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Common gamma-chain (γc) cytokine-based fusion proteins for applications in immunotherapy

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Common gamma-chain (γc) cytokine-based fusion proteins for applications in immunotherapy

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Abstract

Common gamma-chain (γc) cytokine-based fusion proteins for applications in immunotherapy

By Spencer Ng

Cytokines are protein messengers that can induce immune cells to activate, proliferate, differentiate, and engage in their effector functions. They may also directly inhibit any of these processes and instruct immune cells to undergo senescence and cell death. One family of cytokines with a wide array of immunomodulatory properties is known as the common gamma-chain (γc) group of cytokines. Comprising of interleukins-2, 4, 7, 9, 15, and 21, the γc cytokines are so named because they share the use of a common gamma-chain receptor (CD132). Each of these cytokines uses a ligand-specific alpha-chain (αc) for binding in addition to the γc, creating a heterdimeric receptor. IL-2 and IL-15 also share the use of a common beta-chain (βc, CD122), to form a heterotrimeric receptor. IL-2 was the first of these cytokines to be discovered as a T cell growth factor and has since become the only cytokine approved by the Food and Drug Administration (FDA) for use in cancer immunotherapy. Despite their immunostimulatory capabilities, γc cytokine monotherapy has had mediocre success in clinical trials for cancer, chronic viral infections, and autoimmune ailments.

In order to improve upon the efficacy of γc cytokine therapy, we have modified them by creating fusion proteins consisting of a γc cytokine and transforming

growth factor-beta (TGF-β) antagonists (FIST fusion family) for cancer immunotherapy. The rationale for the fusion of a TGF-β antagonist to γc cytokines is the observation that TGF-β secreted by tumors can actively suppress the immune response and dampen the effect of cytokine-based therapies. In particular, we find that the fusion of IL-15 to a TGF-β antagonist (FIST-15) potently stimulates natural killer cell-mediated anti-tumor immunity. We further explore the effects of FIST-15 in the setting of acute viral infection and a model of hepatic fibrosis.

A second family of fusion proteins consisting of γc cytokines and granulocyte/monocyte-colony stimulating factor (GMCSF; GIFT fusion family) were similarly designed to augment immunological responses against cancer. However, the fusion of GMCSF to IL-15 resulted in a protein (GIFT-15) with immunosuppressive properties. We have found that GIFT-15 acts primarily on B cells, converting them to a regulatory phenotype (B_{regs}). We demonstrate that B_{regs} may be therapeutically exploited in autoimmune conditions, where inappropriate activation of the immune response results in pathology.

Taken together, we find that modification of γc cytokines by creating fusions with TGF-β receptor antagonists or GMCSF can result in the formation of new proteins with unique immunobiological properties. The use of such γc-derived fusion proteins can be used to enhance the efficacy of cytokine-based immunotherapies.

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Chapter 1

Introduction

1.0.0 Overview of common gamma-chain interleukins

Cytokines are small protein molecules that act as hormone messengers for the immune system. They can exhibit a wide range of effects on the immune response, including the induction of activation, differentiation, and/or proliferation of immune cells. Conversely, cytokines can act to suppress immune responses either directly acting on immune cells or activating regulatory subsets of immune cells, which dampen proinflammatory responses. The wide-ranging effects of cytokines have made them a target of study for many decades, beginning with the discovery of interferons in the mid-20th century [1-3].

While cytokines have been grouped into families based on function, more traditionally, they have been classified according to their structure. Type 1 cytokines, which share a four alpha-helical bundle crystal structure, form the largest of known cytokine families [4]. The Type 1 family of cytokines is further subdivided into short-chain and long-chain cytokines, which vary in the length of the alpha-helices within their respective structures [5]. Type 2 cytokines differ from Type 1 cytokines due to structural differences, with type 2 cytokines exhibiting more than the typical four alpha-helices seen in Type 1 cytokines [6, 7].

