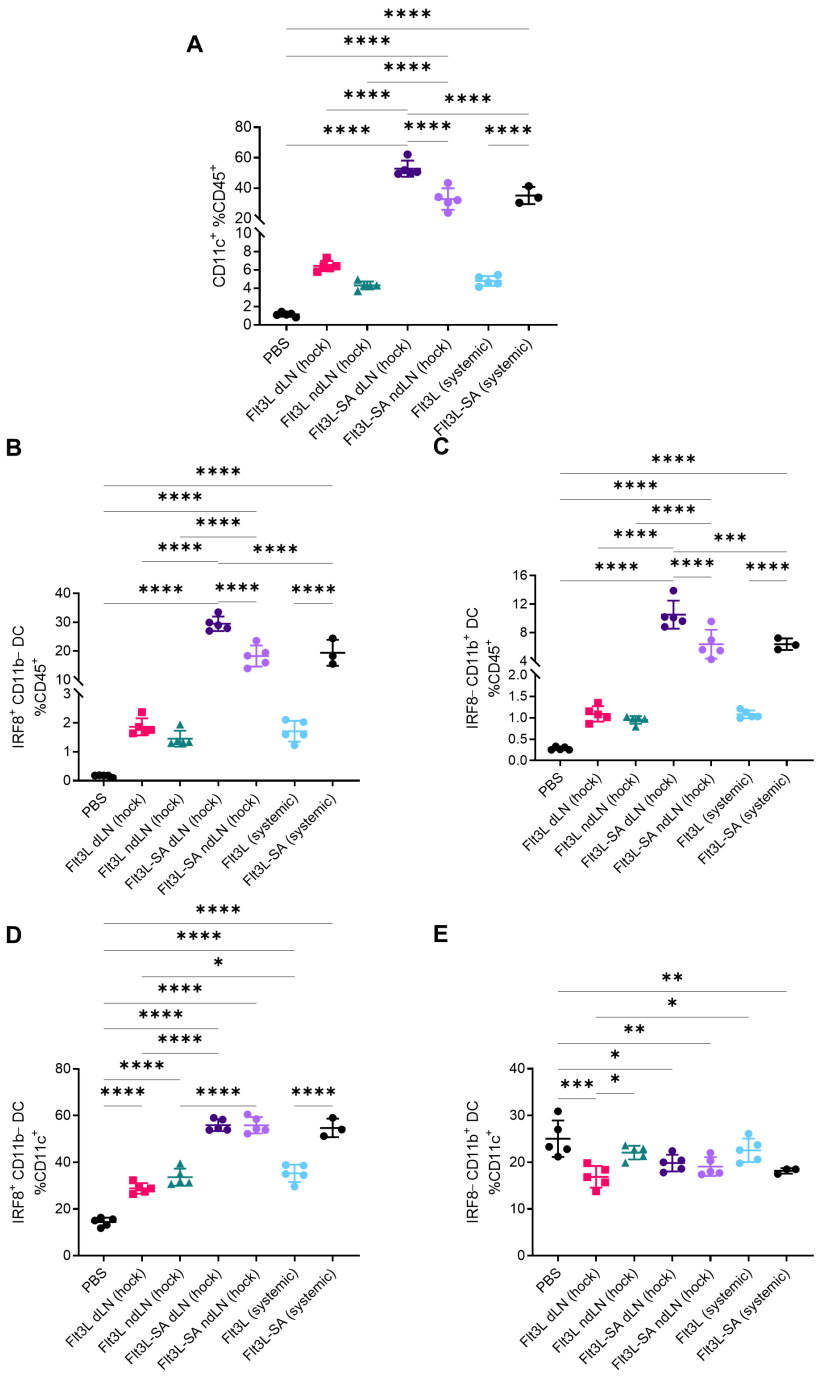

Figure 2.10: ***In Vivo* Bioactivity and Dosing Location Lymph Node Dendritic Cell Characterization | a** Total DC's as proportion of the hematopoietic ($CD45^+$) compartment for each treatment method. **b-c,** Analysis of cDC1 ($IRF8^+$) and cDC2 ($CD11b^+$) DCs as proportion of total hematopoietic compartment and **d-e,** proportion of total $CD11c^+$ cells. Each data point represents one mouse, with error bars for SD. Significance calculated via one-way ANOVA with Tukey's multiple comparison.

Looking next in the lymph nodes for a glimpse at both local and systemic responses, we again see general, significant increases in DCs in all mice treated. As was the same in the spleen, DC expansion was stronger in mice treated with the SA variant as compared to the WT. However, we do note a slight but significant boost in DCs in the LN draining the injection as compared to both the non-draining LN and the systemic injection, with no difference between the non-draining LN and the systemic injection groups (**Fig. 2.10A**). The cDC1 and cDC2 populations both followed the same increases as in the bulk DC population (**Fig. 2.10B-C**), but just as we saw in the spleen, the cDC1 showed preferential expansion after treatment regardless of injection location whereas there were minimal meaningful differences in the cDC2 population as a percent of the total DCs (**Fig. 2.10D-E**). From these data, we concluded that: injection route only had minor differences in efficacy overall, the dose given exceeded what was necessary, and the addition of the SA domain to Flt3L showed major benefit compared to the WT Flt3L.

Pharmacodynamics of Flt3L-SA treatment

Once we knew both the bioactivity and the pharmacokinetics of the engineered Flt3L-SA, we next sought to examine the pharmacodynamics. To do so, we treated C57BL/6 mice with a single, 10μg molar equivalent dose in a range from 20 to 4 days before euthanasia (**Fig. 2.11**) to analyze the immune components of the spleen, including DCs and T cells, representative gating seen in **Fig. 2.12**.

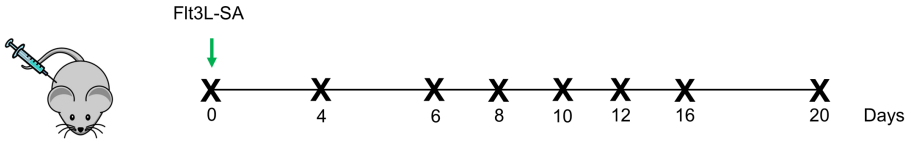

Figure 2.11: **Cellular Pharmacodynamics Timeline |** Mice were treated subcutaneously on day 0 with 10μg molar equivalent Flt3L-SA and euthanized at the days indicated with an X. Splenic contents were then isolated and analyzed by flow cytometry. Representative of timeline for figures 2.13-2.17.

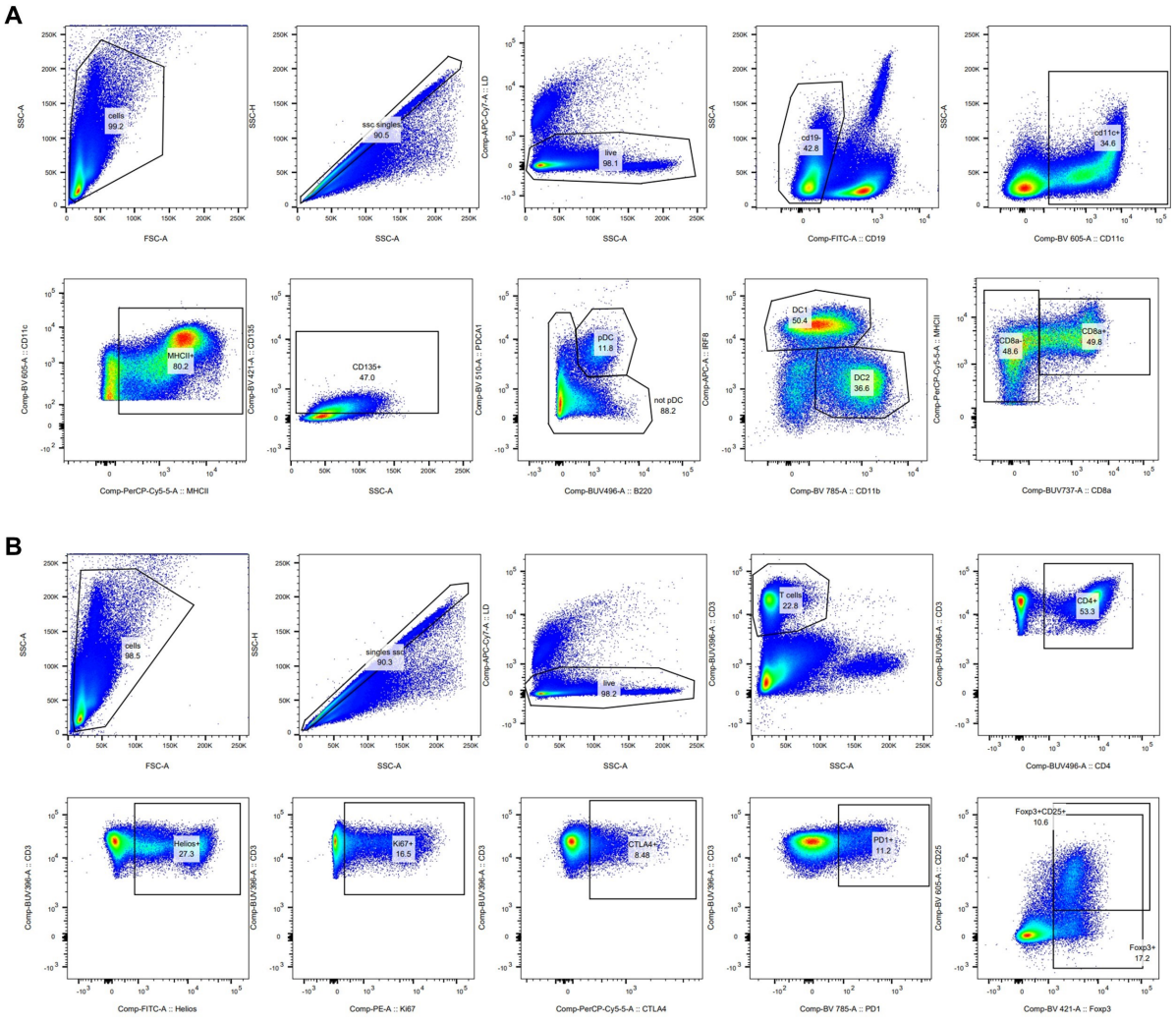

Figure 2.12: **Representative gating for Figure 2.13-Figure 2.17 | a,** Gating for Myeloid populations and **b,** gating for Treg populations

We first analyzed how expression of CD135, the receptor for Flt3L changed on DCs overtime following treatment (**Fig. 2.13A**). Interestingly, we see that receptor expression is down-regulated 4 days after treatment, in coordination with the injected treatment still being present, as seen in **Fig. 2.6B**. However, once systemic drug is substantially reduced, 6-8 days after treatment, expression is greatly upregulated such that over 40% of DC stain positive for CD135, and this value doesn't reduce back to physiologic levels until about 16 days after treatment. These effects are likely due to receptor internalization following signaling, acting as a negative feedback loop. Once the drug is cleared, however, signaling slows and thus surface receptor expression increases. Next, analyzing the changes in the DC compartment, we see peak increases in DCs on day 6 following treatment, seen as both total counts per spleen as well as percentage of total live cells, with roughly 20 million DCs per spleen making up 12% of total splenocytes, and a return to physiologic levels around day 12-16 post treatment (**Fig. 2.13B**). This phenomenon is especially obvious when the data are visualized normalized to saline controls, where there is an average 8-fold increase in total DCs on day 6 post treatment (**Fig. 2.13C**).

We next characterized the individual subtypes of $CD11c^{+}MHCII^{+}$ DC: plasmacytoid DC (pDC) defined by expression of B220 (pink), type 1 classical DC (cDC1) defined by expression of the transcription factor IRF8 (teal), and type 2 cDC (cDC2) defined by expression of the integrin CD11b (purple).(194; 195; 222–224) Similar to what we see with total DCs, we see increases in both cDC populations with peaks in total numbers and overall splenic percent reached on day 6 following treatment; however, we note that pDCs seem to actually peak 10 days following treatment (**Fig. 2.13D-E**). These effects are even more pronounced when normalized to saline treatment: cDC1 show an average 50-fold increase 6 days following treatment, pDC show a 40 fold increase in splenic composition 10 days following treatment, and cDC2 peak at an 8 fold increase 6 days following treatment (**Fig. 2.13F**).

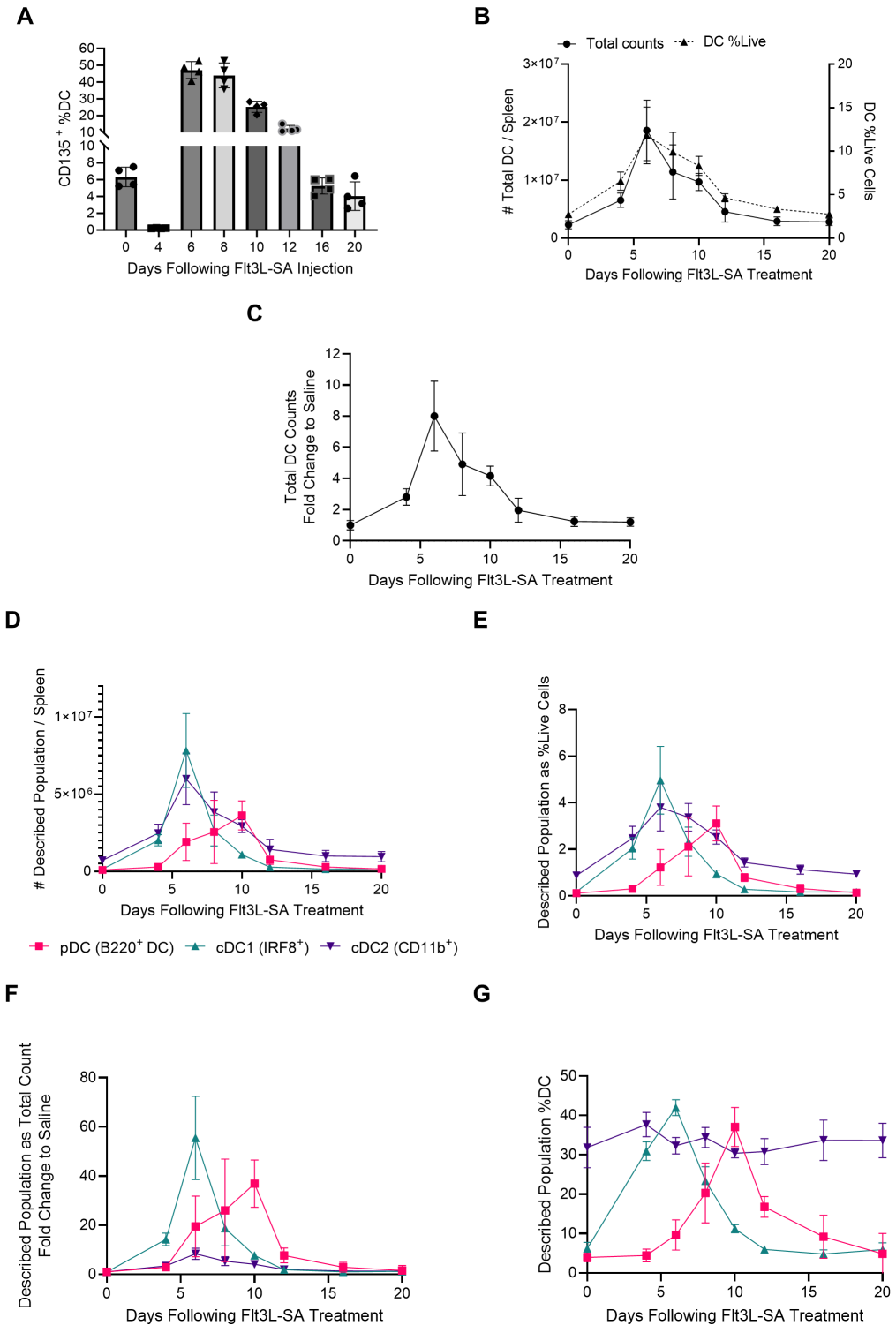

Figure 2.13: **Dendritic Cell Splenic Pharmacodynamics following Flt3L-SA Treatment | a,** CD135 expression on $CD11c^{+}MHCII^{+}$ cells post single Flt3L-SA injection. (Continued on following page)

Figure 2.13: Continued **b,** Total DC counts per mouse spleen (left axis) as measured as percent of live cells (right axis) multiplied by the number of live cells isolated. **c,** DC counts per spleen normalized to day 0 counts to demonstrate fold change from untreated.**d-g** Subdivisions of DCs over time as marked by B220 (pDC), IRF8(cDC1), and CD11b (cDC2) as total number per spleen (**d**), percent of live cells (**e**), fold change in numbers compared to untreated (**f**), and as a percent of total DCs (**g**). Each point in **a** represents one mouse with SD, **b-g** represent n=4 with error bars for SD.

Interestingly, even though all DC compartments seem to increase in response to treatment, when values are normalized to see how much of the DC compartment each subset comprises, we note that the relative numbers of cDC2 remain unchanged throughout the experiment, whereas cDC1 show a large increase by day 6 and a return to baseline around day 12, whereas pDC percentage peaks on day 10 post treatment (**Fig. 2.13G**). This phenomenon suggests a stochastic differentiation of cDC2, where generation of progenitors maintain a stable percentage of this compartment, whereas cDC1 are preferentially differentiated by FLT3L signaling through the CD135 receptor. Indeed, Flt3L has been shown sufficient for all DC development, but necessary for development of cDC1.(218; 225–227) Additionally, the delay in pDC development may be due to loss of CD135 early into the Common Lymphoid Progenitor (CLP) differentiation cascade, whereas the Common Myeloid Progenitor (CMP) pathway maintains CD135 expression throughout the cDC differentiation process.(228–230)

We also sought to determine if DC phenotype had changed at peak expansion. To do so, we compared expression of costimulatory receptors (CD40, CD80, and CD86) as well as coinhibitory receptors (PD-L1 and CD200) via flow cytometry on splenic DCs 6 days following treatment and compared to untreated mice and mice injected with Monophosphoryl-Lipid A (MPLA), a known TLR4 agonist.(231) Interestingly, we see a significant decrease in the expression level of CD40 in mice treated with Flt3L-SA compared to untreated, but no difference for mice treated with MPLA (**Fig. 2.14A**). We also see a significant rise in DCs staining positive for CD80 as well as the gMFI for CD80 in mice treated with Flt3L-SA; however, we also note significant increases in CD86 for both Flt3L-SA and MPLA treated groups,

suggesting that the MPLA was in fact working as intended (**Fig. 2.14B-C**). Looking next at inhibitory markers, we see a major increase in PD-L1 expression and percent positive in only the MPLA treated group (**Fig. 2.14D)**, which could potentially be a marker of early or recent activation rather than simply inhibition.(232–234) We also note a significant decrease in CD200 expression in Flt3L-SA treated mice (**Fig. 2.14E**). Overall, these data suggest a potential for a slightly activated phenotype on the DCs after Flt3L-SA treatment. In the same experiment, we also sought to determine if other major populations were changing in proportion in response to Flt3L-SA treatment. To this end, we see no significant changes in T cell, B cell, nor monocyte components of the spleen following therapeutic intervention, when normalized to the non-DC compartment of the spleen (**Fig. 2.15A-C**). We take this data to show that there are not unintended, off-target effects on other bulk compartments of the immune system.

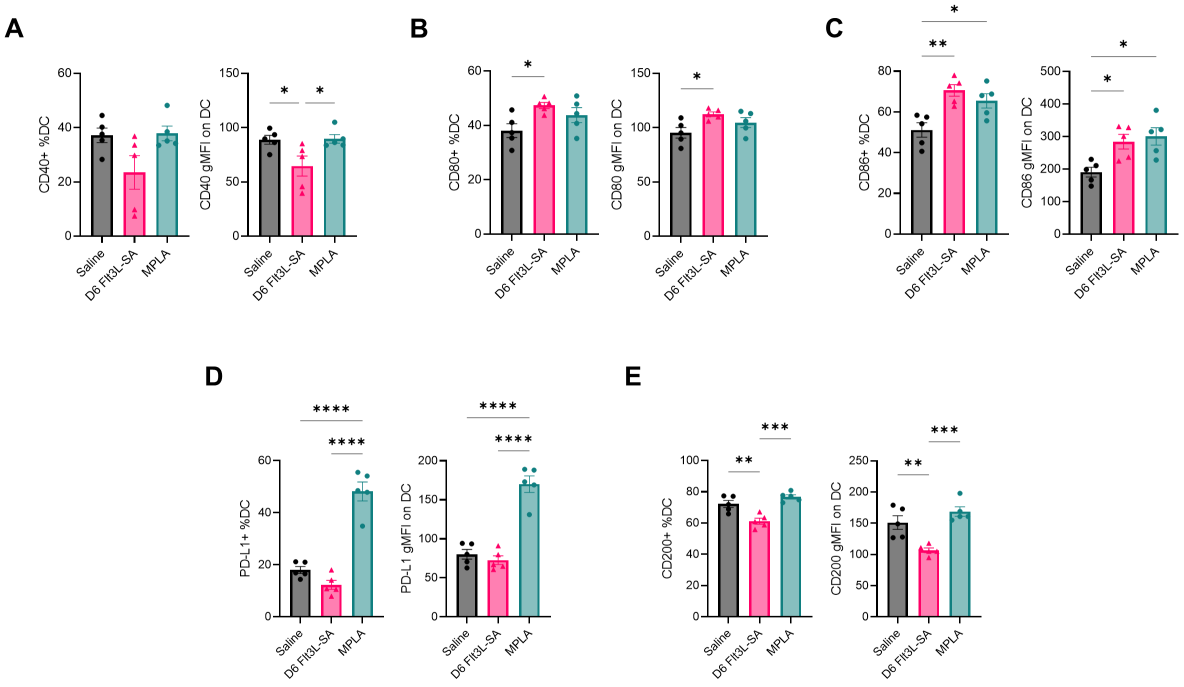

Figure 2.14: **Dendritic Cell Characterization at Peak Expansion from Flt3L-SA Treatment | a-c,** Phenotypic characterization of markers of immune activation, including CD40 (**a**), CD80 (**b**), and CD86 (**c**), on bulk splenic DCs 6 days after Flt3L-SA treatment in comparison to DCs from mice treated subcutaneously with MPLA 24 hours before euthanasia. **d-e,** Phenotypic expression of immunosuppressive markers on splenic DCs including PD-L1 (**d**) and CD200 (**e**). Each data point represents one spleen with error bars on percent positive graphs (left) representing SD, and error bars on gMFI graphs representing SEM. Significance calculated via one-way ANOVA with Tukey's multiple comparison.

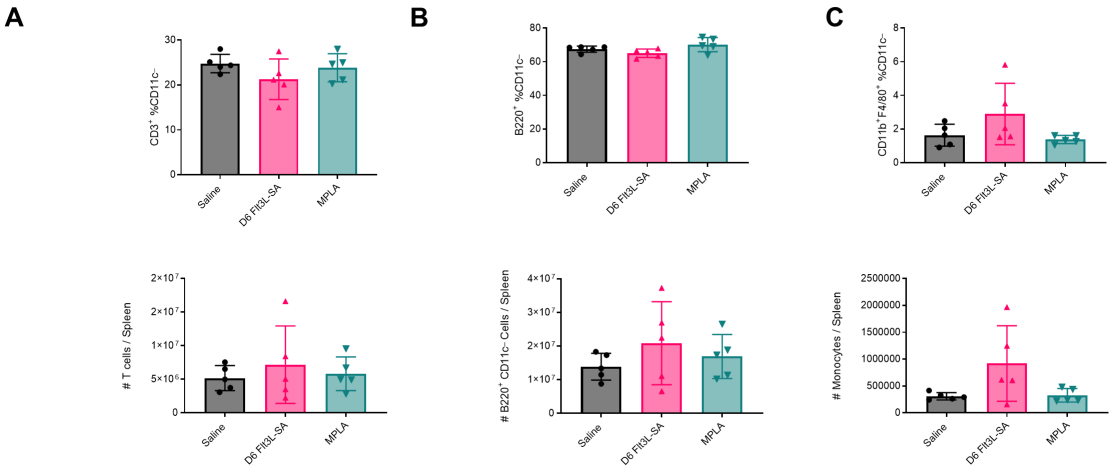

Figure 2.15: **Non-Dendritic Cell Populations Do Not Show Changes due to Flt3L-SA Administration | a,** T cells ($CD3^+$) **b**, B cells ($B220^+$) **c**, and Monocytes ($CD11b^+F4/80^+$) do not significantly change at peak DC expansion when normalized to CD11c- populations (top), or in total counts (bottom, calculated as population as a percent of live cells multiplied by total live counts per spleen). Each point represents one mouse with error bars corresponding to SD.

With the DC compartment characterized after a single injection, it is also reported that DC expansion following Flt3L treatment corresponds with expansion of Tregs.(204; 207; 235; 236) With this in mind, we sought to characterize how the T cell compartment also changes following Flt3L-SA treatment, with a focus on Tregs, in the same experiment as described in **Fig. 2.11**. First and foremost, we see a peak expansion of $CD4^+Foxp3^+CD25^+$ Tregs 8 days following treatment with a 20% increase over saline treated mice. Furthermore, it is recently becoming more appreciated that $CD4^+Foxp3^+CD25^-$ cells act as Treg precursors in the periphery.(46–49; 237–239) The transcription factor Helios then stabilizes Treg functions and prevents off target, pro-inflammatory programs from initializing.(50; 51) In this regard, we notice both $Helios^+$ and $Foxp3^+$ cells expand at similar timescales, with a peak increase of about 30% 10-12 days post Flt3L-SA intervention and a return to saline levels by day 20 (**Fig. 2.16A**). This timescale also corresponds to general $CD4^+$ T cell expansion where we note a significant increase in $Ki67^+$ $CD4^+$ T cells 10 days following treatment (**Fig. 2.16B**).

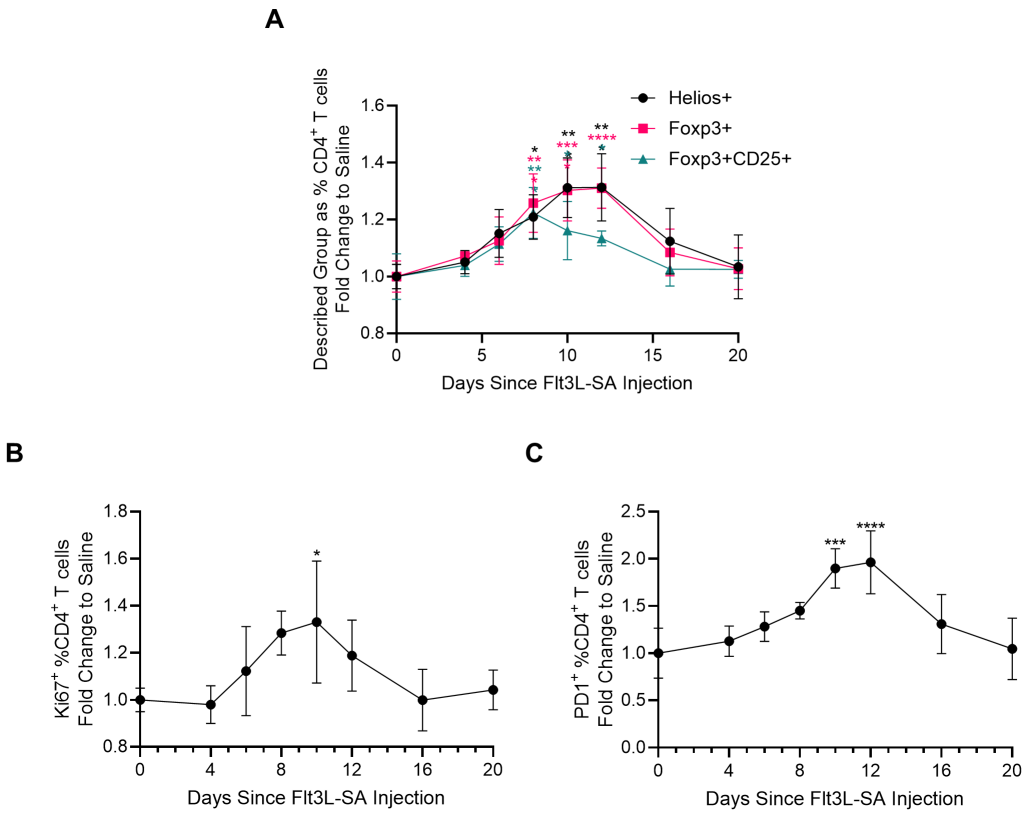

Figure 2.16: **Flt3L-SA Changes the CD4$^+$ T Cell Compartment Towards Tolerance and Dysfunction | a,** Splenic fold change compared to saline (day 0) in the proportion of the CD4$^+$ T cell compartment comprised of Helios$^+$ cells, total Foxp3$^+$ cells, and CD25$^+$Foxp3$^+$ cells (Tregs) after a single s.c. injection of Flt3L-SA at day 0. **b,** Splenic fold change in total CD4 T cells expressing Ki67 or **c,** PD-1 compared to saline treated mice. Each point represents n=4 mice with error bars representing SD. Significance denotes a one-sided t-test comparison between that time-point and day 0 with color denoting the population of interest.

Finally, we note an increase in expression of the co-inhibitory receptor PD-1 on CD4$^+$ T cells following treatment with Flt3L-SA. This data shows nearly a doubling in the percent of the CD4 compartment expressing this receptor by days 10-12 after treatment and again a return to saline levels by day 20 (**Fig. 2.16C**). PD-1 shows an interesting dichotomy as both a marker of exhaustion but also as a marker of recent activation and antigen recognition. At this time, we are unable to differentiate how much of an effect PD-1 expression has on suppressing the function of these cells, but we believe that this may be more of a marker that the T cells we see expanding are doing so due to an increase in availability of antigen:MHCII

complex and recent activation rather than as a marker of terminal exhaustion.(235; 240–249)

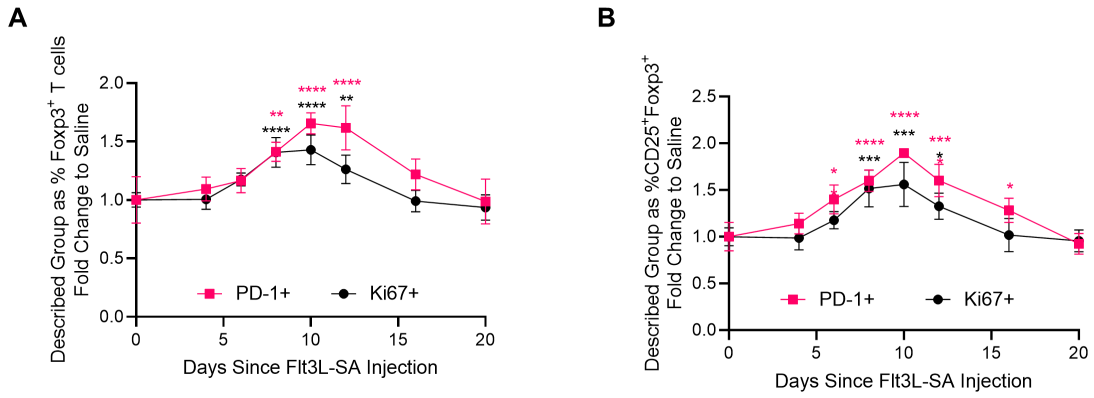

Figure 2.17: **Flt3L-SA Alone Expands Suppressive T Cells | a,** Relative amount of Ki67 or PD-1 expression in Foxp3$^+$ and **b,** Tregs (defined as Foxp3$^+$CD25$^+$) denoted as fold change compared to day 0. Each point represents 4 mice with error bars of SD. Significance denotes one-sided t-test between that timepoint and day 0 with color corresponding to the appropriate graph.

We finally dive into characterization of the Foxp3$^+$ and Foxp3$^+$CD25$^+$ populations. In agreement with what we note for the total CD4$^+$ T cells, we see that Foxp3$^+$ cells peak expression of Ki67 and PD-1 within days 8-12 post treatment, with PD-1 expression continuing to increase slightly later than Ki67 (**Fig. 2.17A**), supporting our claims of PD-1 as a marker of recent activation rather than a marker of terminal exhaustion. In the Foxp3$^+$CD25$^+$ population, Ki67 expression follows a similar trajectory as the Foxp3$^+$ population; however, the population staining positive for PD-1 seems to increase to a higher extent in the population also staining CD25$^+$ (**Fig. 2.17B**), potentially signifying a lower barrier of activation for cells expressing CD25.

2.3.3 Establishment of a Therapeutic Regime of Flt3L-SA Induced Tolerance

We next sought to develop and characterize the response to a therapeutic regime involving Flt3L or Flt3L-SA. Knowing the pharmacokinetics of Flt3L-SA (**Fig. 2.6**), we chose repeated, subcutaneous injections 5 days apart as demonstrated in **Fig. 2.18**. We chose to

characterize the DC and Treg compartments of the spleen as a measure of systemic changes as well as characterizing the effects in the individual mesenteric draining lymph nodes, to account for localized responses within each section of the gut. We chose to analyze the gut draining lymph nodes as previous observations in other experiments noted significant growth in these secondary lymphoid organs in response to treatment, and we wondered if changes in these organs could inform potential other avenues of therapeutic entry (representative gating for these experiments in **Fig. 2.19**).

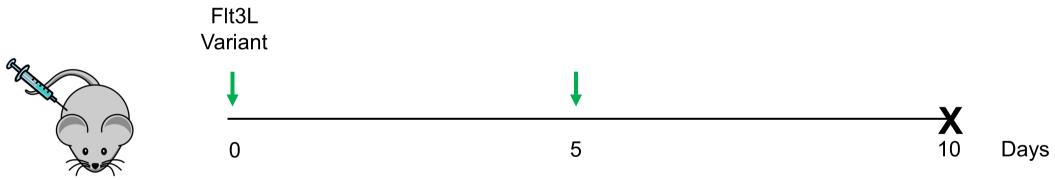

Figure 2.18: **Flt3L Therapeutic Regime Timeline** | Mice were treated two times s.c. 5 days apart with 10μg EQ of either Flt3L or Flt3L-SA and then euthanized on day 10. Treatment regime represents experimental timeline for figures 2.20-2.27.

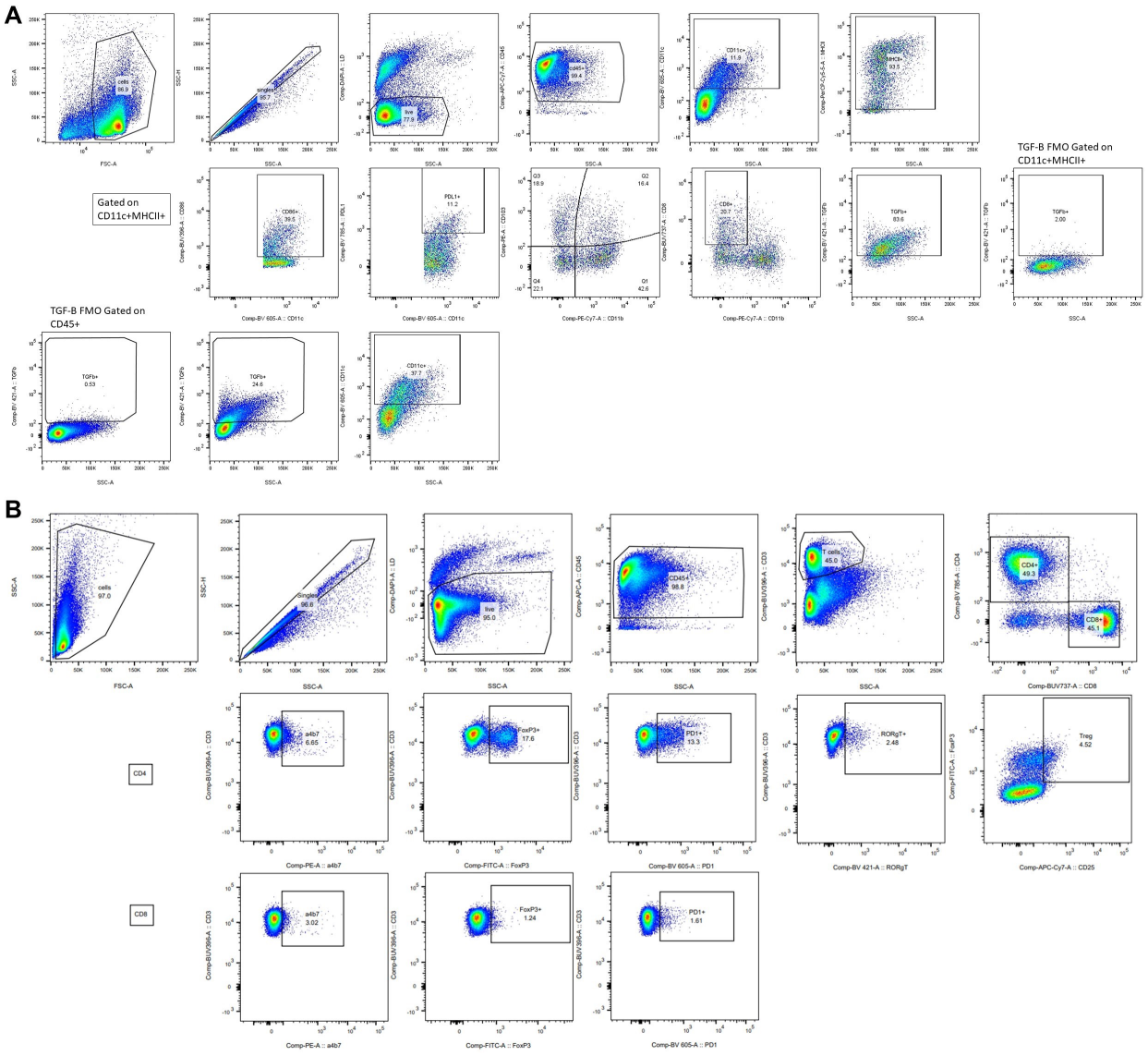

Figure 2.19: **Representative gating for Figure 2.20-Figure 2.27 | a,** Gating for Myeloid populations and **b,** gating for Treg populations

DC Phenotype and Composition Following Flt3L-SA Regime

We first characterized how the splenic DC compartment changed after treatment. With this treatment regimen, we see no differences between the WT Flt3L treatment and saline (roughly 1% of the splenic content); however, we note an increase to nearly 5% of the spleen comprised of DCs corresponding to an estimated 4 million DCs in the spleen (**Fig. 2.20A**).

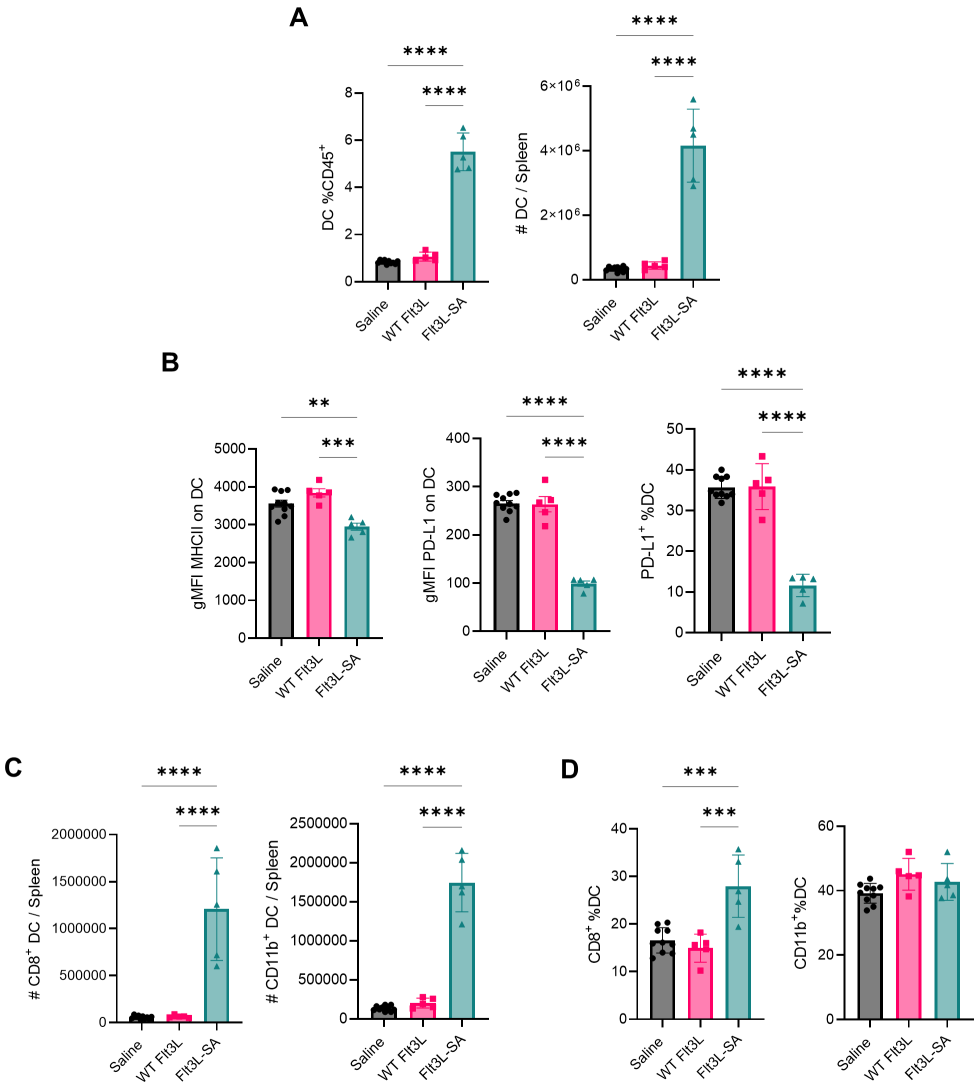

Figure 2.20: **Flt3L-SA but not Flt3L Significantly Increases Splenic DCs with a Preference for cDC1s | a,** DC ($CD11c^{+}MHCII^{+}$) expansion after treatment as proportion of $CD45^{+}$ cells and total estimated cells per spleen. **b,** Phenotypic characterization of DCs in the spleen denoted by MHCII and PD-L1 expression and percent of total DCs staining positive for PD-L1. **c,** Total numbers of cDC1 ($CD8^{+}$) and cDC2 ($CD11b^{+}$) per spleen as well as **d,** percent of the total DC parent population. Each data point represents one mouse with error bars on percent positive graphs representing SD, and error bars on gMFI graphs representing SEM. Significance calculated via one-way ANOVA with Tukey's multiple comparison.

Looking phenotypically at these generated DCs, we note a significant reduction in expression of MHCII and PD-L1 as shown by gMFI as well as a reduction in the percentage of DCs

staining positive for PD-L1 in mice treated with Flt3L-SA (**Fig. 2.20B**). Finally, we see a significant increase in the estimated counts of both $CD8^+$ cDC1s as well as $CD11b^+$ cDC2s (**Fig. 2.20C**); however, as seen in the pharmacodynamic study, when the cells are normalized to percentage of total DCs, we see an increase in the percent of DCs with a cDC1 phenotype and no difference in the percent of DC displaying a cDC2 phenotype (**Fig. 2.20D**). We take these data to suggest that treatment with Flt3L-SA causes an increase in splenic DC content, as seen previously, with a decrease in activation phenotype as demonstrated by PD-L1 and MHCII expression; however, due to the reduced number of treatments compared to most regimes involving Flt3L, we don't see response to the WT Flt3L variant.

In this study, we also examined the TGF-β expression of the DC compartment. TGF-β is a highly pleiotropic and immunosuppressive cytokine involved in many processes including the generation and maintenance of Tregs,(57; 86; 88; 237; 239; 250; 251) and as such we believe that it might be a key signal linking the DC proliferation to Treg expansion. To study this, we stained the cells for the inactive form of TGF-β, naturally expressed as a fusion to the Latency Associated Peptide (LAP). After treatment with Flt3L-SA, we see a significant increase in splenocytes expressing LAP, nearly doubling the percent found in saline treated mice (**Fig. 2.21A**). Additionally, we note that of these cells expressing LAP, we see a significant increase in how many of those are DCs, making up over 40% of LAP expressing cells (**Fig. 2.21B**); however, when looking at what percentage of DCs express LAP, we see no differences between groups (**Fig. 2.21C**). We take these data to show that therapeutic intervention with Flt3L-SA decreases the activation of DCs as well as increasing the amount of TGF-β within the spleen.

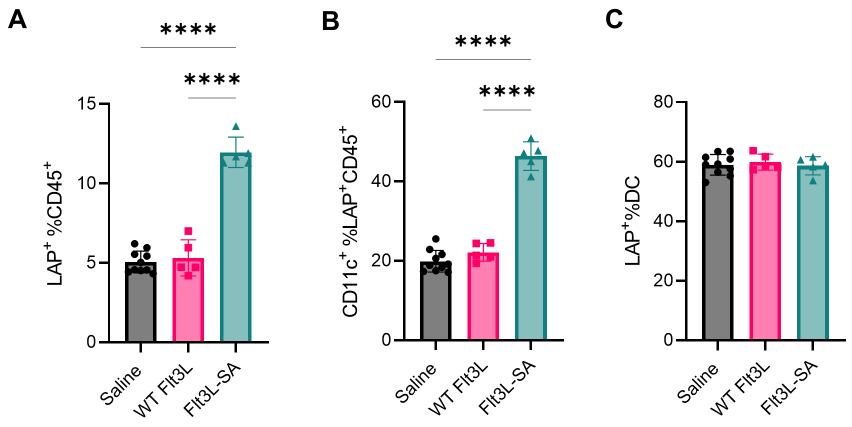

Figure 2.21: **Flt3L-SA Increases Splenic LAP Expression | a,** TGF-β expressing cells, as measured via flow cytometric staining for the LAP precursor, out of total CD45$^+$ cells. **b,** Proportion of LAP expressing cells with a DC phenotype, and **c,** proportion of DCs expressing LAP. Each data point represents one mouse with error bars representing SD. Significance calculated via one-way ANOVA with Tukey's multiple comparison.

We next characterized the effects of Flt3-LA treatment on cells in the lymph nodes draining the intestines, known as the mesenteric dLN. Whereas in the spleen we note about a 5x increase in DC cellularity, in each of the mesenteric dLN we note an increase from nearly 1% DC content to 20% of the lymph node being comprised of DCs when treated with Flt3L-SA (**Fig. 2.22A**). Whereas in the spleen we noted that the DCs primarily take on a cDC1 phenotype, in each of the mesenteric dLN we note that the primary subtype seems to consist of CD11b$^+$, cDC2 rather than any of the other types examined (CD8$^+$, CD11b$^+$CD103$^+$, or CD103$^+$) (**Fig. 2.22B-E**). Furthermore, as it has been noted that the intestines go from a pro-tolerogenic phenotype to a more pro-immunogenic phenotype moving from the duodenum to the colon,(252) we also sought to characterize the inflammatory potential of the lymph nodes draining each of the sections of intestine following treatment. We saw only minimal changes in overall MHCII expression on DCs with regards to which section drained and treatment (**Fig. 2.23A**); however, we do note in the saline treated group that transversing from proximal to distal dLN we see an increase in both PD-L1 and CD86 expression, indicative of increasing inflammatory potential based on the expression of the

aforementioned markers of inflammation.