Of the short-chain, type 1 cytokines, the most well-studied and evaluated for their effects on the immune system are the common gamma-chain (γc) interleukins. The known members of γc interleukin family consist of IL-2, 4, 7, 9, 15, and 21. These γc interleukins are so named because they share the use of the common

gamma-chain receptor (CD132) for ligand binding and signaling [8]. In addition to CD132, binding of each individual interleukin is dependent on a ligand-specific alpha-chain (αc), creating a heterodimeric receptor complex. Additionally, IL-2 and IL-15 share the use of a common beta-chain (βc, CD122), binding to a heterotrimeric complex. Expression of the 65 kDa γc is predominately restricted to cells of the hematopoietic lineage, particularly lymphocytes [9].

IL-2 was the first member of the γc cytokine family to be discovered. It was initially named T cell growth factor (TCGF) or blastogenic factor (BF), due to its potent ability to induce T cell proliferation. Research surrounding how IL-2 functioned and bound to its cognate receptor led scientists to the discovery of the common gamma-chain [10, 11]. The gene encoding a component of the IL-2 receptor (known then as IL-2Rγ) was found to be mutated in patients with X-linked severe combined immunodeficiency disease (XSCID) [12]. Although patients with XSCID exhibit a total lack of T cells, NK cells, and dysfunctional B cells, individuals (and mice) lacking IL-2 expression generally showed relatively normal T cell and NK cell development [13, 14]. This seemingly incongruous observation led scientists to conclude that other cytokines besides IL-2 also utilize the γc and that these other cytokines were more important for the development of normal T cells and NK cells [8, 15]. This led scientists to eventually discover the remaining members of the γc family of interleukins, IL-4 (1982) [16], IL-7 (1988) [17], IL-9 (1990) [18], IL-15 (1994) [19, 20], IL-21 (2000) [21].

An important property of short-chain γc interleukins is that they bind to receptors devoid of intrinsic kinase activity [22]. That is, neither the γc, the ligand-specific αc, nor the IL-2/15Rβc receptor molecules have any kinase activity of their own. Instead, the γc and associated αc's are associated to Janus kinases (JAKs) that undergo phosphorylation during a ligand binding event [23]. Ligand binding brings JAKs associated with the different receptor chains in close proximity to one another, resulting in transphosphorylation and activation of JAKs. The JAKs will further phosphorylate specific tyrosine residues on the cytoplasmic tail of their associated receptor chains, creating docking sites for signal transducers and activation of transcription (STAT) proteins. JAK proteins then phosphorylate and conserved Tyr residue on the C'-terminus of STATs, causing them to undergo a conformational change, which allows them to dimerize with other STAT proteins through the conserved Src-homology domain 2 (SH2 domain), and to translocate into the nucleus where they bind to regulatory elements and initiate unique transcriptional programs. To date, four JAK proteins (JAK1, JAK2, JAK3, and TYK2) and seven STAT proteins have been described in mammalian cells [24]. Because certain JAK kinases will preferentially associate with certain ligand-specific αc's and certain STAT proteins will preferentially associate with certain JAK kinases, fidelity and specificity of the signal transduced remains relatively consistent despite the very high number of permutations in signal output possible given the combinations of all JAKs and STATs [25]. It is also worth noting that in addition to the JAK/STAT pathway, there is also evidence to

suggest that γc cytokines can act on MAP-kinase (Ras/Raf/MEK/Erk) and PI3 kinase (PI3K) pathways as a form of non-canonical signaling [26, 27].

With this overview, we turn our attention to an overview of cancer immunotherapy, particularly how various immune effector subsets are involved in the pathogenesis of cancer, as well known strategies for tumor immune evasion. The latter part of this overview focuses on the role that tumor-derived transforming growth factor-beta (TGF-β) has on the immune system, as it provides the rationale for the creation of the FIST family of fusion proteins. We then turn to a review of the individual γc cytokines and their fusion to granulocyte/macrophage-colony stimulating factor (GMCSF; GIFT fusion family) and their applications in immunotherapy.