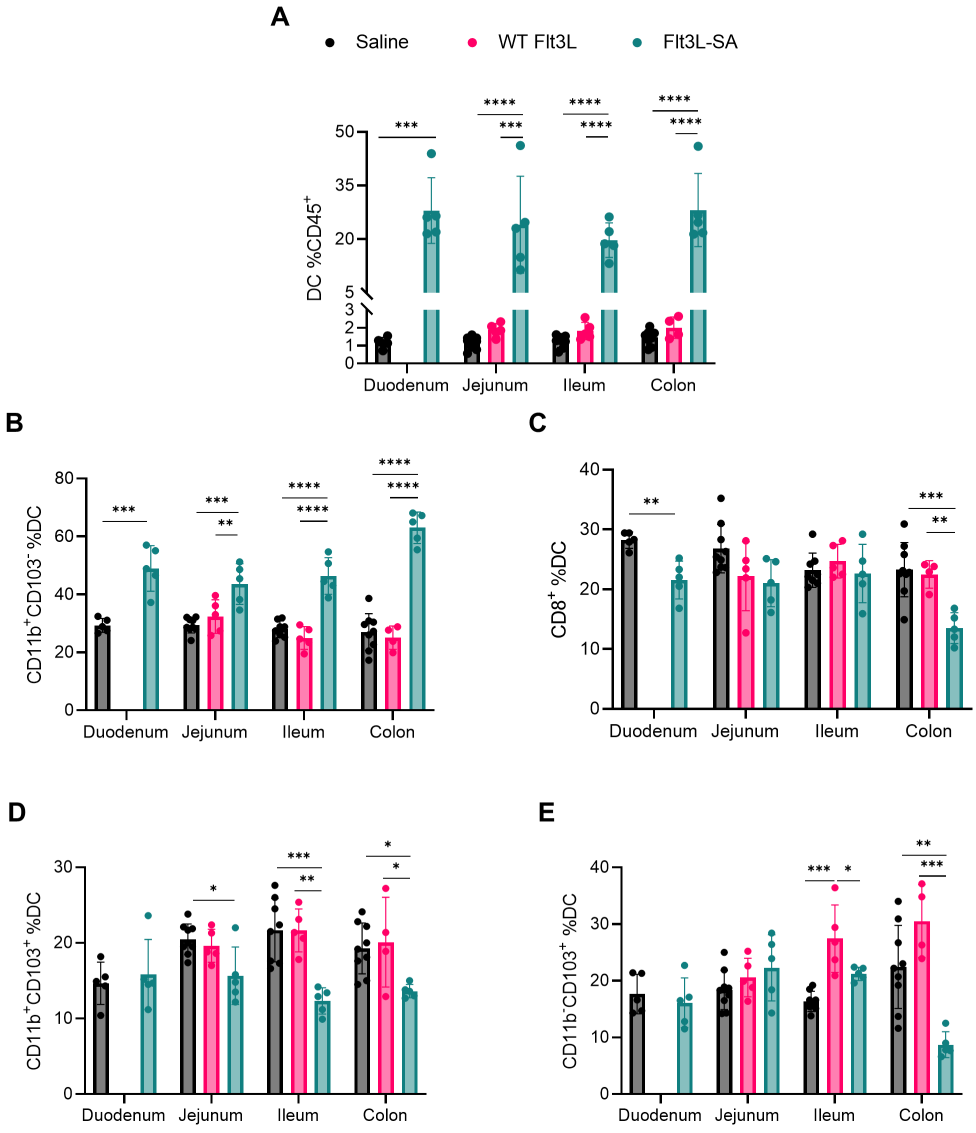

Figure 2.22: **Flt3L-SA Demonstrates Massive DC Expansion in Gut dLN, Primarily a cDC2 Phenotype | a,** Expansion of CD11c$^+$MHCII$^+$ cells in lymph nodes draining the labeled section of the gut. **b-e,** phenotype of expanded DCs in each region dLN as labeled as (**b**) cDC2 , (**c**) lymphoid resident cDC1, (**d**) gut dLN cDC2, or (**e**) migratory cDC1. Each data point represents one mouse with error bars representing SD. Significance calculated via one-way ANOVA with Tukey's multiple comparison within each section dLN.

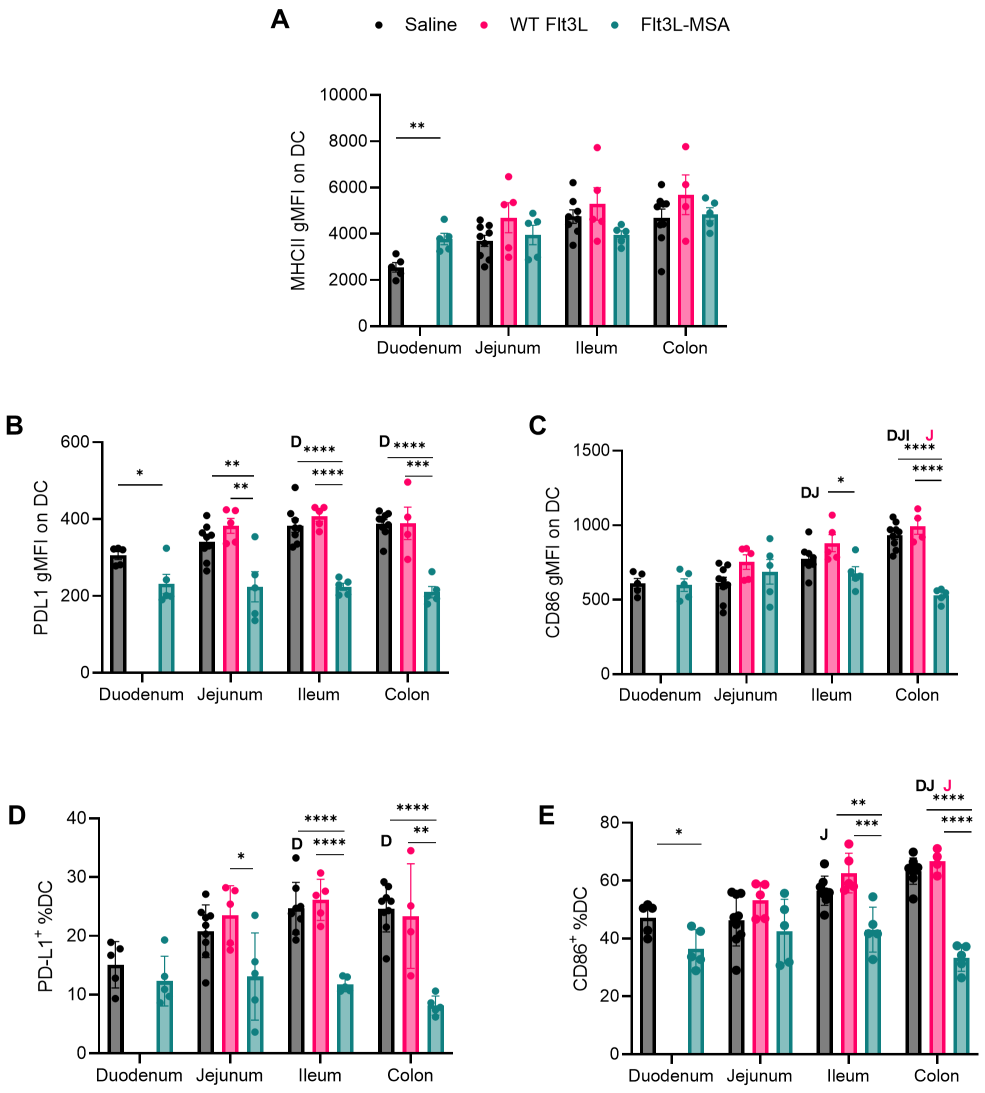

Figure 2.23: **Flt3L-SA Downregulates CD86 and PD-L1 Seen in Distal Gut vs Proximal Gut | a,** MHCII expression on CD11c^{+}MHCII^{+} cells as measured by gMFI as organized by section of gut drained by the lymph node. **b,** Co-inhibitory expression on DCs as transversing the gut draining lymph nodes as measured by gMFI. Letters indicate significant differences comparing that group to the section beginning with that letter (Duodenum vs. Ileum dLN $p<0.05$, Duodenum vs. Colon $p<.01$). **c,** Costimulatory receptor CD86 expression on DCs as measured by gMFI of that color on DCs. Significance within one treatment comparing different draining sections denoted by color of the letter corresponding to the dLN. For saline treated mice: Ileum v.s Duodenum $p<0.05$, Ileum vs. Jejunum $p<0.01$, Colon vs. Duodenum $p<0.0001$, Colon vs. Jejunum $p<0.0001$, Colon vs. Ileum $p<0.05$. For WT Flt3L treated mice, Colon vs. Jejunum $p<0.05$. **d,** Co-inhibitory expression as denoted as percent of DCs staining above the FMO for PD-L1. Letters represent significant (Continued on the following page.)

Figure 2.23: (Continued) differences between that dLN and the dLN beginning with the letter. Duodenum vs. Ileum or vs. Colon dLN are both $p<0.01$. **e,** Costimulatory receptor expression as denoted as percent of DCs staining above the CD86 FMO. Signficant differences in inflammation between dLN within one treatment group denoted by the first letter of the dLN with color representing treatment group. For saline treated mice: Ileum vs. Jejunum dLN $p<0.05$, Colon vs. Duodenum $p<0.001$, Colon vs. Jejunum $p<0.0001$. For WT Flt3L treated mice, Colon vs. Jejunum $p<0.05$. Each data point represents one mouse with error bars on gMFI graphs (**a-c**) representing SEM and error on **d,e** representing SD. Significance calculated via one-way ANOVA with Tukey's multiple comparison comparing treatment groups within each gut dLN section or calculated within one treatment group against all sections of gut dLN (ie: comparing saline vs. WT Flt3L vs. Flt3L-SA all within the colon dLN or comparing the Duodenum, Jejunum, Ileum, and Colon dLN within the saline treated mice).

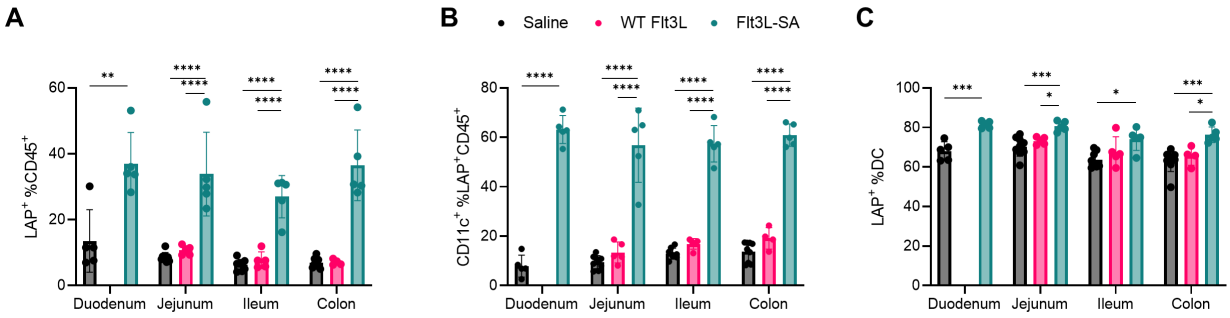

Figure 2.24: **Characterization of TGF-β Expression by DCs in Gut dLN | a** TGF-β expressing cells, as measured via flow cytometric staining for the LAP precursor, out of total $CD45^+$ cells. **b,** Proportion of LAP expressing cells with a DC phenotype, and **c,** proportion of DCs expressing LAP. Each data point represents one mouse with error bars representing SD. Significance calculated via one-way ANOVA with Tukey's multiple comparison.

In mice treated with Flt3L-SA this effect is lost and we note a significant decrease in these inflammation markers in all distal gut dLN compared to the saline treated controls (**Fig. 2.23B-E**). Finally, as we saw in the spleen, we see significant increases in TGF-β expression in all of the gut dLN, of which the TGF-β expressing cells were primarily comprised of DCs, with only minor changes in what percent of the DCs stained positive for TGF-β (**Fig. 2.24A-C**). These data show major changes in the DC composition of the gut dLN and potentially signify a shift to a more tolerogenic or anti-inflammatory phenotype in the more distal gut.

Regulatory T cell Expansion and and T cell Phenotype Following Flt3L-SA Regime

As we have already established that Flt3L-SA treatment expands Tregs, we next looked to determine how the Treg and bulk T cell population changed following a therapeutic regime, starting first with splenic characterization. In general, we note no differences in splenocytes when comparing mice treated with WT Flt3L as compared to splenocytes from saline treated mice, likely due to the suboptimal dosing regimen when compared to other studies using Flt3L. We do note a decrease of T cells as a proportion of the total splenocytes with Flt3L-SA, which is likely due to the DC expansion; however, when looking at total T cell counts in the spleen, we actually note a significant increase as compared to saline treated mice (**Fig. 2.25A**). Similar trends are seen in the $CD8^+$ T cell compartment, where as a portion of total T cells, $CD8^+$ are significantly reduced in Flt3L-SA treated mice compared to saline, but no significant differences are seen when looking at total $CD8^+$ counts in the spleen (**Fig. 2.25B**). The decrease in $CD8^+$ T cells out of total T cells is likely due to the expansion of $CD25^+Foxp3^+$ Tregs, where in all factors measured, fraction of total T cells, fraction of $CD4^+$ T cells, and absolute counts per spleen, we see significant increases only in mice treated with Flt3L-SA (**Fig. 2.25C**). We also previously noted an increase in expression of PD-1 after a single treatment and next characterized expression in a therapeutic regime. In all T cell subsets (total T cells, $CD8^+$ T cells, $CD4^+$ T cells, and $CD25^+Foxp3^+$ Tregs), we see a significant increase in the percent of those expressing PD-1 in Flt3L-SA treated mice; in addition, we also note higher levels of PD-1 expression, as measured by gMFI, as we dive deeper into the population with the highest expression on $CD25^+Foxp3^+$ Tregs, and no significant differences in expression levels on $CD8^+$ T cells (**Fig. 2.25D-G**). We again take the increased expression levels and percentages staining positive for PD-1 here to signify a recent antigen exposure thus leading to recent activation and expansion, rather than to signify signs of terminal exhaustion, in accordance with literature showing that PD-1

upregulation is correlated with strength of TCR signaling.(235; 253–257)

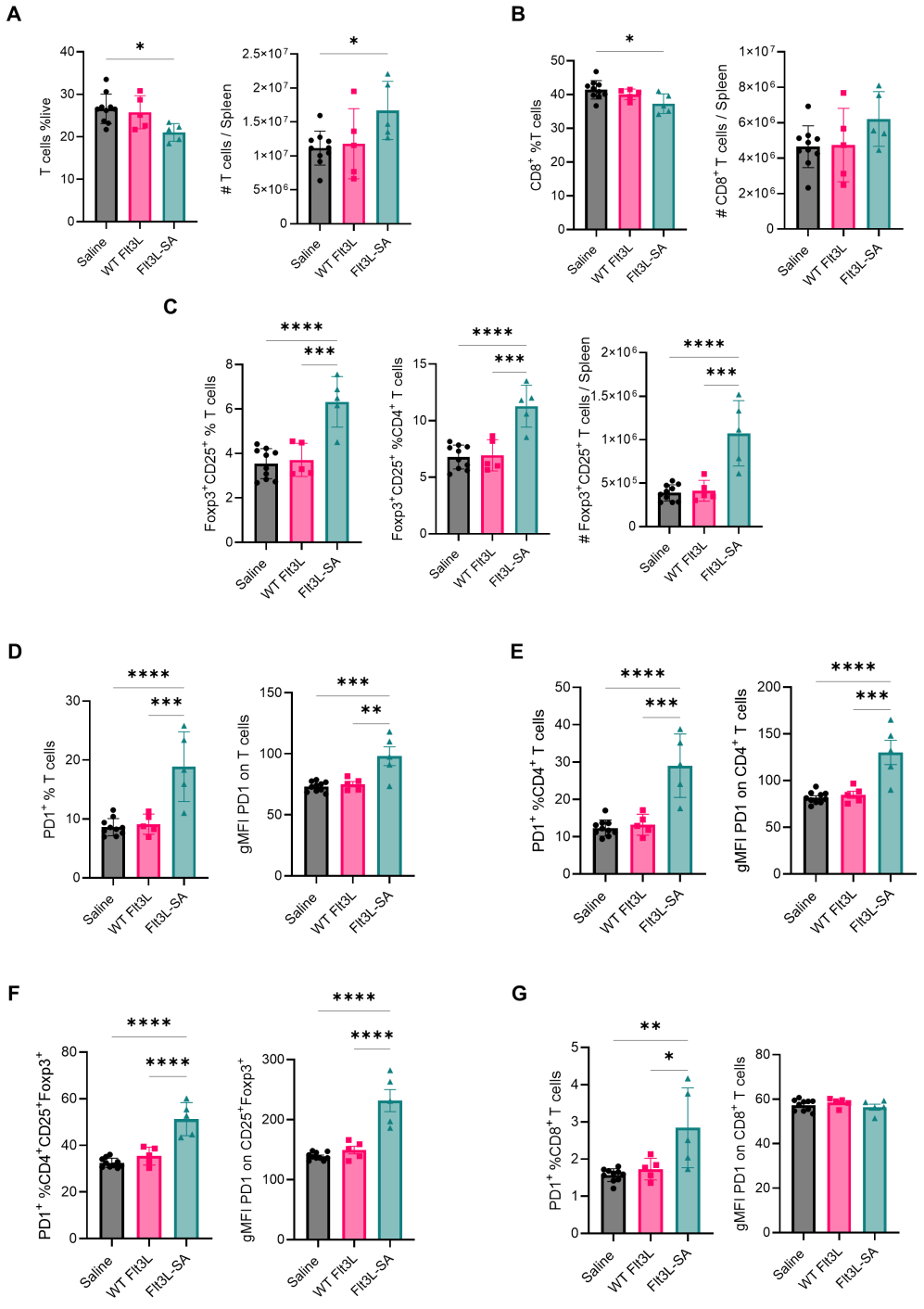

Figure 2.25: **Splenic T Cell Characterization After Flt3L Therapeutic Regime** | (Continued on following page.)

Figure 2.25: Continued. **a,** T cells ($CD3^+$) as proportion of total live cells (left) and total counts per spleen (right).**b,** $CD8^+$ T cell representation as proportion of total T cells and total number per mouse spleen. **c,** Treg ($Foxp3^+CD25^+$) cells as proportion of total T cells, proportion of $CD4^+$ T cells, and estimated number of cells per spleen. **d-g,** Co-inhibitory receptor expression on **d** Total T cells, **e** $CD4^+$ T cells, **f** Tregs, and **g** $CD8^+$ T cells represented as percent positive of the parent population as well as gMFI of expression. Each data point represents one mouse with error bars on gMFI graphs representing SEM and all others representing SD. Significance calculated via one-way ANOVA with Tukey's multiple comparison.

We also looked to characterize the T cell compartment of the gut dLN, with the added benefit of staining for markers related to the mucosal immune system, primarily Rorγt and integrin $\alpha_4\beta_7$, a cell adhesion molecule used for homing to the gut tissue.(87) In all examined dLN, we see significant increases in both $Foxp3^+$ and $Foxp3^+CD25^+$ as a proportion of total $CD4^+$ T cells in mice treated with Flt3L-SA (**Fig. 2.26A-B**); however, we also note significant increases in Rorγt^+ T cells in the jejunum and colon dLN in mice treated with Flt3L-SA (**Fig. 2.26C**). While Rorγt^+ T_{H17} cells tend to instigate inflammation, they are in fact important to maintaining gut health by secreting cytokines related to barrier functionality, including IL-17, IL-22, and IL-23.(258; 259) In addition, Tregs co-expressing Rorγt in addition to Foxp3 have been found to restrain excessive inflammation caused by T_{H17} cells.(260–262) We see significant increases in Rorγt^+ Tregs after treatment with Flt3L-SA in the jejunum and colon dLN, with the largest increases in the colon dLN, where these cells have been shown especially effective at controlling inflammation(261) (**Fig. 2.26D-E**). However, when we normalize to see Rorγt^+ as a percent of total Tregs, we see no differences with treatment (**Fig. 2.26F**), suggesting proliferation of established Rorγt^+ Tregs rather than *de novo* generation of this population. Finally, we looked at PD-1 and $\alpha_4\beta_7$ expression on T cells to glimpse into the regulatory state and homing of cells found in the gut dLN. In this, we see significant increases in PD-1 expression in all examined dLN in total T cells as well as $CD4^+$ T cells in mice treated with Flt3L-SA, but a significant increase in Treg expression of PD-1 is only seen in the colon dLN after treatment (**Fig. 2.27A-C**). Similar

to PD-1 expression, $\alpha_4\beta_7$ expression is significantly increased on all T cells and on CD4$^+$ T cells in Flt3L-SA treated mice in all dLN with the largest increase noted in the colon dLN (**Fig. 2.27D-E**). We, again, only see significant increases in $\alpha_4\beta_7$ expression after treatment with Flt3L-SA on Tregs in the colon dLN. Overall, we take these data to suggest increased antigen recognition in the gut and gut dLN following treatment with Flt3L-SA, leading to T cell proliferation and eventual homing back to the initial organ after passing through the lymphatics and lymph system.

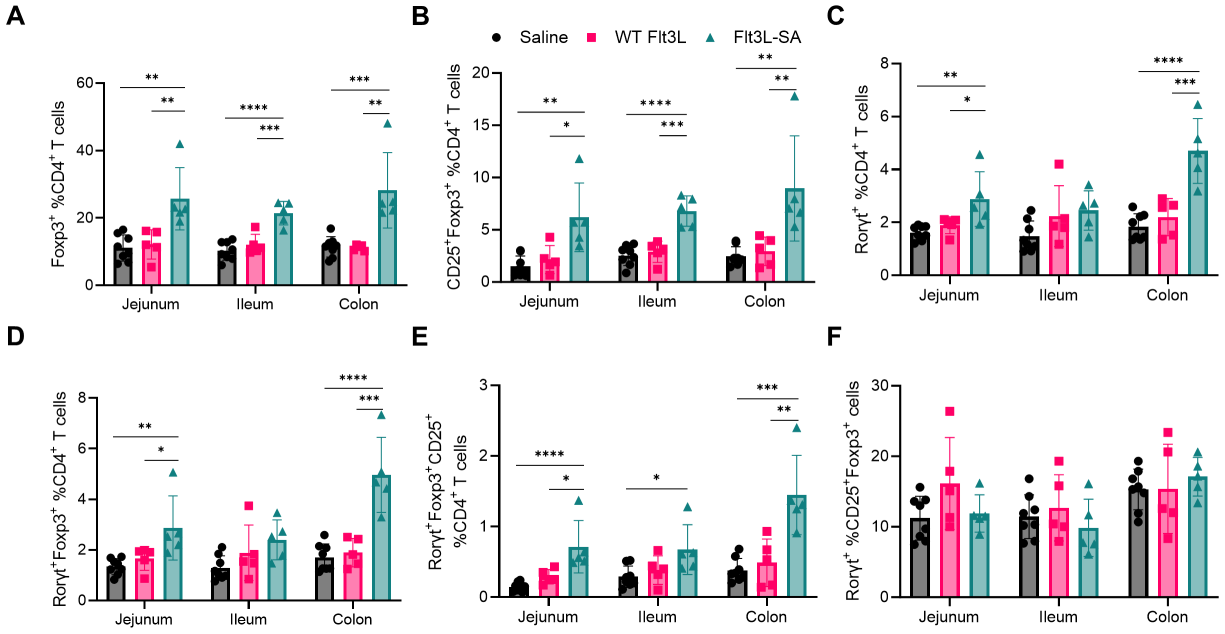

Figure 2.26: **Treg Characterization of Gut dLN After Flt3L Therapeutics | a,** Proportion of infiltrating CD4$^+$ T cells expressing the transcription factor for Treg fate, Foxp3 in the lymph nodes draining the represented section of intestine. **b,** Conventional Treg (CD25$^+$Foxp3$^+$) cell infiltration into the gut dLN. **c,** Rorγt expressing cells in the gut dLN, including T_{H17} cells and Rorγt$^+$ Tregs. **d,** T cells coexpressing Rorγt with Foxp3 in the gut dLN and **e,** Rorγt$^+$Foxp3$^+$CD25$^+$ T cells infiltrating gut dLN. **f,** Proportions of Rorγt$^+$ now normalized to total Foxp3$^+$CD25$^+$ Tregs as transversing the gut. Figures **a-e** are out of total CD4$^+$ T cells in each dLN, while **f** represents count out of total Tregs. Each data point represents one mouse with error bars representing SD. Significance calculated via one-way ANOVA with Tukey's multiple comparison within each organ dLN.

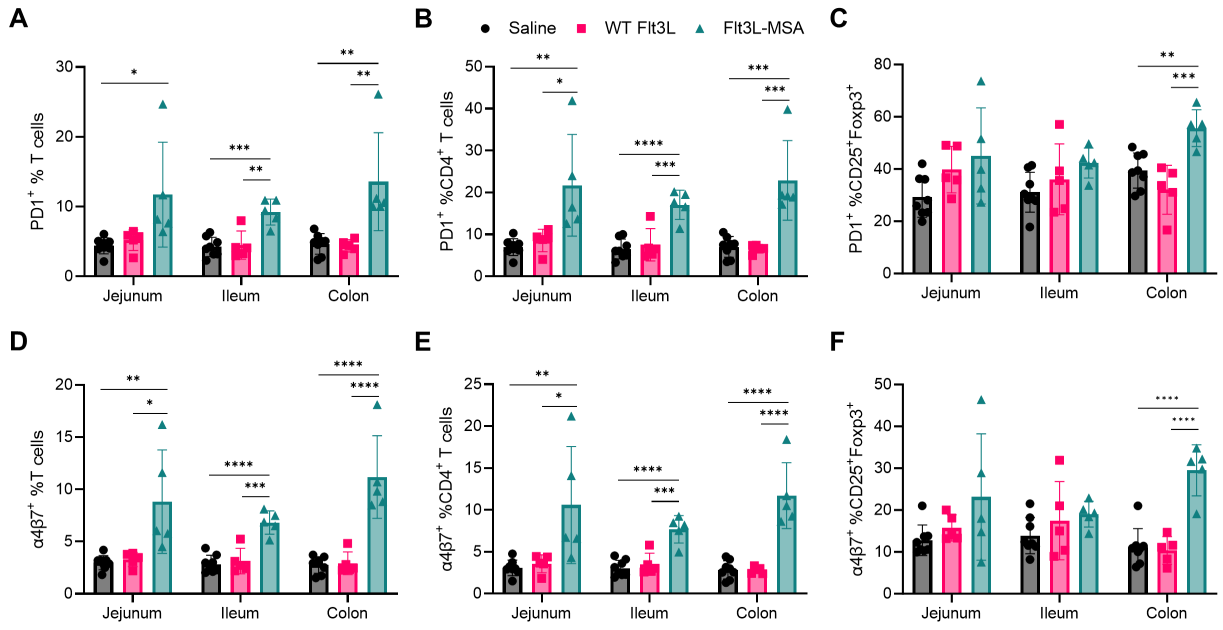

Figure 2.27: **Activation and Homing Cues on T Cells Following Flt3L Treatment | a-c,** T cell expression of the early activation/exhaustion marker PD-1 on **a,** all T cells, **b,** $CD4^+$ T cells, and **c,** $CD25^+Foxp3^+$ Tregs. **d-f,** Expression of the gut lamina propria homing integrin $\alpha4\beta7$ on **d,** total T cells, **e,** $CD4^+$ T cells, and **f,** $CD25^+Foxp3^+$ Tregs. Each data point represents one mouse with error bars representing SD. Significance calculated via one-way ANOVA with Tukey's multiple comparison within each organ dLN.

Synergy of Flt3L-SA with PD-1 Blockade in Enhancing Treg Expansion

With the results acquired in our previous studies, it seems as though PD-1 and TGF-β may play an important and unobserved role in the talk between DCs and Tregs following Flt3L intervention. With this in mind, we sought to perform the same therapeutic intervention as previously established with the addition of blocking antibodies against PD-1, pan TGF-β, or isotype control (representative gating **Fig. 2.28** and treatment schematic in **Fig. 2.29A**), with the goal of seeing how blockade affects Treg proliferation in response to Flt3L-SA treatment.(263–265) In this instance, we see no differences with blockade in $CD4^+$ T cell content in the spleen (**Fig. 2.29B**); however, we do note that the only group which did not see significant increase in Ki67 staining in $CD4^+$ T cells when comparing with or without Flt3L-SA treatment was the group receiving TGF-β blockade (**Fig. 2.29C**). We also see no

loss of Foxp3^{+}CD25^{+} Treg expansion after Flt3L-SA treatment in any group (**Fig. 2.29D**), but interestingly, when only looking at T cells expressing Foxp3, we actually see a significant boost in these cells when Flt3L-SA is combined with PD-1 blockade (**Fig. 2.29E**). This could potentially suggest a synergistic effect between the Flt3L-SA and PD-1 blockade in generating Treg precursors.

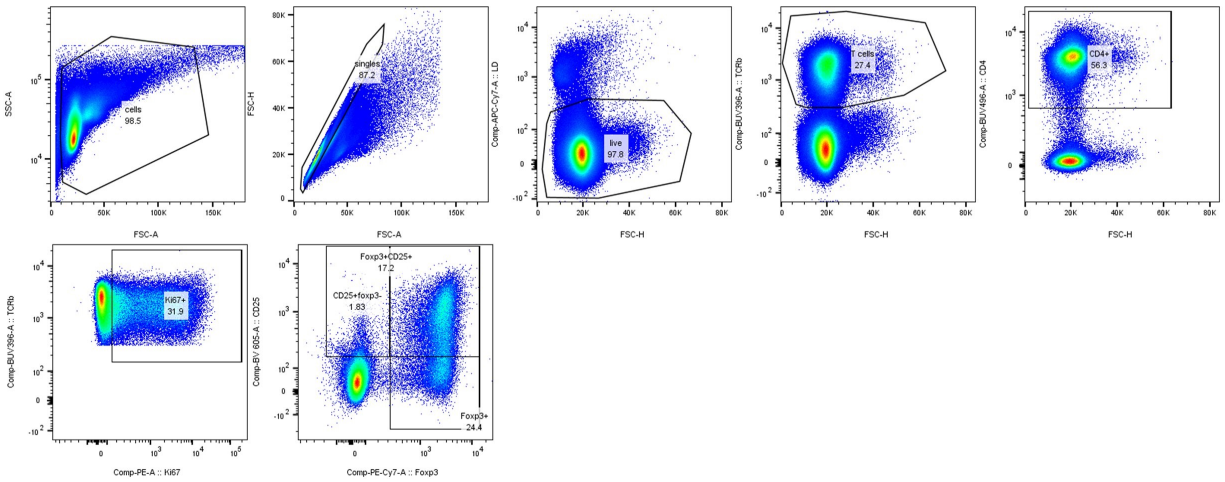

Figure 2.28: **Representative Gating for Cytokine Depletion**

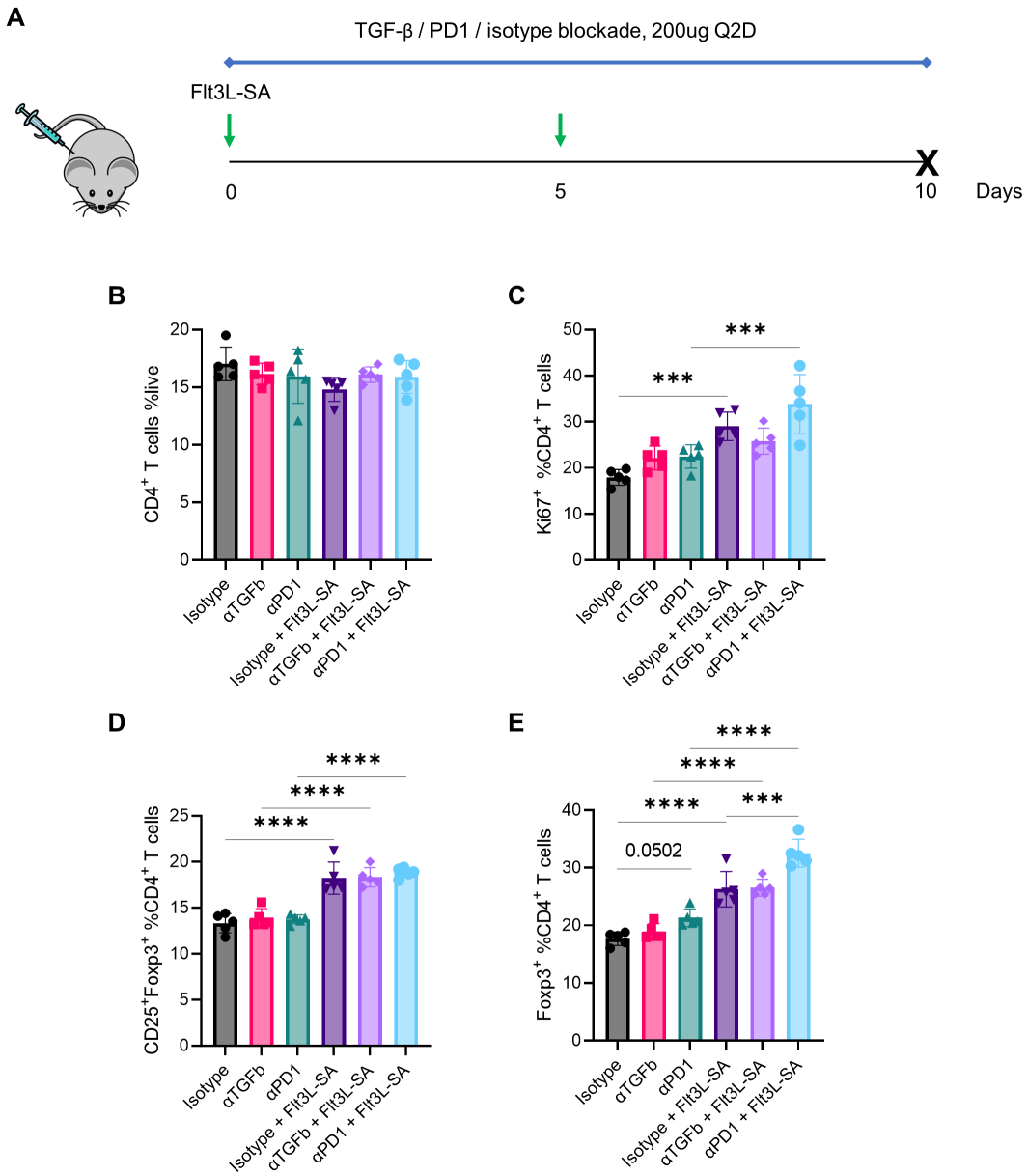

Figure 2.29: **Blockade of Potential Mechanisms Leading to Flt3L-SA Induced Treg Expansion | a,** Schematic representing treatment regime for Flt3L-SA (10 μg molar equivalent given on day 0 and day 5) as well as antibody treatments, 200 μg, every other day before euthanization and splenic harvest on day 10. **b,** Splenic infiltration of $CD4^+$ T cells and **c,** Ki67 staining on those same cells demonstrating recent proliferation. **d-e,** Splenic infiltration of (**d**) conventional $CD25^+Foxp3^+$ Tregs as well as (**e**) infiltration of $Foxp3^+$ $CD4^+$ T cells. Subfigure **b** normalized to total live cells, while **c-e** represented out of total $CD4^+$ T cells. Each data point represents one mouse with error bars representing SD. Significance calculated via one-way ANOVA with Tukey's multiple comparison.

2.4 Discussion

Herein, we have described the generation and characterization of a novel molecule capable of exploiting the innate immune system and antigen presentation pathways to direct adaptive immunity. Considering first the biochemical differences between the native form and the engineered Flt3L, we note no major differences in the receptor affinity for either construct (**Figure 2.3**), but a slight reduction in bioactivity (**Figure 2.5**) suggesting a difference in ability of the protein to induce dimerization and signaling via the receptor, potentially due to steric hindrances in protein:protein interactions. With this and the enhanced pharmacokinetics of the Flt3L-SA construct (**Figure 2.6**) in mind, there may be a mechanism for the addition of the bulky albumin domain to induce a tissue tropism for this molecule when treated *in vivo*. In other words, the activity of the molecule may be enhanced in areas where endogenous Flt3L is expressed, such as the bone marrow, as there could be less steric hindrances in a Flt3L:Flt3L-SA interaction than there would be in a Flt3L-SA:Flt3L-SA interaction. Whereas in the case of treating with the native Flt3L, there are many challenges, the least of which being clearance by the kidneys due to size, the addition of the albumin domain not only enhances retention of the drug but also demonstrates a vast improvement in activity *in vivo*, overcoming the requirement of daily injections and still having enhanced proliferation over the native form (**Figures 2.7-2.10**).

Following the more molecular studies of our construct, we then demonstrated the pharmacodynamics of the novel molecule (**Figures 2.11-2.17**). We note peak splenic DC expansion six days following treatment, with these taking a primarily cDC phenotype, followed shortly with peak pDC expansion 10 days after treatment. While both cDC populations expand, when we normalize as percent of total DCs we see no differences over time in the cDC2 population, suggesting that there is a stochastic process in DC differentiation that maintains the cDC2 fate. We do, however, note an expansion in the proportion of DCs with a cDC1 phenotype after treatment, in accordance with the fact that Flt3L is a known signal in cDC1

differentiation.(225–227)

In these studies, as has been seen in previous work by other labs, we also show an upregulation of suppressive adaptive responses in relation to Flt3L administration.(204; 207; 235; 266) Working off of those studies, where antigen presentation by the generated DCs was essential to Treg proliferation, we also see evidence of antigen presentation leading to such proliferation, such as cellular expansion and PD-1 expression (**Figures 2.16-2.17 and 2.25**). From this, we form the hypothesis that the enhanced DC presentation capacity leads to enhanced exposure of the immune system to autoantigens or potentially to commensal-derived antigens, especially considering the extreme increase in DC numbers in the gut draining LN (**Figures 2.25 and 2.26**). In addition to cellular readouts of tolerance (such as decreased MHCII and CD40 on DCs as well as other markers of mRegDC fates(206)), we also note a very significant increase in LAP expression after Flt3L-SA treatment, a large portion of which is expressed by the DC compartment (**Figures 2.21 and 2.24**). This TGF-β expression may further contribute to the expansion of the Treg compartment due to antigen recognition concurrent with cytokine presentation by the APC.(86; 250; 267) With this in mind, in addition to the apparent PD-1 signaling axis, we also sought to determine if blockade of these pathways were potential avenues for preventing such suppressive expansion. While we were unable to fully determine if the TGF-β blockade was functional, we do note a potential synergistic pathway where treatment with Flt3L-SA along with PD-1 blockade may actually enhance this expansion (**Figure 2.29**).

Flt3L has been used previously in studies related to enhancing presentation of tumor associated antigens(187; 196–198; 203) especially related to instances of irradiation,(194; 195) which adds further DAMPs to the milieu to generate adaptive related killing of tumor tissue. In these cases, Flt3L treatment is combined with PD-1 blockade as a means to enhance $CD8^+$ T cell killing;(194; 195) however based on our data, we suggest that there may be better blockades, such as those against TIM3, Lag3, or CTLA4, to give in addition to

Flt3L (or Flt3L-SA) to enhance tumor clearance without also giving rise to more pro-tumor Tregs.(268) Furthermore, previous evidence points to a tolerogenic use of Flt3L including for type 1 diabetes prevention as well as prevention of inflammatory bowel disease, the effects of which were attributed to Treg expansion.(204; 235; 269; 270) We herein provide evidence that these effects may also be attributed to the expansion of tolerogenic DCs themselves or at minimum a decrease in pro-inflammatory displaying DCs (as evidenced by expression of MHCII, PD-L1, and CD86) entering the gut dLN with the effect becoming more pronounced traversing from the proximal small intestine to the distal colon dLNs (**Figure 2.22**).

This chapter serves to demonstrate the biochemical and medicinal properties of the novel cytokine fusion Flt3L-SA. With these effects in mind, the following chapter will cover various applications of Flt3L-SA in therapies involving both the innate and the adaptive responses.

2.5 Materials and Methods

Animals

C57BL/6 mice were purchased from Jackson Labs and housed at the University of Chicago Animal Facility. All procedures were approved by the University of Chicago Institutional Animal Care and Use Committee and performed on protocol 72449. Injections were diluted to the appropriate concentration in 50μL sterile saline for S.C. injections or 100μL for I.V. or I.P. treatments. Mice were euthanized via CO_2 asphyxiation as approved by UChicago ARC.

Protein Production and Purification

Plasmids encoding Flt3L or Flt3L-SA were diluted into OptiPro transfection media to a concentration of 50μg/mL. Linear, 25kDa polyethylemine was then also diluted to a concentration of 100μg/mL before slowly adding the PEI mixture to the DNA to a final ratio of 1:1

(v:v). This mixture was incubated at room temperature for 10 minutes without agitation to allow DNA/PEI complexation. After complexation, 1mL of transfection reagent was slowly added with agitation to 25mL of HEK293F cells grown to a concentration of 1e6 cells/mL. Transfected cells were then incubated at 37°C for one week with constant shaking before protein harvesting. At harvest, cells were removed by centrifugation at 4000G for 10' before filtering the supernatant through 0.22μm filter. Filtered supernatant was then pH adjusted to about pH of 8 using 1M Tris at a pH of 9. This supernatant was then loaded over a HisTrap HP (Cytiva) column on a GE AKTA Avant FPLC. Once loaded, the column was then washed using 50mM imidazole to remove loosely bound proteins and then the protein was eluted with 400mM imidazole. Protein eluted was further purified over a superdex 200pg column (Cytiva) in PBS, pooling only pure fractions of the correct size. The final protein was then tested for LPS using a HEK TLR4 cell line with quantiblue to confirm endotoxin free samples before aliquoting and freezing.

SDS-PAGE

Protein was prepared according to buffer instructions for reducing and non-reducing preparations before boiling for 15 minutes. 10μg of protein in sample buffer was then added to each lane of a stain-free gel (Biorad 4568096) before running until the dye front reached the bottom using TGS buffer. Gel activation and imaging occurred on a ChemiDoc XRS+.

CD135 Binding ELISA

High-bind plates (Corning 9018) were coated by incubating overnight at 4°C with 10nM recombinant CD135 (R&D 768-F3-050) in 50nM sodium bicarbonate buffer at pH 9.2. Following coating, plates were washed in PBST before blocking in 1x reagent diluent (R&D DY995) for 2 hours at RT. Samples were then diluted to the indicated concentration in triplicate using the same reagent diluent. Blocked wells were washed and then sample was

added and incubated for one hour. Sample was removed before addition of the detection antibody (R&D 841481) at the recommended concentration and incubated for 1 hour followed by washing and addition of Streptavidin-HRP (R&D 890803) at the indicated concentration for a 30' incubation. Wells were then washed before the addition of TMB (Millipore ES-001). TMB was incubated while protected from light until saturation was reached, at which point the reaction was stopped using 10% Sulfuric Acid. Quantification occurred by reading the absorbance at 450nm and 570nm. Data was then plotted as the absorbance at 450nm minus absorbance at 570nm.

BMDC Generation

BMDC were generated according to a modified Lutz protocol. (271; 272) Briefly, bone marrow was flushed from the long bones of healthy 6–15 week-old C57BL/6 mice into RPMI 1640 media and filtered over 100um filters. On day 0, 3 million nucleated cells were plated in 10mL of a modified Lutz media (RPMI 1640 supplemented with 10%FBS, 1%Pen/Strep/L-glutamine, 50uM beta-mercaptoethanol, 25mM HEPES, 20ng/mL GM-CSF, 200ng/mL Flt3L) in 100mm non-tissue culture treated petri dishes. Cells were fed by the addition of 10mL of the initial media (with GM-CSF and Flt3L) on day 3. On day 6, media was refreshed by removing 10mL and centrifuging the cells before resuspension in 10mL of fresh, complete media (GM-CSF and Flt3L). Non-adherent cells were harvested on day 9 for use and starved for 2 hours in incomplete RPMI 1640.

Phospho-ERK1/2 Western Blot

1 million starved BMDCs were resuspended into 100μL of incomplete RPMI. 100μL of 4μM Flt3L or Flt3L-SA in incomplete RPMI was then added to each tube for the indicated time, at the end of which 1mL of ice-cold PBS was added and cells were placed on ice before centrifugation at 4°C. Supernatant was then removed and cells were lysed using RIPA

buffer (Thermo 89901) with protease and phosphatase inhibitor added (Thermo A32959, 1 tablet/10mL RIPA). Cells were incubated in the RIPA buffer for 5 minutes on ice before centrifugation at 17,000 G for 10 minutes. Supernatant was taken and total protein quantified via BCA (Thermo 23227). Cell lysate was prepared in fresh reducing running buffer and run on SDS-PAGE as previously described. Protein was transferred from the gel to a PVDF membrane using a wet transfer system before blocking the membrane with 5% BSA solution in TBST. Membrane was probed using 1:5000 dilution of anti-pERK1/2 (Biolegend 369502) for 2 hours followed by HRP-conjugated anti-mouse (CST 7076S). Detection occurred using Clarity substrate (Biorad 1705060) before stripping the membrane with Restore Stripping Buffer (Thermo 21059). The procedure was then repeated using 1:5000 dilution of anti-total ERK (Biolegend 686902) and HRP conjugated Anti-Rat (Jackson Immuno 112-036-003) at 1:10,000 to probe for total ERK1/2.

Phospho-flow for phospho-ERK1/2

Starved BMDCs were plated in a 96 well plate at 500,000 cells in 100μL of incomplete RPMI. Warmed cytokine solution was then added to each well as a 2x concentration in 100μL of incomplete RPMI before incubation for exactly 5 minutes. At the end of the incubation period, 50μL of warmed 5x Lyse/Fix buffer (BD 558049) was added to each well before incubation at 37°C for 10 minutes. Cells were then washed with PBS before resuspension in ice cold Perm Buffer (BD 558050) and a 15 minute incubation on ice. Cells were then washed twice in FACS buffer (PBS + 2% FBS + 1mM EDTA) before staining with anti-pERK (as described in methods for flow cytometry staining) and acquisition on a BD Fortessa.

Pharmacokinetic Study

Mice were treated one time via S.C. injection and blood was collected into Lithium-Heparin coated tubes at the indicated timepoints following treatment and plasma isolated following

centrifugation at 10,000G for 10' before freezing. Once all timepoints had been collected, Flt3L content in the plasma was quantified via ELISA (R&D Dy427) using Flt3L standard for mice treated with WT Flt3L and using an equimolar Flt3L-SA standard for Flt3L-SA treated mice. Plasma was diluted 100-10,000x and the lowest dilution for each timepoint which did not oversaturate the standard was taken and converted before accounting for dilution.

Preparation of Single-cell Suspensions from Whole Organs

After euthanasia, spleens and lymph nodes were harvested into 0.5mL of complete DMEM and placed on ice. Once all tissues from all mice were removed, spleens were pushed through a 70μm filter and washed with incomplete DMEM. Suspensions were then centrifuged at 1750 RPM for 7 minutes before resuspension in 3mL of ACK lysis buffer (Thermo 1049201) followed by a 5' incubation before dilution in incomplete DMEM. Cells were then centrifuged as previously stated before counting and a final resuspension in complete DMEM at a concentration of 20 million cells/mL. Lymph nodes were processed by the addition of Collagenases D and IV at a final concentration of 1mg/mL each enzyme (Roche 11088866001 and Worthington LS004188, respectively). Lymph nodes were incubated at 37°C for 45' before processing in a manner similar to spleens without the ACK lysis step.

Inhibition of Protein Transit for LAP staining

Conditions where LAP staining was performed had a pre-incubation step in GolgiPlug (BD 555029) and GolgiStop (BD 554724) to increase sensitivity of the staining. Briefly, 2 million isolated cells were plated in Complete RPMI (RPMI + 10% FBS + 1% P/S) with 1x each inhibitor according to manufacturer instructions. Cells were then incubated at 37°C for 4 hours before proceeding with flow staining, as outlined below, using the CytoFix/CytoPerm kit for intracellular staining.

Staining for Flow Cytometry

2 million isolated cells were plated per sample before washing with plain PBS. Viability stain was diluted in plain PBS at 1:500 with the addition of Fc Block before adding 50μL/well and incubation on ice for 15'. Viability dye was quenched by washing with FACS buffer (PBS + 2% FBS + 1mM EDTA) before addition of surface staining antibodies in a 1:1 dilution of Brilliant Stain Buffer (BD 563794) in FACS buffer. Surface staining occurred for 20' at RT before washing in plain PBS and subsequent fixation. Samples not requiring intracellular staining were fixed for 20' on ice using 2% PFA in PBS. Samples requiring only intracellular cytokine staining were fixed for 20' on ice using the BD Cytofix/Cytoperm kit (BD 554714) before washing and intracellular staining for 1hr-O/N at 4°C. Samples staining for nuclear factors (with or without cytokine staining) were fixed using the Foxp3 Transcription factor staining set (Thermo 00-5523-00) for 45-60' on ice before intracellular staining for 1hr-O/N at 4°C. After fixation and intracellular staining, cells were washed and resuspended in FACS buffer for data acquisition on a 5 laser BD fortessa.

Data Analysis

All data was plotted and analyzed on GraphPad Prism v10 software using statistic tests noted in the figure legends. Flow cytometric data was analyzed using FlowJo v10.

2.5.1 Antibodies Used in Chapter 3

Table 2.1: Antibodies, Vendors, Localization, and Dilutions for Chapter 2.

Antigen	Color	Vendor	Catalog	Localization	Staining Dilution
$\alpha4\beta7$	PE	Biolegend	120606	Surface	1:200
B220	BUV496	BD	612950	Surface	1:200

Continuation of Table 2.1					
Antigen	Color	Vendor	Catalog	Localization	Staining Dilution
CD103	BV605	Biolegend	121433	Surface	1:200
CD103	PE	BD	561043	Surface	1:200
CD11b	Percp-Cy5.5	BD	550993	Surface	1:200
CD11b	BV785	Biolegend	101243	Surface	1:200
CD11b	PE-Cy7	Biolegend	101216	Surface	1:200
CD11c	PE-Cy7	BD	558079	Surface	1:200
CD11c	BV605	Biolegend	117333	Surface	1:200
CD135	BV421	BD	562898	Surface	1:100
CD19	FITC	Biolegend	152404	Surface	1:200
CD25	BV605	Biolegend	102035	Surface	1:200
CD25	BV421	Biolegend	102054	Surface	1:200
CDϵ	BUV396	BD	563565	Intracellular	1:200
CD4	BUV496	BD	612952	Surface	1:200
CD4	BV785	BD	740844	Surface	1:200
CD45	BV785	BD	564225	Surface	1:200
CD45	APC-Cy7	Biolegend	103116	Surface	1:200
CD45	APC	Biolegend	103124	Surface	1:200
CD8α	BUV737	BD	612759	Surface	1:200
CD86	BUV396	BD	564199	Surface	1:200
CTLA-4	Percp-Cy5.5	Biolegend	106316	Surface	1:200
Foxp3	BV421	Biolegend	126419	Nuclear	1:50
Foxp3	FITC	BD	560403	Nuclear	1:200
Foxp3	PE-Cy7	Invitrogen	25-5773-82	Nuclear	1:50

Continuation of Table 2.1					
Antigen	Color	Vendor	Catalog	Localization	Staining Dilution
HELIOS	AF488	Biolegend	137223	Nuclear	1:200
IRF8	APC	Invitrogen	17-9852-82	Nuclear	1:200
Ki67	PE	Biolegend	652404	Nuclear	1:200
LAP	BV421	Biolegend	141408	Intracellular	1:200
MHCII	APC-Cy7	Biolegend	107628	Surface	1:200
MHCII	Percp-Cy5.5	Biolegend	107626	Surface	1:200
PD-1	BV785	Biolegend	135225	Surface	1:200
PD-1	BV605	Biolegend	135219	Surface	1:200
PDCA1	BV510	BD	747607	Surface	1:200
PD-L1	BV785	BD	741014	Surface	1:200
Rorγt	BV21	BD	562894	Nuclear	1:200
TCRβ	BUV396	BD	742485	Surface	1:200
Viability	eFluor455	Invitrogen	65-0868-14	Viability	1:500
Viability	eFluor780	Invitrogen	65-0865-14	Viability	1:500
pERK1/2	PE	Biolegend	369506	Phospho fix/perm	1:100
pan TGF-β	N/A	BioXCell	BP0057	*in vivo* neutralization	
PD-1	N/A	BioXCell	BP0273	*in vivo* neutralization	
IgG1 isotype control	N/A	BioXCell	BP0083	*in vivo* neutralization	
End of Table					

CHAPTER 3

IMMUNOTHERAPEUTIC APPLICATIONS OF FLT3L-SA

3.1 Abstract

Biologic drugs, such as enzyme replacements or cytokine-blocking treatments, have enabled major benefit to numerous patients which could otherwise require intense immunosuppressive regimes to continue to live, leaving them vulnerable to infection. While life-changing for many, these foreign proteins can instigate immune reactions leading to a potential high failure rate, dependent on the drug. When failure occurs, some patients can change drugs, if another exists on the market (e.g. TNF-α inhibitors); however, for patients on enzyme replacement or hemophiliacs on clotting factors where there are not alternatives, the only real viable options are broad suppression or antibody depletion mechanisms, which also leave patients immunosuppressed.(273) In this chapter, we address two potential options to leverage T cell education and prevent autoimmunity to foreign proteins, both utilizing combination therapies with Flt3L-SA (described in depth in Chapter 2). The first method described is work combining low-dose antigen tolerance induction with Flt3L-SA to change the B cell education pathway, potentially via inducing Treg and T follicular regulatory cell differentiation to significantly reduce anti-drug antibody responses. The second methodology seeks to provide a preliminary framework to combine Flt3L-SA treatments with oral antigen delivery to modify the T cell compartment.

3.2 Introduction

Biologics usage and approval is on the rise as more and more protein drugs meet FDA guidelines and become the preferred treatment modality for many autoimmune conditions as well as usage in patients seeking enzyme replacement.(274; 275) Since these drugs are, by definition, foreign proteins, the body can react to them in deleterious ways leading to a high rate of failure(276–279) – due to either development of infusion reactions to the drugs or the formed antibodies inhibiting the active function of the drug – for these life-

saving treatments. Previous clinical methods to address these anti-drug antibodies (ADA) have involved immunosuppression using drugs like methotrexate or B cell depletion using rituximab; however, these options are undesirable due to the systemic effects leaving the patient susceptible to secondary infection. Previous work in our lab however has sought to modify the initial presentation of the antigen to boost tolerogenic mechanisms.(78–80) Additionally, other groups have looked at combining low-dose antigen in a tolerogenic regime with other drugs,(280) such as encapsulated rapamycin,(207; 281; 282) or in nanoparticles made with phosphatidyl serine to induce tolerogenic APC uptake,(283) which has proven beneficial in reduction of ADA formation and specifically the formation of "inhibitors" in the case of hemophiliacs.

Flt3L is an interesting therapeutic route due to its role in DC genesis. Most recent work involving Flt3L has actually been in the realm of promoting immunity in cases of cancer therapies or vaccination regimes;(187; 188; 192; 194–198; 203) through generating DCs, primarily type 1 cDCs, Flt3L may be capable of promoting antigen presentation to less accessible protein epitopes or to neoantigens in the tumor environment. However, earlier work (as well as that discussed in chapter 2) has demonstrated the innate capability of Flt3L treatments to induce tolerance and Treg proliferation.(204; 207; 270) This chapter demonstrates two potential options to initiate a tolerogenic response to foreign proteins using the previously described Flt3L-SA. The first section directly explores the combination of Flt3L-SA with a low-dose antigen regime to promote Treg generation and decrease ADA reactions, without additional immunosuppressive agents as have been used in the past. This section also provides some insight to potential mechanisms by which low-dose antigen reduces the immune burden of foreign proteins, an understudied field itself.

The second section of this chapter provides preliminary work looking into modification of the initial antigen response through gastric delivery. Due to the necessity of the body to remain unreactive to food antigens, oral immunotherapy (OIT) has been long studied as a

means of generating tolerance to both auto-antigens and foreign antigens.(284) Recent work has attempted to determine mechanistically how oral antigen exposure is prevents immunity; with this study in mind, we seek to determine if the enhanced antigen presenting capability in the gut induced by Flt3L-SA treatment as well as the Treg expansion and TGF-β expression if further able to generate immunosuppressive reactions.

3.3 Therapeutic Application of Flt3L-SA for Prevention of Anti-Drug Antibodies

Knowing that we are generating tolerogenic environment with Treg expansion and DC phenotype, we first sought to use our Flt3L-SA platform in conjunction with low dose antigen to use as a prophylactic treatment in prevention of ADA. This work builds on research exemplifying Flt3L use in conjugation with rapamycin induces pDC mediated Treg expansion and thus ADA suppression.(207) Herein we show combination of Flt3L-SA with antigen alone (no rapamycin) is the only instance of significant antibody reduction in a model akin to enzyme replacement therapy using the FDA approved, highly immunogenic rasburicase (recombinant uric acid oxidase enzyme).

3.3.1 Results

It is well known that low-dose antigen escalation is a viable option for prevention of immunologic reactions to foreign proteins; however, this hit-or-miss approach to tolerance induction is risky when considering life-saving enzyme replacement therapeutics, especially in the cases of metabolic orphan diseases that only have one or two clinically available options. To attempt to address this, we developed a model of ADA generation to Rasburicase (**Fig. 3.1**). In this model, BALB/c mice are pretreated with antihistamine (diphenhydramine) s.c. 45 minutes prior to being challenged IV with 100μg of rasburicase (denoted by red arrows).

This challenge occurs weekly for four weeks and models what would happen at an infusion center, where antihistamines would be given prophylactically to prevent an anaphylactic response.(285) To test the efficacy of tolerance using low-dose antigen, mice in tolerized groups are treated once weekly with 5μg rasburicase for three doses (blue arrows) leading up to the challenge regime. Finally, testing this model in combination with Flt3L-SA (with the knowledge of the cellular dynamics of this drug from Chapter 2), mice are treated with Flt3L-SA five days prior to the first tolerizing dose of antigen and followed with another dose one week later (yellow arrows). Treatment on this timeline, we assume, allows antigen exposure at peak DC expansion as well as subsequent tolerogenesis from the later injections at peak Treg expansion. To track the development of antibodies over time, mice are bled weekly (red droplets) for ELISA before euthanasia on day 46 to check cellular responses to the antigen (denoted by an X on the timeline).

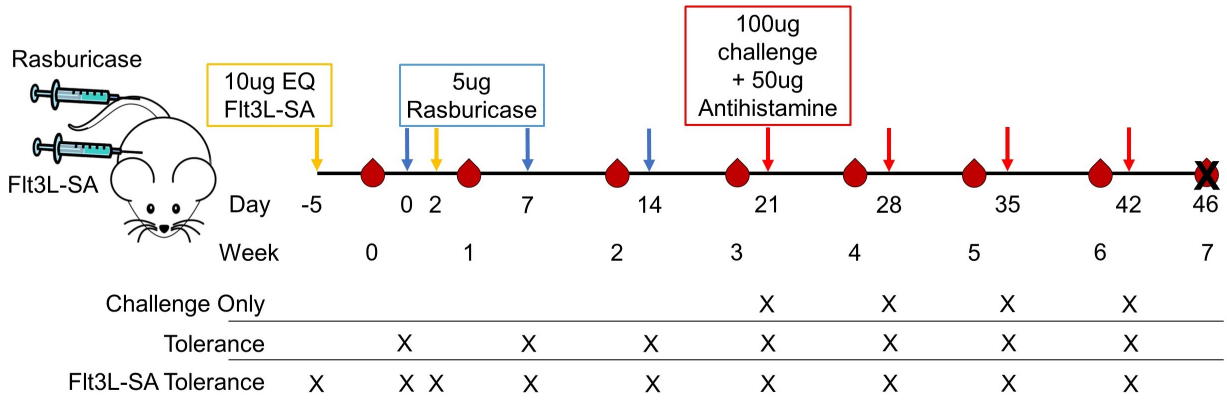

Figure 3.1: **Schematic Representation of Antidrug Antibody Timeline** | Ten week old balb/c mice are treated at the timepoints indicated by the colored arrows with the treatment of the same color. The x on the timeline indicates which groups receive which treatments and how each differs with the names corresponding to those in the following graphs. Additionally, blood samples were taken at the times indicated by red droplets on the timeline; the week number underneath each drop corresponds to the week indicated in 3.2 with a terminal bleed upon euthanasia on day 46.

We first looked at the soluble markers of antigen recognition, antibodies in the plasma. Following over time, we note similar increases in antibody formation between mice pre-

treated with Flt3L-SA and those without with IgG titers first rising following the second administration of low-dose antigen. Antibody levels in the Flt3L-SA group seem maintained following the third dose of antigen, whereas mice without Flt3L-SA co-treatment continue to increase antibody load, although only slightly. As expected, mice which did not undergo a tolerogenic regime already show IgG formation after only one challenge (week 4 time-point), albeit only slightly, with further formation following the second challenge. By the end of the experiment, only the IgG titer of mice treated with Flt3L-SA in the tolerogenesis phase was significantly different from challenge alone, with no significant differences in either direction for the group without Flt3L-SA (**Fig. 3.2A**). These effects are recapitulated by measures of Area Under the Curve for the ELISAs at each time-point (**Fig. 3.2B**). In the first repeat of this experiment, we were able to observe noticeable anaphylaxis-like symptoms – hunched, cold, difficulty breathing, and isolating behaviors (286) – within one hour following the final challenge dose, even with a large dose of antihistamine in the mice. We then attempted to determine if the IgG titer measured prior to the final dose related to likelihood of having such a deleterious response. When we look at week 6 titer and ignore treatments, only looking at whether the mice had a reaction or not, we note that reactions only happen when the mice reach antibodies measured as a titer at or above 4 (**Fig. 3.2C**). Using this knowledge, we considered titers greater than or equal to four as a likely seroconversion to pathogenic levels of IgG. Plotting these seroconversions, we note significant differences between the challenge only group and the Flt3L-SA tolerance group and a near significant trend between the low-dose tolerance group and the Flt3L-SA tolerance group (**Fig. 3.2D**). Additional corroboration of this observation comes from the second repeat of this experiment, where records of these anaphylaxis-like reactions were taken down. In this, we see only minor benefit to low-dose antigen alone, whereas qualitatively, we were able to double the benefit of low-dose treatment by adding Flt3L-SA to the regime, where six mice showed no observable reactions with Flt3L-SA and only three mice in the low-dose group were spared (**Fig. 3.2E**).

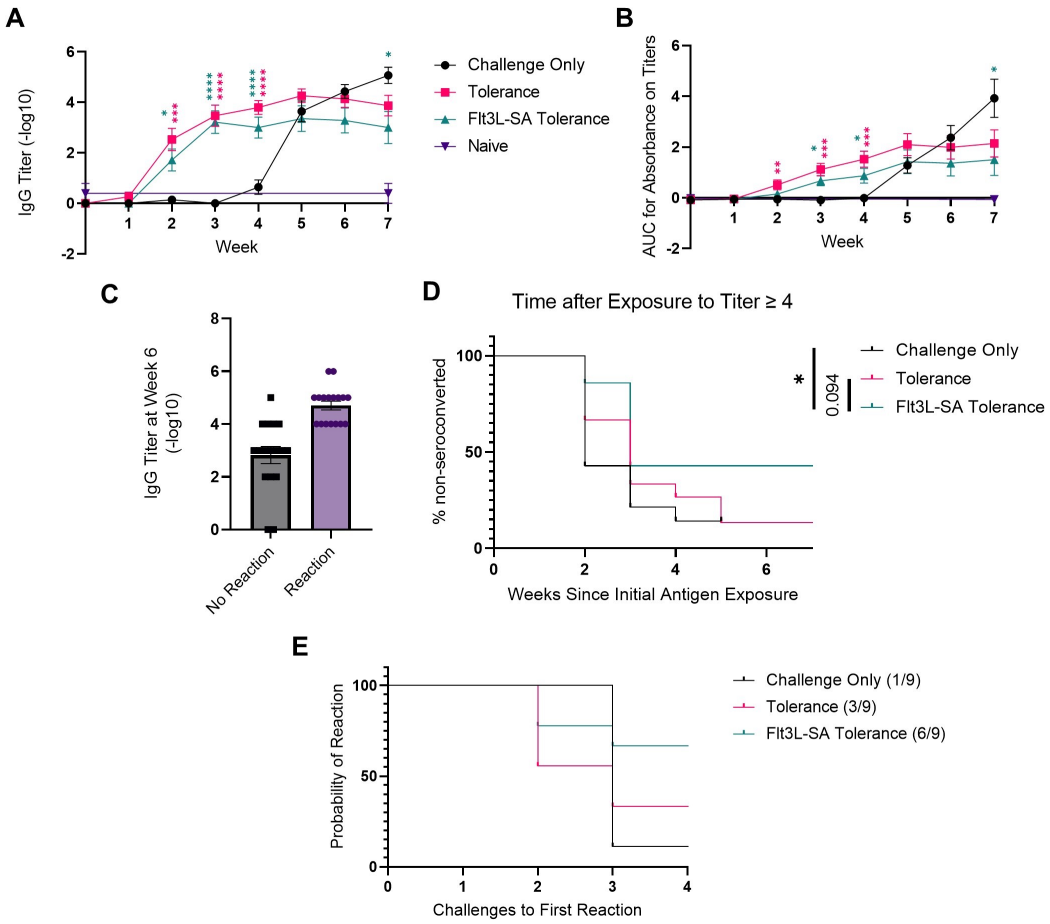

Figure 3.2: **Antidrug Antibody Quantification and Determination of Pathogenic Levels Thereof | a,** Total IgG titer measured over time with **b** representing AUC for the ELISA absorbance versus titer curves. **c,** Plots of measured titer for the week 6 time point corresponding to mice which displayed visual infusion reactions. **d,** Time to seroconversion considering titer of 4 the point of pathogenic amounts of ADA. **e,** Number of challenges until mice displayed visual symptoms of infusion reactions with labels demonstrating number of mice which made it through the last challenge without a reaction. Samples in **a, b, d** represent two pooled experiments with n=5 for naive and n=14-15 for the other 3 groups; **c** represents the first experimental replicate with n=36 mice and **d** representing the second experimental replicate with n=9 per treatment. Significance in **a, b** represents one-way ANOVA with Tukey's multiple comparison for that timepoint with significance against the challenge only group and color represents group and error bars for SEM. **d** significance is by Mantel-cox log-rank test between each group.

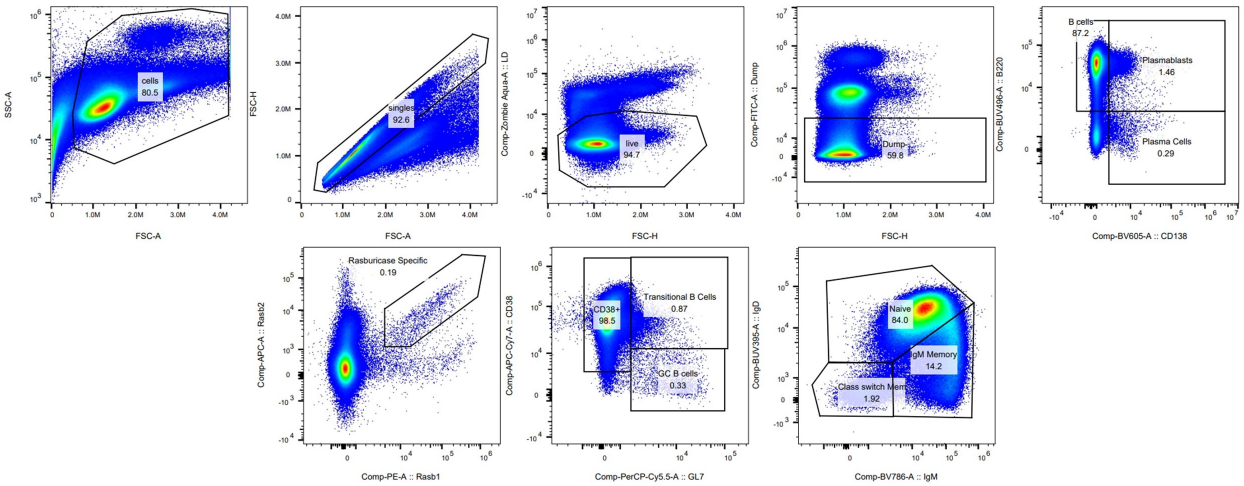

Figure 3.3: **Representative Gating for Figure 3.4-Figure 3.6**

Upon euthanasia, spleens were taken from these mice to allow for cellular characterization of the response following the challenge regime (representative B cell flow gating in **Fig. 3.3**). Characterizing the bulk B cell response first, we importantly observe no differences in total B cells (**Fig. 3.4A**), making this measurement a relevant normalization of how other populations are changing. Next, we see significant increases in the percent of B cells with a germinal center (GC) phenotype ($CD38^{-}GL7^{+}$) as well as total count in the challenge only group (**Fig. 3.4B**), indicative of a continued affinity maturation response in the challenge group. The next group of interest to us was that of cells displaying a phenotype called by others a "memory precursor" ($CD38^{+}GL7^{+}$), which are indicative of a cell capable of bypassing the GC response and going straight to an early memory phenotype.(287) Of this population, we only observe a near-significant trend towards increasing this population in the tolerance regime, with no differences noted in the Flt3L-SA tolerance group (**Fig. 3.4C**). With this in mind, we also phenotyped the memory B cell response, as denoted by CD38 expression and lack of IgD.(80) In accordance with the early response B cells, we see a significant increase in the population of total memory B cells when normalized to total B cells in only the tolerance group, however this effect is only near significant when translated to count (**Fig. 3.4D**).

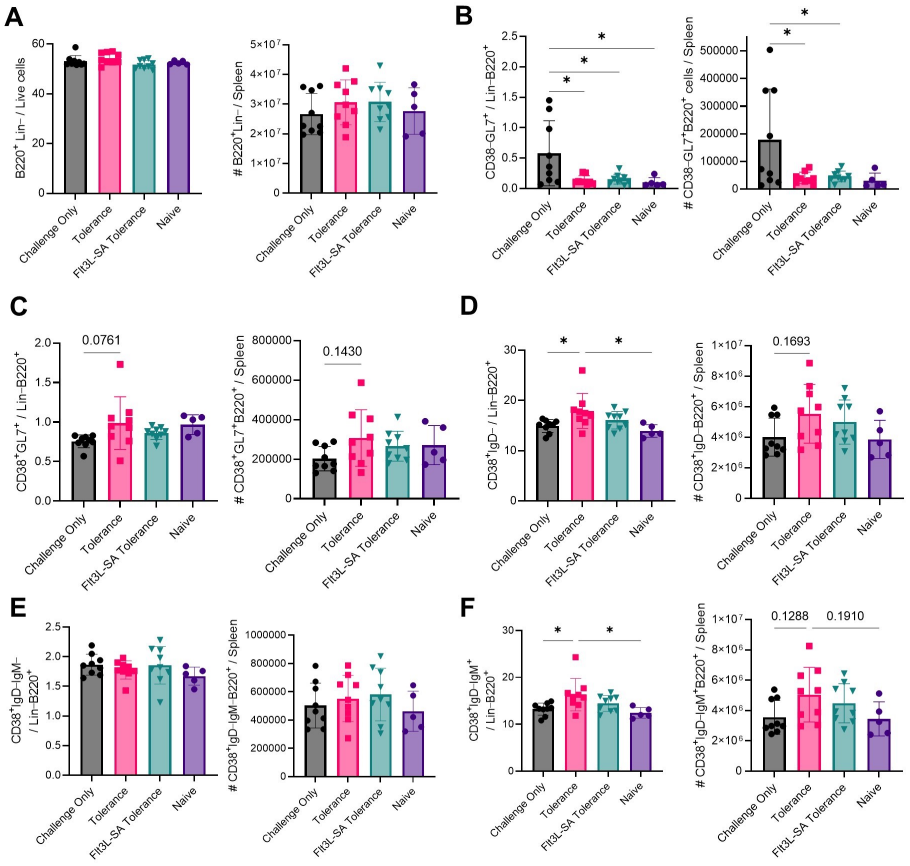

Figure 3.4: **Flow Cytometric Quantification of Splenic Bulk B Cell Populations After Challenge | a,** Total B cells in the spleen, **b,** Germinal Center B cells, **c,** memory precursors, **d,** total memory cells, **e,** class-switched memory, and **f,** IgM$^+$ Memory. Lin represents CD11c, F4/80, Gr-1, CD4, CD8α, and streptavidin. Representative of one experimental replicate with each point representing one mouse and error bars for SD. Statistics calculated using one-way ANOVA with Tukey's multiple comparison.

Diving into these populations, we note no differences in the population of IgM- class-switch memory B cells (**Fig. 3.4E**), leaving this difference to be primarily observed in the IgM$^+$ memory B cell group (**Fig. 3.4F**). Interestingly, these observations would seem to indicate at minimum a continued GC response in the group which did not receive tolerizing doses as well as a change in the memory compartment due to the addition of Flt3L-SA to the regime.

Using fluorescent probes of comprised of biotinylated antigen and labeled streptavidin,(2; 80; 288–294) we are also capable of probing the antigen specific B cell response, including

looking at naive B cells potentially capable of recognizing our antigen (**Fig. 3.5A**). Looking first at the B cell population binding these probes, we see a significant increase in the B cell population binding antigen in the group without tolerization and only a near significant decrease in the number of those cells when compared to the group which concurrently received Flt3L-SA (**Fig. 3.5B**). Diving deeper into this population to compare the expression of costimulatory markers between antigen specific cells and the bulk population, all treated mice show significant increases in CD40 expression on antigen specific B cells as compared to the bulk, indicative of these cells looking for T cell help to continue the immune response; however, when staining for CD80, only antigen specific B cells from mice treated with Flt3L-SA showed a no significant change in CD80 expression as compared to their cognate bulk population (**Fig. 3.5C**). Lack of CD80 signaling could indicate a hyporesponsiveness and lack of IgG secretion as well as a defect in T cell cross-talk with these cells.(295) We next looked into the dividing the population of antigen specific B cells via surface markers with naive ($CD38^{+}IgD^{+}IgM^{+}$) B cells as the first population of interest. As expected, the majority of antigen specific cells in the naive mice were of this phenotype with the only treatment that did not seem to be significantly different being the Flt3L-SA treated group. These values, however, did not translate to increased numbers of naive antigen specific cells, suggesting instead that this difference is due to expansion of other antigen specific populations (**Fig. 3.5D**). The next population we were interested in probing was that of B cells in the GC reaction (defined by expression of GL7 and loss of CD38). As previously noted, the bulk GC reaction was increased in mice without any tolerization (**Fig. 3.4B**); however, when we look at what percent of those cells stain positive with the Rasburicase probes, we note significant decreases between the challenge control and mice receiving Flt3L-SA in the tolerance regime but not those without Flt3L-SA. We do see significant decreases between all groups and the challenge only when probing what percent of antigen-stained cells display a GC phenotype as well as total counts of antigen specific GC B cells (**Fig. 3.5E**).

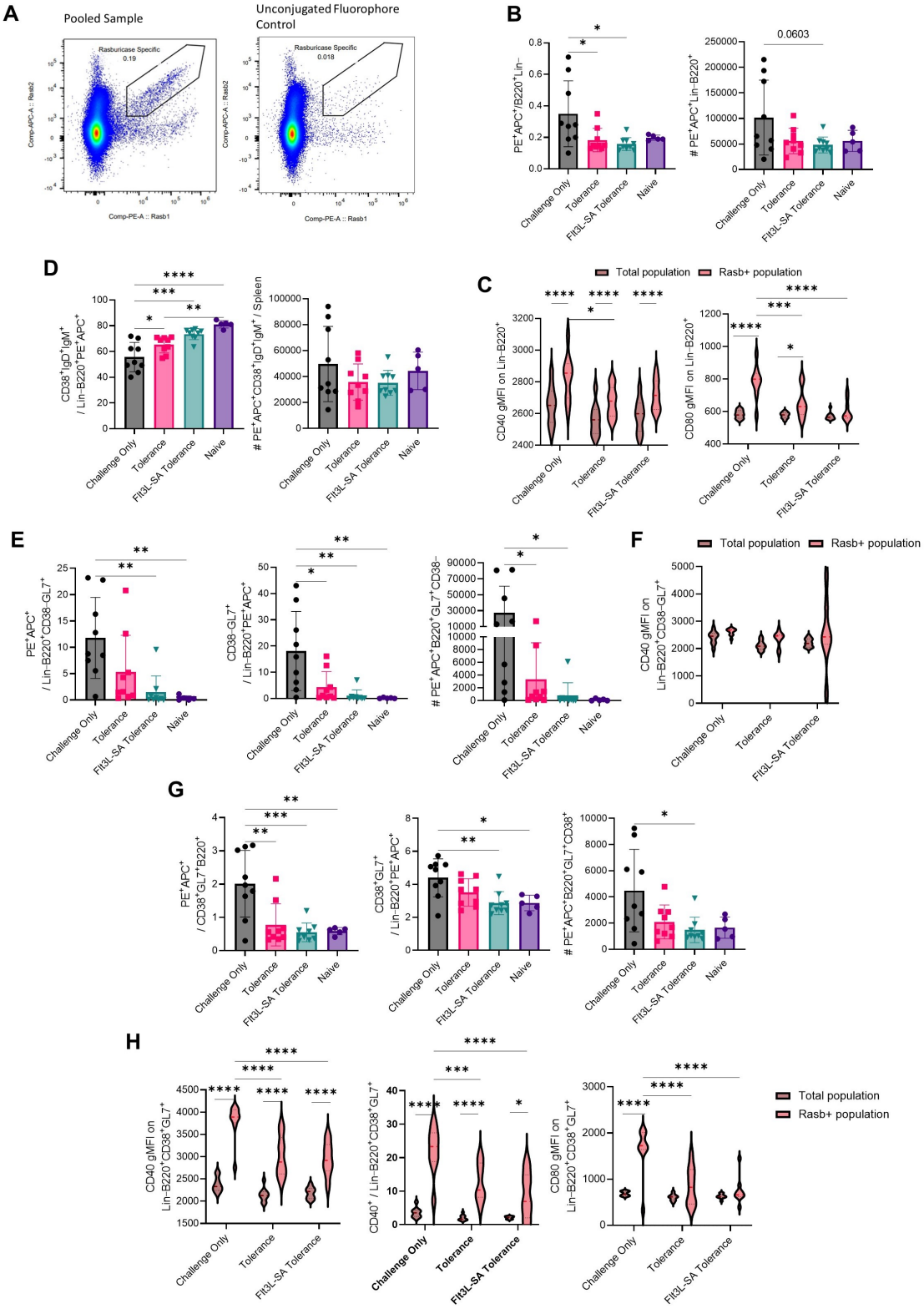

Figure 3.5: **Antigen Specific B Cell Responses and Characterization** | Figure description continued on next page.

Figure 3.5: Continued: **a,** Representative staining for Rasburicase specific B cells (left) and sample with PE- and APC-streptavidin added as non antigen controls (right). Previously gated on singles, live, Lin (CD11c, F4/80, Gr-1, CD4, CD8α, and streptavidin)-, B220$^+$. **b,** Quantification of antigen specific B cells and **c,** expression of costimulatory markers. Quantification of naive B cells as a proportion of total antigen specific cells as well as representation in the spleen. **e,** Quantification of antigen specific Germinal Center B cells and **f** expression of CD40 on those cells and the bulk population. **g,** Antigen specific memory precursor cells represented and **h** expression of costimulatory signals on those same cells. Significance for graphs in **b, d, e, and g** calculated via one-way ANOVA with Tukey's multiple comparison and each point represents one mouse with bars for mean and SD. Significance in **c and h** calculated with two-way ANOVA with Tukey's multiple comparison and **f** using a mixed effects model. gMFI represents geometric mean fluorescent intensity.

Due to the nature of these cells searching for T cell help, we see no significant differences between any groups for expression for one of the major receptors involved in this interaction, CD40 (**Fig. 3.5F**). Finally, we probed the cell population previously described as a memory precursor potentially involved in non-GC reactions, as defined by coexpression of GL7 and CD38.(287) We see significant decreases in the proportion of this population staining with antigen in all groups compared to the challenge group, but when looking at this phenotype as a proportion of all antigen specific B cell, we lose significance compared the challenge and the tolerized group. Furthermore, we only see a significant decrease in the counts of this population comparing the challenge group to the tolerization regime with Flt3L-SA (**Fig. 3.5G**). Interestingly enough, these cells all seem to be searching for T cell help, even though they have been described in the past as not requiring a GC reaction.(287) All groups demonstrate significant upregulation of CD40 on antigen specific precursors when compared to the bulk population of these cells, but the addition of a tolerization regime decreases these levels on antigen specific cells. As expected, these effects are recapitulated when looking at percentages of these cells staining positive for CD40; however, in changing focus to CD80 expression, only the group which had no tolerization demonstrates a significant increase in CD80 expression (**Fig. 3.5H**).(296) These data in hand, we seem to see some interesting effects occurring in the antigen specific B cell population related to how low-dose antigen

affects B cell education and signaling, including how these cells interact with T cells. We have additionally probed to see how these effects are modified by the addition of Flt3L-SA in the tolerogenesis regime.

We next sought to characterize how the memory component of the B cell compartment was changed by therapeutic tolerization. Overall, we see a significant decrease in the percent of total memory cells (defined by CD38 expression and the lack of IgD, signifying antigen experience) binding antigen in both groups receiving tolerization. However, we see a significant increase in the proportion of antigen specific cells staining phenotypically as memory in only the tolerized group without Flt3L-SA, and we see near significant trends in decreasing the number of antigen specific memory cells with the addition of Flt3L-SA (**Fig. 3.6A**). We are further able to divide these populations into IgM memory cells and class-switch memory (CSM) based on the expression of IgM. The IgM memory compartment shows the same trends as those seen of the total memory, with significant decreases in antigen specific IgM memory due to tolerization, but increases in the percentage of antigen specific cells with an IgM memory phenotype in the group tolerized without Flt3L-SA and no significant changes in terms of cell counts (**Fig. 3.6B**). We do however see interesting effects of antigen tolerization on markers of T cell/B cell communication in these cells. For instance, we see significant upregulation of CD40 on antigen specific IgM memory cells in the challenge group and the tolerance group without Flt3L-SA as compared to the bulk IgM memory population in those same mice; however, both groups which received antigen tolerance demonstrate a significant decrease in CD40 expression on the antigen specific compartment. In contrast to this, only mice which did not receive antigen tolerance demonstrated an increase in CD80 expression on antigen specific IgM memory cells (**Fig. 3.6C**). CSM cells, on the other hand, show significant reductions with the addition of a tolerance regime, regardless of metric (**Fig. 3.6D**), and also demonstrate increases in both CD40 and CD80 (**Fig. 3.6E**).

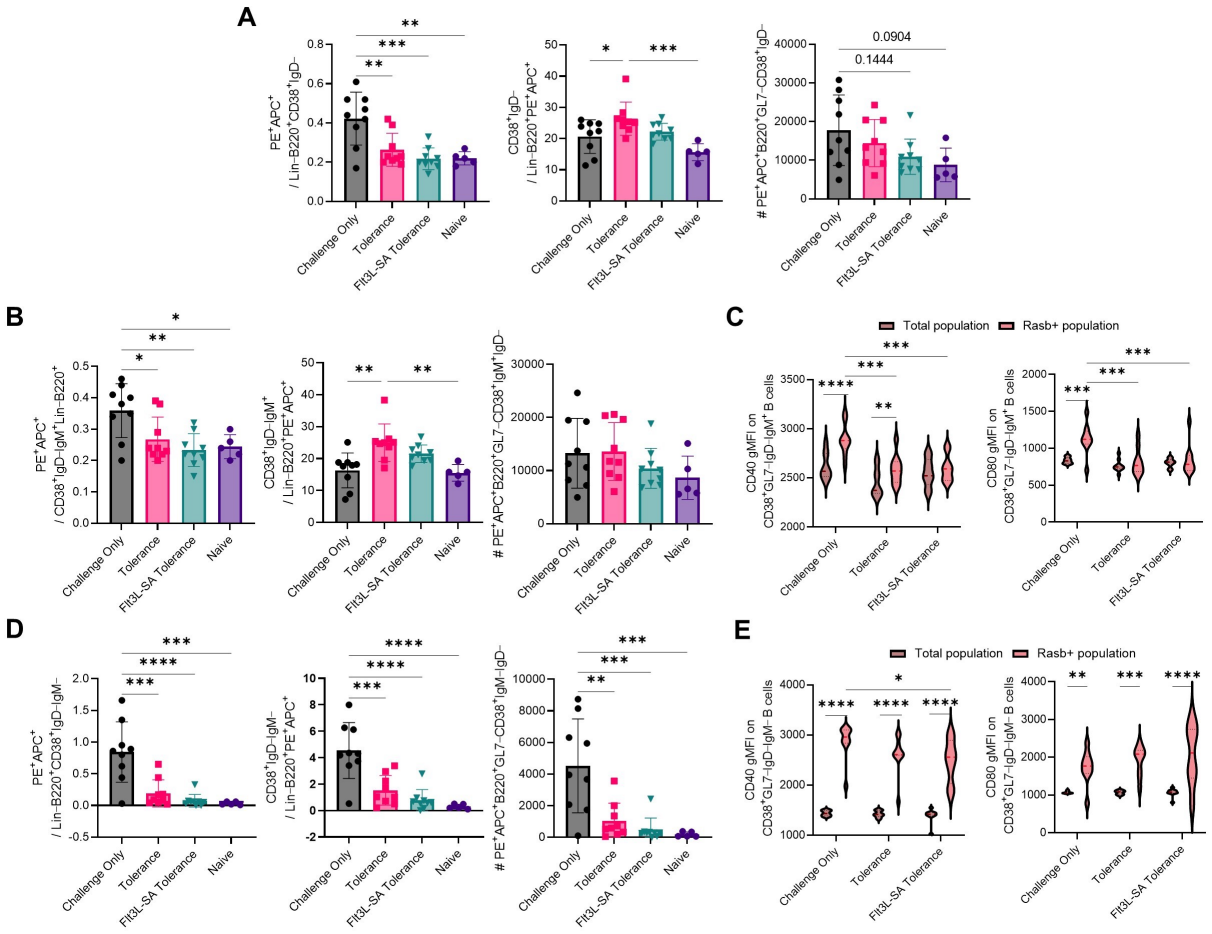

Figure 3.6: **Antigen Specific Memory B Cell Responses and Characterization | a,** Quantification of total antigen specific memory B cells. **b,** Quantification of antigen reactive IgM^+ memory B cells and **c** expression of costimulatory markers on those populations. **d** Quantification of antigen specific, class-switch memory B cells with **e** costimulatory marker expression. Each point represents one mouse with bars for mean with SD. Significance for **a, b, and d** calculated using one-way ANOVA with Tukey's multiple comparison. Significance for **c** calculated with two-way ANOVA with Tukey's multiple comparison and **e** using a mixed effects model. gMFI represents geometric mean fluorescent intensity.

In addition to looking at B cells via flow cytometry, we also performed ELISPOTs to look at antigen specific, IgG secreting cells. Due to the toxic nature of the uric acid oxidase protein, ELISPOT plates were coated with αIgG capture antibody before plating splenocytes in triplicate and 18-hour culture. Antigen specificity was then probed using the same biotinylated Rasburicase used in the fluorescent probes. Example wells can be seen in **Figure**

3.7A. Quantification of these spots shows a significant decrease in IgG secreting splenocytes with the addition of a tolerization regime (**Figure 3.7A**). Correlating these spots against previously mentioned measurements, namely antigen specific cells as a percent of class switch memory (**Figure 3.7C**) and IgG in the plasma at euthanasia as quantified by area under the curve on the ELISA (**Figure 3.7D**), demonstrates a high correlation of R^2 equal to 0.8381 and 0.7053, respectively. These strong correlations provide justification and credibility to the aforementioned measurements of antigen specific reactions.

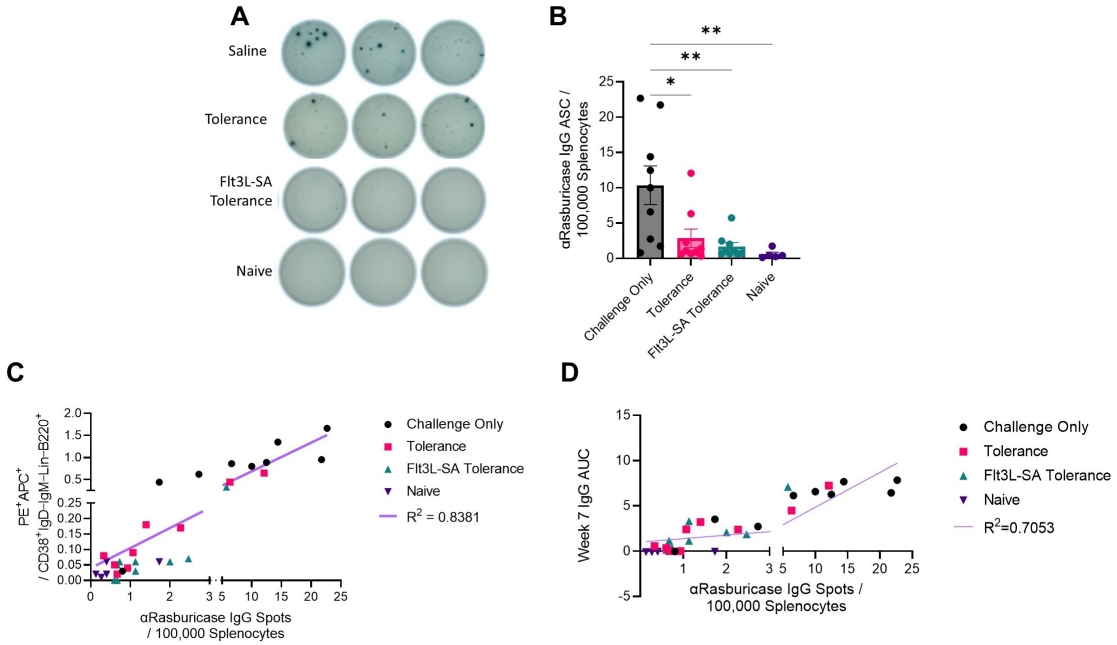

Figure 3.7: **IgG Secreting Cell Quantification via Elispot and Correlation with Previously Stated Metrics of Antigen Response | a,** Representative wells for one mouse for each treatment with 3e5 cells plated per well. **b,** Quantification of spots with each point representing the average of the three wells per one mouse. Bars represent mean with SEM. Significance calculated using one-way ANOVA with Tukey's multiple comparison. **c,** Correlation of the values from **b** against **3.6d** with a simple linear regression. **d** Correlation of values from **b** against **3.2b** with a simple linear regression.

Due to the extensive communication between T cells and B cells during immune responses to foreign proteins, we next sought to characterize the T cell response occurring at the time of the measured B cell response (representative T cell flow gating in **Fig. 3.8**); however,

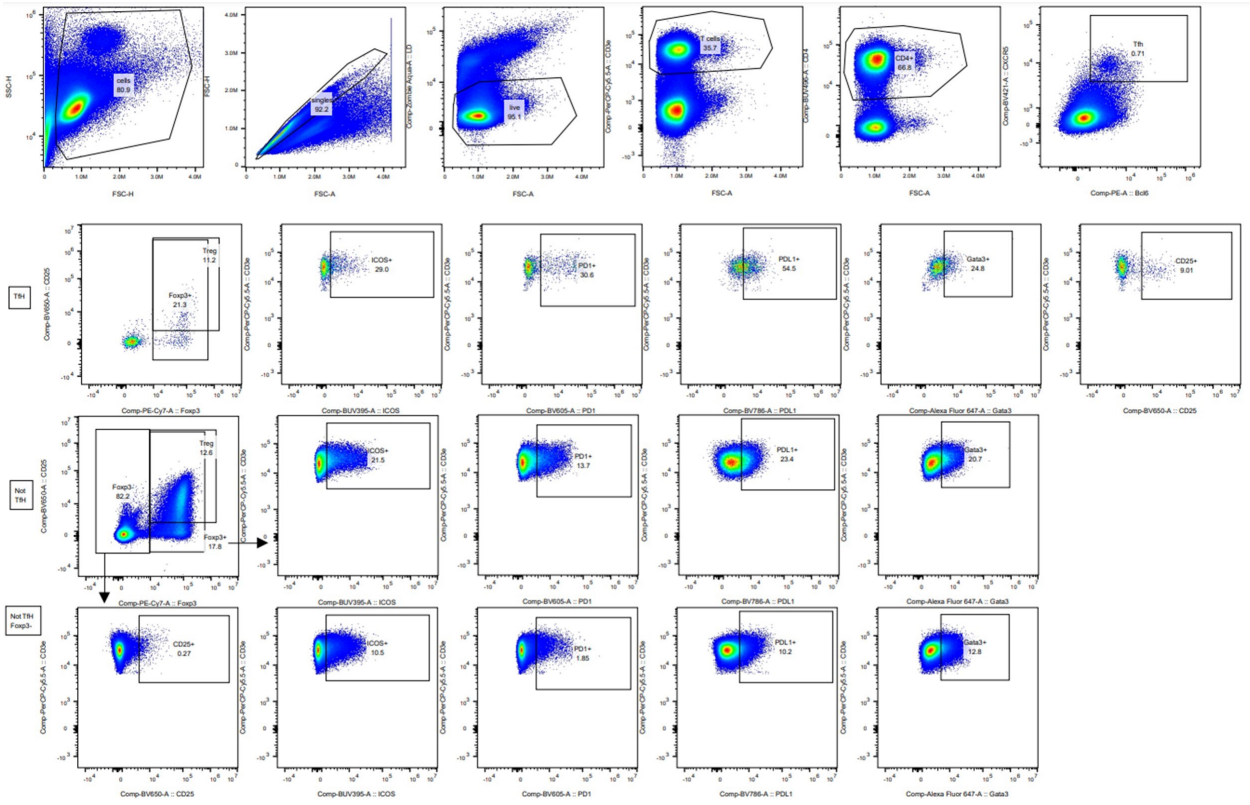

Figure 3.8: **Representative Gating for Figure 3.9**

due to the nature of T cell reactivity to only peptide:MHC complexes, we were unable to probe the antigen specific response and can only make claims based on the bulk population. To this end, we first characterized the follicular helper T cells (Tfh) in the spleens of treated mice.(44) We see a significant decrease of these cells as a percent of total CD4 T cells in only the Flt3L-SA treated group compared to the challenge group, but this effect is not seen as total counts per spleen. Further characterizing activation status of these cells, we note a significant increase in expression of ICOS and PD-1 on Tfh cells from challenge only mice in comparison to the tolerance and naive groups, but no significance in relation to the Flt3L-SA group (**Figure 3.9A**). Looking next at bonafide Tregs or Foxp3 expressing cells (no coexpression of Tfh markers), we see a near significant increase in the percentage of Tregs ($Foxp3^{+}CD25^{+}$ cells) of total $CD4^{+}$ T cells in the group which received Flt3L-SA which is also seen as total number of these cells per spleen (**Figure 3.9B**); of these cells, we see no

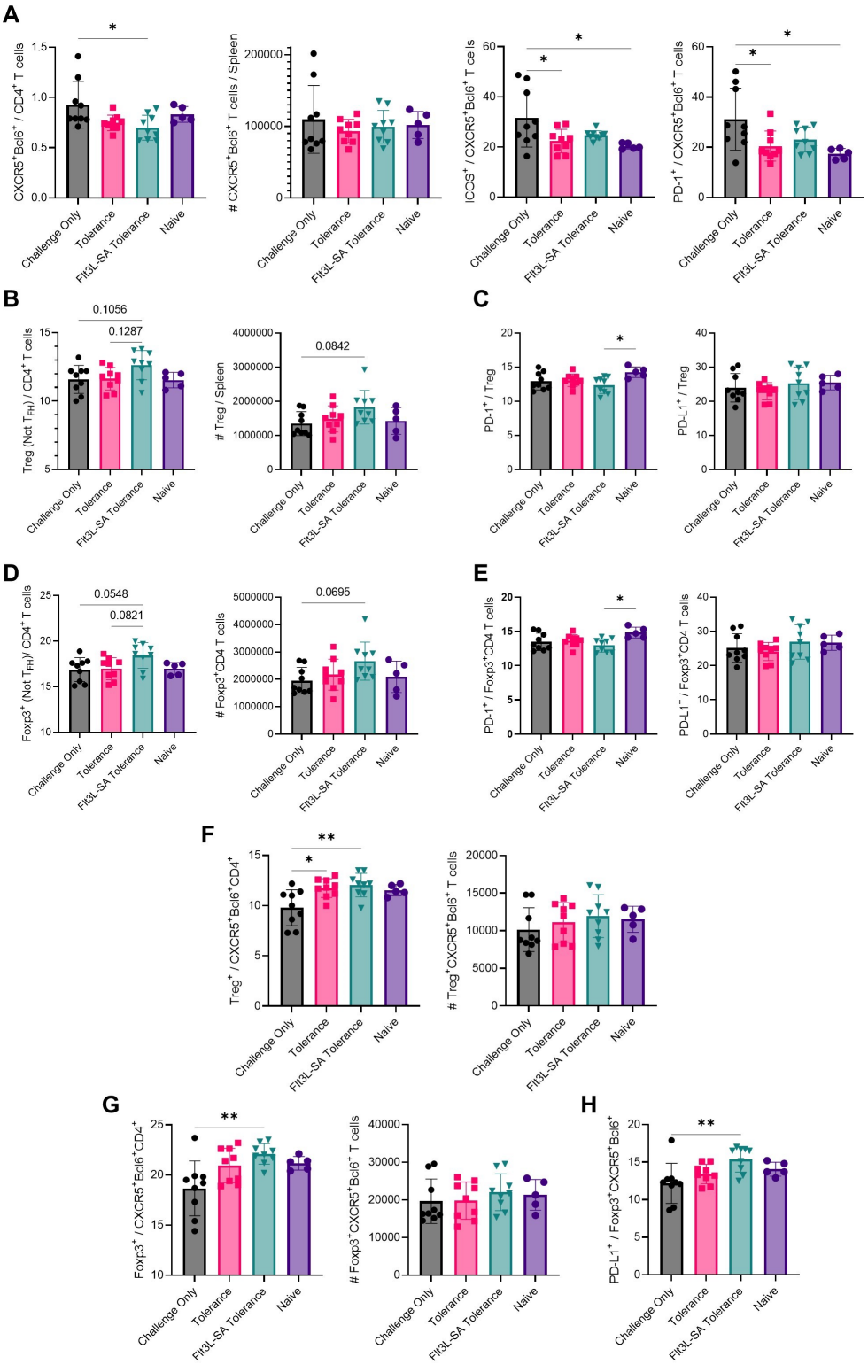

Figure 3.9: **Splenic Tfh, Treg, and Tfr Responses Following Challenge** | Figure description continued on next page.

Figure 3.9: Continued: **a,** T follicular helper cell (defined by CXCR5 and Bcl6 expression) characterization in the spleens of mice following challenge regime. **b,** Treg ($CD25^{+}Foxp3^{+}$) characterization pre-gated as not expressing CXCR5 and Bcl6 with **c** PD-1 and PD-L1 expression on the same cells. **d, e** Similar characterization as before but on $Foxp3^{+}$ cells (confirmed not expressing Tfh markers). **f** Percent of Tfh cells expressing Treg markers CD25 and Foxp3 as well as **g** expressing only Foxp3. **h,** Percentage of the cells in **g** co-expressing PD-L1. All points represent one mouse each with bars for mean with SD. Significance calculated using one-way ANOVA with Tukey's multiple comparison correction.

significant differences in expression of receptors of the coinhibitory PD-1/PD-L1 pathway (**Figure 3.9C**). In a similar way, we see near significant increases in total Foxp3 expressing $CD4^{+}$ T cells in the Flt3L-SA treated group as percentage and total count (**Figure 3.9D**), but no significant differences in PD-1 or PD-L1 expression on these cells (**Figure 3.9E**). We next probed the Tfh compartment for cells which may be considered T follicular regulatory (Tfr), which express markers of Tfh lineage as well as those of the Treg lineage. In this, we see significant increases in the percent of Tfh cells expressing Foxp3 and CD25 in mice treated with a tolerizing regime compared to mice receiving a challenge alone; however, this effect is not seen in total count (**Figure 3.9F**). If we only use Foxp3 to distinguish these cells, we only see a significant increase in mice treated with Flt3L-SA compared to those which received a challenge, but again this effect is lost in total counts (**Figure 3.9G**). It has been reported that these cells may execute a suppressive function via expression of PD-L1 which then interferes with PD-1 signaling on reactive B cells, thus disrupting the germinal center response.(297; 298) When diving into PD-L1 expression on these $Foxp3^{+}$ Tfr cells, we only see a significant increase in the percent of these cells expressing PD-L1 in mice treated with Flt3L-SA compared to those which received the challenges (**Figure 3.9H**).

3.3.2 Discussion

In this section we've described the antidrug antibody response and proposed two ways to help mitigate this reaction in a model of enzyme replacement therapy. As expected and as

previously published,(80; 299) low dose antigen alone reduces antibodies generated against the foreign protein, but in these cases, only mice which concurrently received Flt3L-SA had a significant reduction (**Fig. 3.2A, B**). Furthermore, by looking at which mice had visible anaphylaxis-like reactions after treatment and cross-referencing that to the antibody levels in the plasma before that treatment, we are able to determine a level of antibody in the blood which is likely pathogenic to cause these symptoms (**Fig. 3.2C**). Considering then a titer greater than or equal to four as a pathogenic seroconversion event, following treated mice to seroconversion we see significant benefit to the addition of Flt3L-SA to the treatment regime (**Fig. 3.2D**). Benefit is also observed when looking just at how many challenge doses it takes to show symptomatology where one-third of treated mice showed benefit of low-dose antigen and that value is doubled with the addition of Flt3L-SA (**Fig. 3.2E**). Interestingly, all of these reactions occurred while the mice were actively under the influence of a powerful antihistamine, suggesting that these pathologies were not in fact due to histamine but some other pathway, potentially that of a Type III hypersensitivity involving antibody complexes, complement fixation, and serum sickness pathology.(300) Alternatively, anaphylactic reactions have been observed as being caused by crosslinking of FcγR on neutrophils and basophils, especially with regards to anti-drug reactions.(301–307)

We next looked at cellular factors in the spleen which may contribute to these differences in antibody levels. While no differences were observed in total, bulk B cells (**Fig. 3.4A**), we see a significant increase in GC B cells in the challenge only group (**Fig. 3.4B**), indicative of an ongoing germinal center and potentially an affinity maturation response.(308; 309) However, in the bulk compartment we note factors that may signify that the low-dose antigen group may bypass the GC reaction through inducing a precursor fate which goes straight to IgM memory (**Fig. 3.4C-F**).(287) We were next able to probe the antigen specific B cell compartment using fluorescent probes made in house, as previously published.(2; 80; 288–294) With this technology, we observe no differences in numbers of antigen specific B cells

between either tolerance regime (**Fig. 3.4B**); however, there may be a difference in how these cells are attempting to talk to T cells evidenced by differences in expression of CD40 and CD80 between the overall B cell population and the rasburicase staining population (**Fig. 3.4C**). In fact, literature suggests that CD80 expression on B cells is involved in initiation and maintenance of GC reactions, Tfh development, as well as formation of plasma cells,(310; 311) potentially signifying that the addition of Flt3L-SA to the therapeutic regime may impair Tfh development and thus provide for a longer lasting benefit than low-dose antigen alone. In accordance with this, we also note a near significant increase in Foxp3 expressing cells as well as $Foxp3^{+}CD25^{+}$ conventional Tregs in mice treated with Flt3L-SA, even long after expected Treg increases from the Flt3L-SA (**Fig. 2.16A**). In addition, while we only see a slight reduction in Tfh cells with the Flt3L-SA treatment (**Fig. 3.9A**), of the cells there a significant proportion of them seem to maintain a regulatory fate as determined by expression of Foxp3 (**Fig. 3.9G**) as well as expression of PD-L1 on these cells (**Fig. 3.9H**). (312–314)

In addition to interrupting the GC reaction, we notice differences between mice tolerized with antigen alone versus those tolerized with Flt3L-SA in the regime when looking at the antigen specific "precursor" and IgM memory populations. Of the former population, we see the addition of Flt3L-SA to the regime significantly impacts the number of these cells present in the spleen, while the ones that persist seem to be phenotypically similar to those from mice which received only the low-dose antigen (**Fig. 3.5G, H**). Furthermore, we provide evidence that low-dose antigen alone may transiently induce tolerance due to the generation of IgM memory cells (**Fig. 3.6B**), but these cells are still capable and actively searching for T cell help as evidenced by expression of CD40 (**Fig. 3.6C**). Literature suggests these cells are capable of re-seeding GC responses upon secondary challenge and may in fact boost such a reaction.(287) Of additional interest to this project in particular, IgM memory cells have been implicated in broad cross-reactive responses against foreign glycan epitopes,(315)

and considering that rasburicase is produced in yeast, there is a potential for these B cell reactions to be against non-protein components of this drug.

Overall, we herein provide evidence of enhanced efficacy of antigen-based therapies by co-dosing Flt3L-SA during tolerogenesis and prevention of pathogenic levels of antidrug antibodies to a known immunogenic, FDA approved enzyme drug. We also provide potential evidence of tolerogenic mechanisms of low-dose antigen, an understudied field on its own. One flaw in this particular model is that mice in fact produce a functional uric acid oxidase enzyme (humans do not have a functional enzyme), and as such we are unable to test whether or not the antibodies generated are neutralizing and thus would prevent enzyme activity *in vivo*. Methods to address this particular challenge and others related to antigen choice will be addressed in chapter 4. Additionally, due to the activity of the enzyme and how understudied this process is, it is difficult to measure the antigen-specific T cell response. Prolonged culture with this enzyme causes cellular death through the production of hydrogen peroxide, making restimulation of T cells *ex vivo* a particular challenge, and as such we were unable to probe the antigen-specific T cell response in this work.

3.4 Combination of Flt3L-SA with Oral Antigen Modifies Primary Immune Education

Considering the massive expansion and effects seen in the gut draining lymph nodes in previous experiments with Flt3L-SA, we hope to utilize this platform to enhance oral immunotherapies (OIT). Peripheral tolerance induction via oral antigen exposure has many knobs and dials to fine tune the immune response following antigen exposure. Strategies involving modifying the antigen dose (high vs. low), (316–321) microbiome and metabolite composition,(322–328) and antigen delivery method (329–336) have all influenced our understanding of how oral tolerance induction may occur.(337) Such studies have recently led to the first FDA approval of a composition for oral tolerance induction to peanut antigens

in peanut-allergic individuals. However, even with research on this topic stemming from the beginning of the 20th century,(338; 339) the exact mechanism by which the approved Palforzia™works is unknown and the induced tolerance seems transient, at best.(90; 340; 341) In one recent study, it was found that oral antigen enforces an anergic-like state on reactive CD4+ T cells with the capability to self-renew this population as well as to go down a Treg fate pathway.(342) In such a way, we use this section to discuss preliminary work focused on enhancing such an effect to prevent future reactions to potential antigens, be it autoimmune or reactions to foreign antigens.(343) Additionally, we hope that research into how to change other aspects of the gut system, such as APC and Treg presence and state, may provide further insight into how oral tolerance is induced and maintained (especially considering the importance of $CD103^+$ cDC1 in inducing iTregs in the gut)(344), with the intention to provide even better routes for oral immunotherapies.

3.4.1 Results

To test the potential ability Flt3L-SA to synergize with oral antigens therapies for tolerance against future, non-oral antigens, we developed the model timeline denoted in **Figure 3.11A**. Briefly, B6 mice received adoptive transfer of ovalbumin reactive OTI and OTII cells the day prior to Flt3L-SA treatment with a repeated administration of Flt3L-SA one week later. Five days following the initial Flt3L-SA treatment, 50mg of ovalbumin was dissolved in 200μL of PBS before intra-gastric gavage with a repeated dose on day 6. To check activity as well as antigen experience, mice were bled on day 9 for flow cytometry before immunization on day 20 with ovalbumin in complete freund's adjuvant (CFA) and euthanasia on day 25. Upon euthanasia, vaccine draining lymph nodes, intestine draining lymph nodes, and spleens were taken for analysis (representative flow gating seen in **Fig. 3.10**). First analyzing the white blood cells from day 9, we see similar DC expansion in the blood in both groups receiving Flt3L-SA, as expected; however, we see a significant increase in expansion

of antigen specific $CD4^+$ T cells (OTII) and $CD8^+$ T cells (OTI) with increased DC presence due to Flt3L-SA (**Fig. 3.11B**). Upon euthanasia after challenge, our main priority was to look at OTI and OTII presence in each of the relevant lymphoid organs as well as Treg differentiation of the OTIIs. Analyzing the vaccine draining site for cellularity first, we only note a significant decrease in OTI proliferation in the group that received OIT alone, whereas the significance was lost with the addition of Flt3L-SA to OIT. For OTII recovery, we note no significant differences with any treatment, which may ultimately be due to variability in the measurements (**Fig. 3.11C**). As for the gut draining lymph nodes, we see decreases in CD8 T cell recovery in both groups receiving OIT, but only have statistical significance without Flt3L-SA (**Fig. 3.11D**). Finally, in the spleen we see no differences nor trends in either T cell compartment (**Fig. 3.11E**). Finally, we sought to compare the bulk Treg compartment to that of the antigen specific, OTII compartment to see if there was a preferential skewing via oral antigen exposure. In the challenge dLN, we note no differences in bulk nor antigen specific $CD4^+$ T cells between any treatment (**Fig. 3.11F**); however, we see non-significant increases in percentage of OTII cells in the gut dLN with a Treg phenotype in the groups which received OIT without differences in the bulk compartment (**Fig. 3.11G**). More systemically, we notice no differences in either metric of Treg characterization in the spleen (**Fig. 3.11H**).

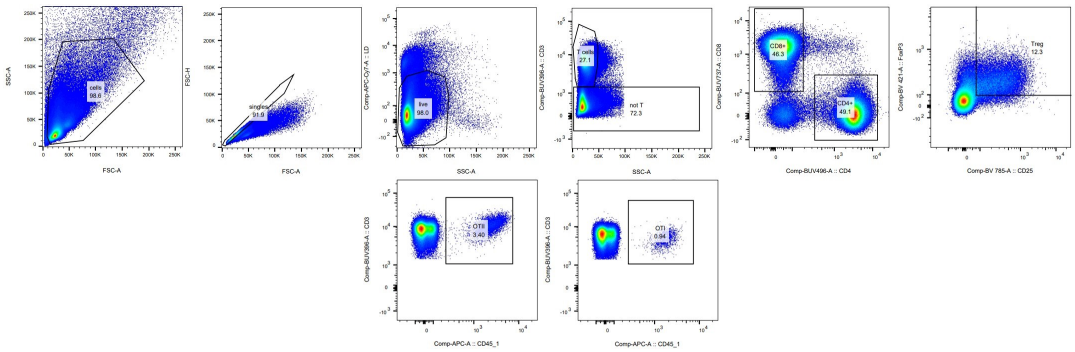

Figure 3.10: **Representative Gating for Figure 3.11 |** Flow gating for pooled sample from experiment in Figure 3.11

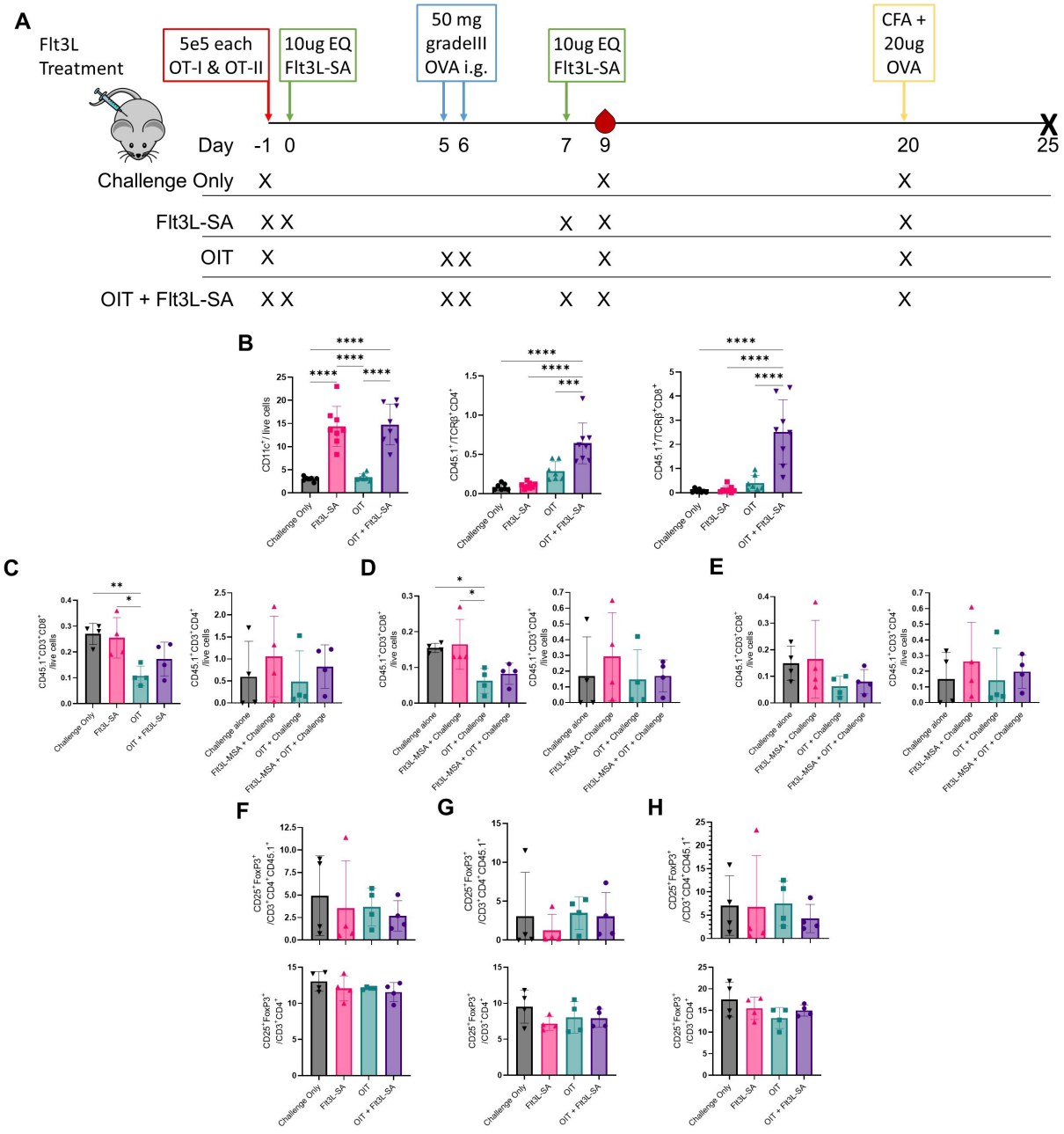

Figure 3.11: **Flt3L-SA with Oral Antigen Increases T Cell Education | a,** Representative schematic of the treatment regime with group names corresponding to group titles in the following graphs. **b,** Cellular measurements found in the blood on day 9, three days post antigen exposure. **c-e** OTI and OTII cellularity in the (**c**) vaccine draining lymph nodes, (**d**) intestine draining lymph nodes, and (**e**) spleen. **f-h,** Treg cellularity as proportion of antigen specific cells (top) or total T cells (bottom) for (**f**) vaccine draining lymph nodes, (**g**) intestine draining lymph nodes, and (**h**) spleen. Data from one experiment with each point representing a single mouse with n=8 per group for **a** and n=4 for **b-h** and bars for mean with SD. Significance calculated with one-way ANOVA with Tukey's multiple comparison.

We left this experiment with more questions than answers and thought to modify the set up ever so slightly to try and address the large within group variability and to probe deeper as seen in **Figure 3.13A**. We first wished to determine if we could see differential effects due to gastric dose and chose to give two different doses of ovalbumin, 10mg and 50mg. Additionally, we worried that the CFA vaccination would be an unrealistic standard to compare to in that inflammation for an autoimmune condition or for allergic responses would never reach such a state as CFA generates. To address this, we modified the vaccine to adjuvant with LPS rather than CFA. As before, we checked white blood cells three days following gastric antigen (representative gating for this experiment can be found in **Fig. 3.12**). Herein, we still see significant DC expansion in all groups receiving Flt3L-SA. Interestingly, we only see significant increases in OTI proliferation in groups receiving both oral OVA and Flt3L-SA, while OTII proliferation shows increases in a dose-dependent manner regardless of the addition of Flt3L-SA (**Fig. 3.13**B). Upon euthanasia, we again took vaccine dLN, intestine dLN, and spleens for CD4 T cell phenotype. Looking first at the vaccine dLN, we note trends toward decreased OTII cellularity in groups receiving oral antigen. Characterizing the OTII cells, we see that oral antigen significantly increases an "anergic" phenotype – $FR4^{+}CD73^{+}$ – on these cells, in accordance with published literature; (326; 342; 345) there are also potential trends in increasing the percent of Tregs developed due to oral antigen as well as significant increases in tox expressing CD4 T cells (**Fig. 3.13C**). In a similar manner to the vaccine dLN, we see evidence of a lack of proliferation of the OTII in the intestine dLN in mice which received OIT, but none are statistically significant from those which did not have a tolerization regime. Characterizing these OTII cells, we see no differences in Treg induction nor CTLA-4 expression, however, we again note increases in potentially anergic cells by staining for CD73. Further interesting, we seem to see an inverse dose-effect in the OTIIs in terms of PD-1 expression where there is a significant decrease in cells expressing PD-1 when given either 50mg of OIT or 10mg of OIT with Flt3L-SA. While not significant,

we see potential trends of increasing cells expressing the exhaustion transcription factor TOX if they had previously been treated with Flt3L-SA (**Fig. 3.13D**). Finally, we again looked at the splenic response to see if OTIIs were systemically different after OIT. Again, we saw no significant differences in numbers of OTII cells in the spleen regardless of treatment; however, this is the first organ where we do in fact see significant changes in OTII education to a Treg fate. Furthermore, we do again see significant differences in the amount of cells displaying an anergic-like phenotype. Also interesting, we note an increase in OTII cells displaying a gut-homing phenotype by expressing the $\alpha 4\beta 7$ integrin in groups which received OIT, but significance against challenge alone is only achieved with the high-dose of oral antigen with Flt3L-SA (**Fig. 3.13E**).

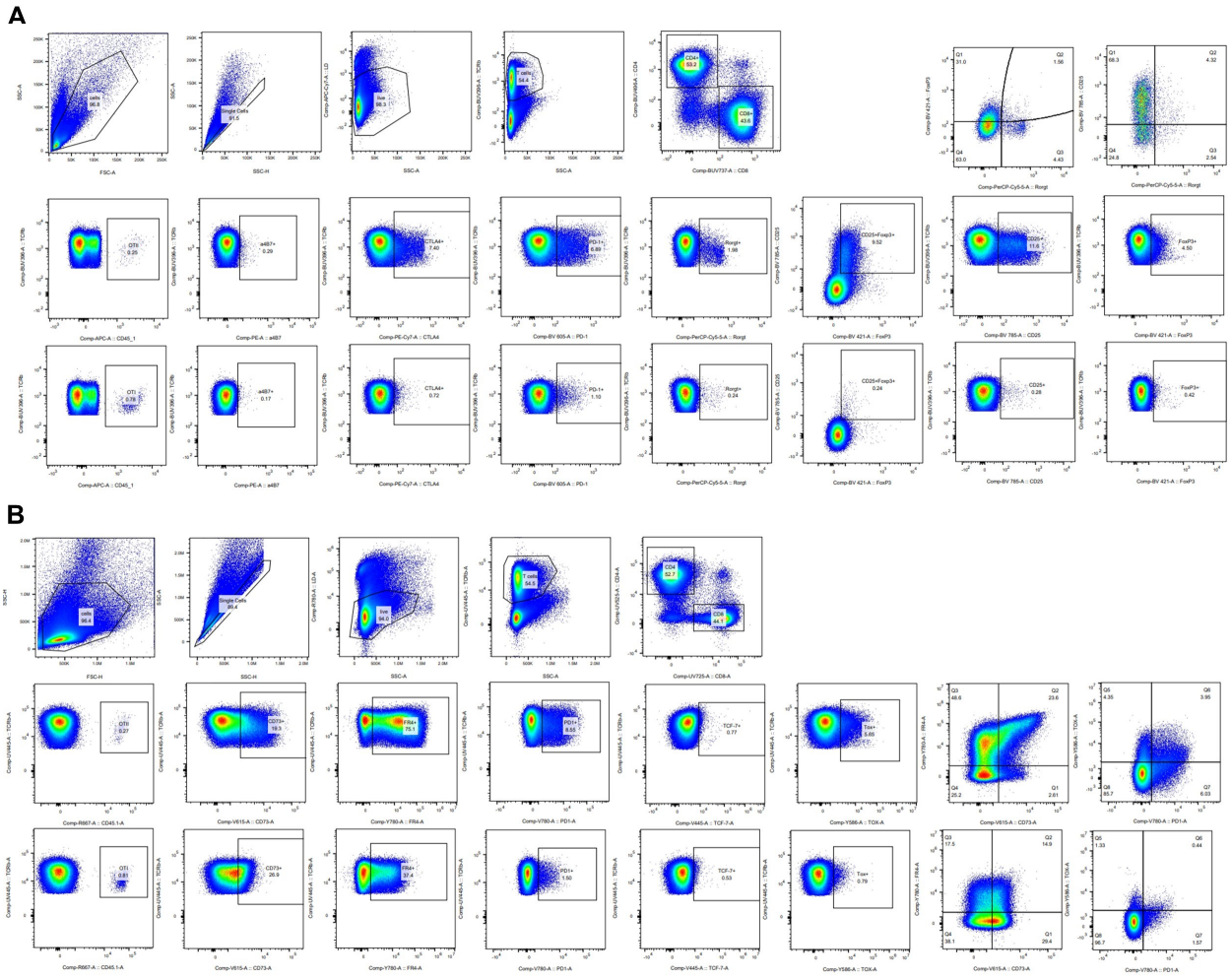

Figure 3.12: **Representative Gating for Figure 3.13 | a** Treg gating panel and **b** exhaustion and anergy staining.

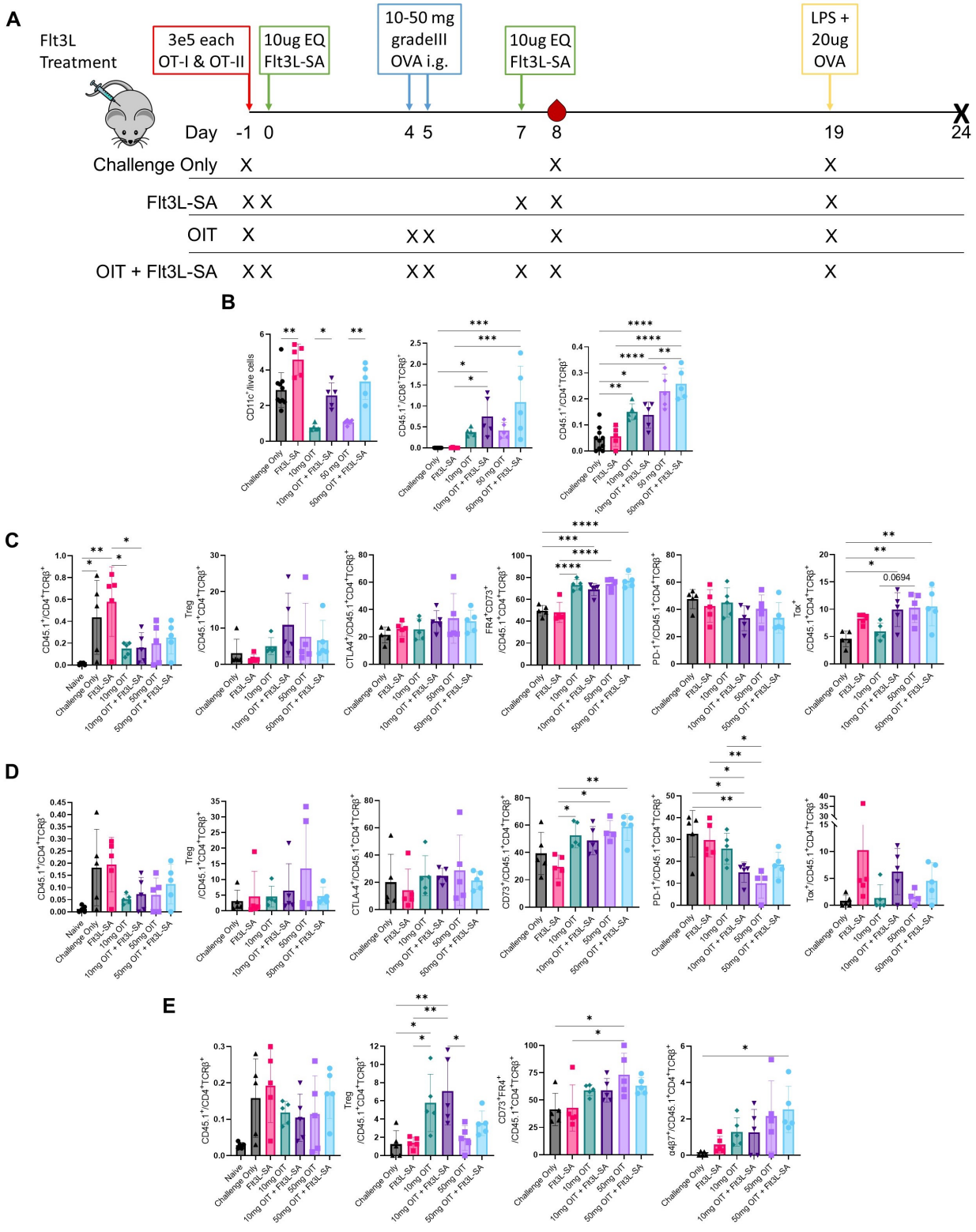

Figure 3.13: **Flt3L-SA Increases Initial T Cell Priming while OIT Changes Displayed CD4$^+$ T Cell Phenotype** | Continued on next page.

Figure 3.13: Continued: **a,** Representative schematic of the treatment regime with group names corresponding to group titles in the following graphs. **b,** Cellular measurements found in the blood on day 9, three days post antigen exposure. **c,** OTII representation in the vaccine dLN (left) and percent representation of different cell phenotypes within that population. **d,** OTII representation in the intestine dLN (left) and percent representation of different cell phenotypes within that population. **e,** OTII representation in the spleen (left) and percent representation of different cell phenotypes within that population. Treg in **c-e** stands for $CD25^{+}Foxp3^{+}$ cells. Data from one experiment with each point representing a single mouse with n=5 per group and bars for mean with SD. Significance calculated with one-way ANOVA with Tukey's multiple comparison.

3.4.2 Discussion

In these experiments, we have noted benefit of the addition of Flt3L-SA to an OIT regime in at least the initial education of T cells, based on exposure and proliferation of reactive T cells in the blood following oral antigen (**Figures 3.11B and 3.13B**. Further characterization of these cells at a peripheral site demonstrates a lack of recall response even with non-oral antigen (**Fig. 3.11C, D**). Although, in the initial experiment, no benefit was observed in terms of Treg differentiation (**Fig. 3.11F-H**). Upon follow up experiment, we note increased OTI proliferation after oral antigen with the combination with Flt3L-SA, even inducing a dose dependence; however, OTII proliferation seems to always have a dose dependence with no added benefit with Flt3L-SA (**Fig. 3.13B**). This potentially suggests that Flt3L-SA increases antigen presentation capacity to such $CD8^{+}$ T cells, which falls in-line with both the preferential cDC1 expansion and the known capabilities of cDC1s to cross-present exogenous antigen to $CD8^{+}$ T cells. (206; 346) The lack of dependence of $CD4^{+}$ T cell proliferation on Flt3L-SA administration may be because of the higher capacity and increased number of cells capable of presenting antigen to $CD4^{+}$ T cells.

Following this to characterize the $CD4^{+}$ T cell compartment after peripheral challenge, we see a systemic decrease in recall response as well as the induction of an anergy-like state, as previously recorded.(342) Additionally, we provide evidence of Flt3L-SA aiding in

generating TOX$^+$ CD4$^+$ T cells in lymph nodes (**Fig. 3.13C, D**. Literature suggests these cells may generate a Tfh phenotype or may generate a more suppressive environment through IL-4 expression or in general forcing immunosuppression as is seen in the bone marrow and tumors.(347; 348)

These data provide interesting preliminary results to see effects of oral therapy in addition to Flt3L-SA treatment. While we chose to focus on CD4$^+$ T cell education and differentiation, considering the effect we see on CD8$^+$ T cells at the initial priming it may be more interesting to focus on CD8$^+$ T cell mediated diseases as we may be able to enforce an exhausted phenotype on these cells. Potentially, pulling these cells away from the tissue they are destroying and bringing them to a more inert and suppressive site, such as the gut, may improve symptomatology. Alternatively, as discussed in 4, we are also actively seeking whether we are capable of modifying the immune response – B cell and T cell mediated – via OIT away from that of a pathogenic state and towards one with less active symptoms.

3.5 Materials and Methods

Animals

C57BL/6 and BALB/c mice were purchased from Jackson Labs and congenically marked CD45.1 OT-I and OT-II mice were bred in-house and housed at the University of Chicago Animal Facility. All procedures were approved by the University of Chicago Institutional Animal Care and Use Committee and performed on protocols 72449 and 72459. Injections were diluted to the appropriate concentration in 50μL sterile saline for S.C. injections or 100μL for I.V. or I.P. treatments. Mice were euthanized via CO2 asphyxiation as approved by UChicago ARC.

Rasburicase Preparation

Rasburicase (Elitek) was prepared according to manufacturers protocol. Briefly, the powdered protein and supplied buffer was brought to room temperature before the addition of 1mL of buffer to each 1.5mg puck of supplied protein under aseptic conditions. Protein was then allowed to dissolve at RT with minimal disturbance for 30 minutes before removal from the glass vial. Protein was then either aliquoted and directly frozen, or directly injected I.V. During the tolerance induction regime, 5μg of protein was diluted into 100μL of sterile saline, while during the challenge regime 66μL of the Rasburicase solution was directly injected into the mice.

Antihistamine Preparation

Diphenhydramine was purchased as a solution from the University of Chicago veterinary office at a concentration of 50mg/mL and protected from light and oxygen. On the day of challenge, a diphenhydramine solution was prepared at 1mg/mL in sterile saline before injecting 50μL S.C. into the flank of the mouse 45 minutes prior to challenge.

Blood Collection

Blood was collected via submandibular venous puncture into Lithium-Heparin coated tubes (Starstedt). For plasma collection, tubes were then centrifuged at 10,000G for 10' before collection of the plasma and subsequent freezing into PCR tubes. For blood samples requiring flow staining, blood was diluted into PBS before centrifugation at 2000G for 5'. Samples were then subjugated to multiple rounds of ACK (Gibco) treatment until the pellet was no longer red. Samples were then stained as previously described.

Anti-Rasburicase Titering ELISA

High-bind plates (Corning 9018) were coated by incubating overnight at 4°C with 10μg/mL rasburicase in 50nM sodium bicarbonate buffer at pH 9.2. Following coating, plates were washed in PBST before blocking in 1x casein buffer (Thermo) for 2 hours at RT. Plasma was then serially diluted into the blocking buffer in 10-fold dilutions beginning at 100x (titer of 2) and finishing at a titer of 9. Blocked wells were washed and then sample was added and incubated for one hour. Sample was removed before addition of HRP-conjugated anti-mouse IgG (Southern Biotech) diluted 5000x in blocking buffer and incubated for 1 hour. Wells were then washed before the addition of TMB (Millipore ES-001). TMB was incubated while protected from light for exactly 18', at which point the reaction was stopped using 10% Sulfuric Acid. Quantification occurred by reading the absorbance at 450nm and 570nm. To quantify a titer as positive, each well had the 570nm reading subtracted from the 450nm reading. Following this, background absorbance and standard deviation thereof was calculated using at least 3 blank wells for each plate. Test wells then had average background subtracted as well as 4x the standard deviation. Any well with a value greater than 0.01 was considered a positive titer, and the final positive titer was reported. Any mice in which the titer of 2 showed no signal were reported as a titer of 0. AUC was calculated as the Riemann sum of the titer vs background-subtracted absorbance curve for each time-point.

Fluorescent Antigen Probe Production

Rasburicase was biotinylated using EZ-link NHS-biotin (Thermo Scientific). Unreacted NHS-biotin was removed using Zeba spin desalting columns, 7kDa MWCO (Thermo Scientific). The extent of biotinylation was measured using the QuantTag Biotin Quantification kit (Vector Laboratories) to ensure 1:1 molar ratio of rasburicase and biotin. Biotinylated rasburicase was reacted for 20 min on ice with 4:1 molar ratio of biotin to streptavidin-conjugated PE or streptavidin-conjugated APC (Biolegend). Streptavidin-conjugated FITC

(BioLegend) was reacted with excess free biotin to form a non-antigen-specific streptavidin probe as a control. Cells were stained for flow cytometry with all three streptavidin probes at the same time as other fluorescent surface markers at a volumetric ratio of 1:400 for the PE and APC probes, and 1:100 for the FITC probe.

Single Cell Preparation and Flow Cytometric Staining

Single cell preparation and flow cytometry staining were performed as described in section 2.5 with the modification of plating 2-4 million cells before flowing on either a 5 laser BD fortessa or a 5 laser Cytek Aurora.

Anti-Rasburicase ASC ELISPOT

Plates (Millipore MAIPSWU) were activated using 70% EtOH for exactly 2 minutes before 3 washes with sterile PBS. Anti-mouse IgG capture antibody (Mabtech) diluted to 15μg/mL in PBS was then added to each well before incubation overnight at 4°C. The next morning, plates were washed with complete media then and incubated at 37°C for at least 2 hours to block. Plates were then washed before adding 300,000 or 150,000 splenocytes/well in triplicate (6 wells/mouse, 3/condition). Plates were incubated at 37C for 18 hours without movement or jostling. After the incubation, plates were washed with PBS and biotinylated-rasburicase (as prepared for the fluorescent antigen probes) was added at 1ug/mL of PBS + 0.5% BSA (w/v) and incubated for 2hr at RT. Plates were then washed with PBS before the addition of 1x streptavidin (Biolegend) in PBS + 0.5% BSA (w/v) and incubation for 1hr at RT. Finally, plates were washed before the addition of TMB (Mabtech) and incubated until spots were visible (10 minutes) followed by quenching the reaction by aggressive washing with DI water. Plates were then dried in the dark before imaging using a CTL ImmunoSpot Analyzer to image, count spots, and perform quality control.

OT-I and OT-II Isolation and CFSE Labeling

Splenocytes were isolated as previously described and red blood cells lysed before $CD4^+$ or $CD8^+$ T cell isolation using STEMCell isolation kits (19852 and 19853) according to manufacturer instructions. Isolated cells were then labeled with CFSE at 5μM prior to resuspension to the desired number of cells in 100μL of sterile, incomplete DMEM for intravenous injection via tail vein.

Antigen Gavage

Grade III Ovalbumin (Sigma) was dissolved to the desired concentration in sterile PBS prior to sterile filtration through a syringe-driven 0.22μm filter. For I.G. delivery, 200μL of solution was then gavaged into each mouse at the indicated timepoints.

OVA Challenges

Endofit Ovalbumin (Invivogen) was diluted to 400μg/mL in sterile saline. For challenges involving CFA, the OVA solution was added in 100μL increments to full volume of CFA, vortexing for 30 seconds between each addition until the full volume was added to reach a 1:1 (v/v) mixture of OVA with CFA. After the final vortexing step, the solution was then vortexed on its side for at least 1 hour on maximum speed to ensure stable emulsification. The emulsification was then injected into anaesthetized mice, at 25μL/hock (100μL total volume, 20μg total OVA). LPS-based challenges used 20μg OVA mixed with 50ng LPS (Sigma) in sterile saline to 100μL total volume (25μL/hock).

3.5.1 Antibodies Used in Chapter 3

Table 3.1: Antibodies, Vendors, Localization, and Dilutions for Chapter 3.

Antigen	Color	Vendor	Catalog	Localization	Staining Dilution
$\alpha 4\beta 7$	PE	Biolegend	120606	Surface	1:200
B220	BUV 496	BD	612950	Surface	1:200
Bcl6	PE	BD	561522	Nuclear	1:100
CD11c	FITC	Biolegend	117306	Surface	1:100
CD11c	BV 421	BD	562782	Surface	1:200
CD138	BV 605	Biolegend	142531	Surface	1:200
CD25	BV 650	Biolegend	102038	Surface	1:200
CD25	BV 605	Biolegend	102035	Surface	1:200
CD25	BV 785	Biolegend	102051	Surface	1:200
CD3ϵ	PerCP-Cy5.5	Biolegend	100328	Intracellular	1:400
CD3ϵ	BUV 395	BD	563565	Intracellular	1:200
CD38	APC-Cy7	Biolegend	102727	Surface	1:200
CD4	FITC	Biolegend	116003	Surface	1:100
CD4	BUV 496	BD	612952	Surface	1:200-400
CD40	BUV 615	BD	751646	Surface	1:50
CD40L	FITC	Biolegend	157005	Surface	1:200
CD45.1	APC	Biolegend	110720	Surface	1:200
CD73	BV 605	Biolegend	127215	Surface	1:200
CD8α	FITC	Biolegend	100706	Surface	1:100
CD8α	BUV 737	BD	612759	Surface	1:200
CD80	BV 650	Biolegend	104732	Surface	1:50
CTLA4	PE-Cy7	Biolegend	106313	Intracellular	1:200
CXCR5	BV 421	Biolegend	145512	Surface	1:100

Continuation of Table 3.1					
Antigen	Color	Vendor	Catalog	Localization	Staining Dilution
F4/80	FITC	Biolegend	123108	Surface	1:100
Foxp3	PE-Cy7	Invitrogen	25-5773-82	Nuclear	1:50
Foxp3	PE	BD	560414	Nuclear	1:200
Foxp3	BV421	Biolegend	126419	Nuclear	1:200
Fr4	PE-Cy7	Invitrogen	25-5445-80	Surface	1:200
Gata3	AF647	Biolegend	653810	Nuclear	1:10
GL-7	PerCP-Cy5.5	Biolegend	144610	Surface	1:200
Gr-1	FITC	Biolegend	108406	Surface	1:100
ICOS	BUV 395	BD	565885	Surface	1:100
IgD	BUV 395	BD	564274	Surface	1:200
IgM	BV786	BD	743328	Surface	1:50
Ki67	PE-Cy7	Invitrogen	25-5698-80	Nuclear	1:200
PD-1	BUV 737	BD	568362	Surface	1:200
PD-1	BV 605	Biolegend	135219	Surface	1:200
PD-1	BV 786	Biolegend	135225	Surface	1:200
PD-1	BV 605	Biolegend	135219	Surface	1:200
PD-L1	BV 786	BD	741014	Surface	1:200
Rorγt	PerCP-Cy5.5	BD	562683	Nuclear	1:200
Streptavidin-dump	FITC	Biolegend	405202	Surface	1:100
TCF-7	BV 421	BD	566692	Nuclear	1:200
TCRβ	BUV 395	BD	742485	Surface	1:200
TOX	PE	Invitrogen	12-6502-80	Nuclear	1:200

Continuation of Table 3.1					
Antigen	Color	Vendor	Catalog	Localization	Staining Dilution
Viability	Zombie Aqua	Biolegend	423101	Viability	1:800
Viability	eFluo780	Invitrogen	65-0865-14	Viability	1:500
End of Table					

CHAPTER 4

FUTURE DIRECTIONS

I've been extremely fortunate to work on these projects with a cohort of incredibly talented individuals. This section will first address directions related to the projects mentioned in the previous chapter before noting some exceptional progress and work not mentioned in this thesis.

4.1 Directions to Progress Flt3L-SA in ADA

As previously mentioned, this work involved use of a recombinant enzyme in wild-type balb/c mice; however, mice naturally express a functional version of this enzyme. Previous work has used a model of these mice which have a genetic knock-out of the uric acid oxidase enzyme, and studies involving these mice with rasburicase could be beneficial to determine a difference between pathogenic levels of antibodies, as determined by the studies in this thesis, versus antibody levels capable of rendering the drug non-functional (via *in vivo* clearance mechanisms or by directly blocking the function of the enzyme). Questions also still remain regarding the exact mechanism of tolerogenesis through Flt3L-SA administration. One could imagine use of a model antigen other than rasburicase, such as ovalbumin, which would allow probing of the B cell compartment as well as the T cell compartment. Such studies could allow us to hone-in closer to a mechanism and exactly determine whether we are capable of *de novo* Treg and Tfr formation through Flt3L-SA; however, most model antigens for which peptide:MHCII probes exist are monomeric, non-enzymatic proteins, which may affect how the antibodies are formed. In any case, if we are able to prove antigen specific Treg generation, we could also consider encapsulation of the antigen in some particulate form to preferentially target DCs while preventing B cell engagement.(81; 349–351) Combining with such a platform may allow us to target antigen to the formed DCs and specifically generate Tregs prior to initial B cell activation, thus synergising to halt the initial GC response entirely. Overall, it may also be interesting to consider models involving other enzyme replacement treatments such as clotting factor deficiencies or metabolic storage diseases –phenylketonuria

or lysosomal storage diseases – for which there are no other treatment options for patients once an allergy is formed.(277; 278; 281) An alternative interest to study for the benefit of these same patients would be prevention of antibodies against adeno-associated viruses as these are used as genetic vectors to enable production of the live-saving biologic within the patient them self, and these viral vectors are increasingly becoming therapeutics of interest.(279)

Considering that many biologics which induce problematic antidrug antibody responses on the market today are cytokine-blocking treatments (primarily TNF-α)(276), it would be interesting to experiment to see if co-administration of Flt3L-SA with these products or biosimiliars could prevent such reactions. Because patients on these drugs are naturally in a more pro-inflammatory state, it would be good to determine whether the DCs and Tregs generated by Flt3L-SA are resistant to activation by such signals. Experiments that could test this may include using the genetic mouse model constitutively expressing human TNF-α which spontaneously develop rheumatoid arthritis. In addition to then seeing the inflammatory state of the DCs and T cells, this same model could be used to test if administration of Flt3L-SA with a TNF-α blocking treatment, such as etanercept, could still prevent reactions, or if it could even work as a monotherapy. If the inflammatory state of these patients proved too strong to overcome with a monotherapy, a consideration might be co-administration with an immunosuppressive agent, such as rapamycin. With the large increase in DC numbers and the research which already shows DCs taking on a tolerogenic phenotype after rapamycin exposure and the benefit of these two drugs in preventing ADAs, this would provide evidence that we could expect synergy. Alternatively, we could also consider co-therapy with Selecta's Immtor platform of nanoparticulate-encapsulated rapamycin.(281) Modification of the size of the nanoparticles allows for specific uptake of rapamycin via APCs decreasing the broad immunosuppression of the drug.

4.2 Directions to Progress Flt3L-SA in Oral and Other Antigen-specific Immunotherapies

One large problem with the studies mentioned in the previous chapter has been the lack of a pathological readout related to gastric delivered antigen. To address this, ongoing work with another student in the lab, Kirsten Refvik, has aimed to look at how we may be able to use Flt3L-SA in conjunction with oral-delivered antigen in the accelerated BDC2.5 transfer model of type one diabetes. Flt3L has been used in the past and shown beneficial in delaying diabetic onset; however, once CD8 T cell involvement is noticed, Flt3L is capable of accelerating the NOD model. Preliminary results in our hands have been intriguing where in models with transfer of four day activated T cells show benefit in Flt3L-SA slightly delaying onset without regard to antigen therapy, but models where naive cells were transferred were accelerated by peptide treatment, agnostic of Flt3L-SA treatment. Mechanisms to understand this effect are currently underway as well as looking at alternative routes of antigen administration to prevent onset before moving to the longer but more translational NOD spontaneous diabetes model. Additional work is currently aimed at experimenting how Flt3L-SA with oral antigen can skew antibody responses away from pathogenic type 2 responses towards more inert antibody subclasses as a potential treatment for food and other allergies. With the profound effects we see in the gut draining lymph nodes, I think that further characterization of the responses occurring in this space as well as in the gut tissue itself could lead to very promising therapies.

4.3 Flt3L Products in Wound Healing, Wound Prevention, and Other Directions

Another incredibly interesting route we've taken in using Flt3L as a therapeutic is in the case of wound regeneration. This project, as a collaboration between myself, Dr. Abigail

Lauterbach, and Kirsten Refvik has explored the understudied realm of DCs in diabetic cutaneous wounds. Herein, we have found that treatment with WT Flt3L reduces the wound burden and promotes regeneration, likely via systemic DC proliferation and recruitment to the wound bed. Additionally, we can reduce the necessary dose for this effect by twenty-fold. We plan to pursue RNA sequencing of DCs and macrophages in the wound to determine what exactly is occurring and attempt to form a transcriptional network of crosstalk between these subsets. Furthermore, in collaboration with Dr. Jason Bugno of the Weichselbaum lab, we are actively working to combine this story with that of radiation-induced oral mucositis – a debilitating and dose-limiting side effect seen in patients undergoing irradiation for head and neck cancers. Studies here demonstrate that treatment with Flt3L-SA prior to irradiation can significantly reduce the oral lesion burden; simultaneously, we see no deleterious effect of the Flt3L-SA treatment on the killing of the tumor itself. This project has been extremely fulfilling and I have been thrilled to work on such an understudied condition (for which no preventative nor alleviating treatment exists) with such an incredible team of people, and I am excited to see this go to submission within the year. Along the lines of wound healing and prevention, I think it could be interesting to see how this treatment could change or even prevent ulceration events either in a diabetic state or otherwise. Unfortunately, I do not know of a good model related to ulceration, and neither Flt3L nor Flt3L-SA can do much to stop Dr. Lauterbach with a biopsy punch. Additionally, Flt3L has been tested in some older models of intestinal inflammation, but as the models improve and become better, I am curious to see how Flt3L-SA could be used in treatment of such intestinal inflammation.

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