1.1.0. Cancer Immunology

The interaction between cancer and the immune system is one that has been a mystery to immunologists and cancer biologists alike. The concept of tumor immunology has evolved since the birth of modern immunology, and the mechanisms that govern this interaction is only beginning to be elucidated. The concept of immunosurveillance in the 1957 formalized by MacFarlane Burnet surmised that the immune system has a critical role in the sensing and destruction of malignancies. Research since then has demonstrated that tumors are not passive in this process of surveillance by the immune system; that they play active roles in modulating the immune response to prevent detection and

destruction. With this enhanced understanding of the tumor-immune system interface, there has been increasing interest in the translation of this knowledge into useful therapies for cancer patients. Clinicians and scientists alike are eager to improve current therapies, or to engineer novel therapies that enhance the immune response against tumors. This review will attempt to summarize current literature on this tumor-immune interface, focusing on the immune system's effector functions against tumors, and the mechanisms by which tumors evade the immune response, with a specific focus on TGF-β and its role in modulating the anti-tumor response of the immune system.

1.1.1 Natural History of Cancer and the Immune System

Cancer represents a heterogeneous group of pathologies primarily characterized by neoplasia, or abnormal growth of cells. Loss of growth control typically arises from mutations in the genes that regulate cell growth and division, resulting in malignant transformation. As these cells grow in an unrestricted manner, they acquire the ability to spread beyond their primary site of growth. In a process known as metastasis, cells from the primary tumor develop the propensity to detach and seed distant organs, and disturb the normal physiology of different organ systems. Frequently, it is metastasis and its disruption of critical organ systems that lead to cancer-associated morbidity and mortality. Cancer represents a significant cause of mortality in the U.S., accounting for nearly 600,000 deaths in 2015, making this pathology the second leading cause of death in the U.S [28]. In 2016, it is expected that over 1.69 million new cancer diagnoses will be made, a trend that has been on the upswing owing to the

increase in U.S. life expectancy [29]. As an individual gets older, their likelihood of developing cancer also increases, due to an increased propensity of accumulating genetic mutations, both from diminished DNA repair capacity and increased exposure to environmental conditions that may predispose to these mutations. What, then, prevents all individuals from developing clinically apparent cancer when the probability of acquiring potentially transforming mutations is so great? Paul Ehrlich, one of the founding fathers of modern immunology had already suspected, at the turn of the century, that the immune system prevents the body from becoming overwhelmed with cancers [30].

In the first half of the 20th century, the focus of modern immunology was quickly diverted from the study of how it might prevent the formation of tumors to the characterization of humoral immunity against pathogens. It was not until the advent of allograft transplantation experiments and firm evidence of cellular-mediated immunity was established that the idea of innate immune protection against tumors was revived. In early transplantation experiments, tumor allografts were met with rejection, suggesting that cell-mediated immunity could reject tumors, like any non-neoplastic tissue. This rejection was later discovered to be mediated by genetic differences in the outbred strains of mice used in these early experiments. With the advent of the syngeneic, in-bred mice, tumor transplantation experiments were met with mixed results. However, it was shown consistently, that inbred strains of mice were protected when immunized with carcinogen-induced tumors, and later challenged with that same tumor. This provided early evidence that immune mediated rejection of tumors a) relied on

cellular components of the immune response, as opposed to humoral components, and b) that these cellular components could target tumors specifically, because syngeneic grafts of normal tissues were accepted by recipient mice, while tumor grafts were rejected. This formed an important basis for a concept advanced by Lewis Thomas and Sir MacFarlane Burnet in 1957 known as the “cancer immunosurveillance theory”, which stated that effector immune cells acted as circulating sentinels, defending the body against tumor cells that could be recognized by as different from normal tissue due to ‘new antigenic potentialities’ expressed by the tumor.

The cancer immunosurveillance theory fell out of favor over the next several decades, even though its hypothesis was well supported by prior experimental evidence. The reason for this was due to inconclusive experiments that tracked spontaneous formation and artificially induced tumor formation in immunodeficient mice. A central tenet of the cancer immunosurveillance theory postulated that a defect in the effector functions of the immune response would likely result in increased spontaneous tumor formation and enhanced susceptibility in tumor formation. Mice rendered immunodeficient via neonatal thymectomy or the use of pharmacological agents and heterologous anti-sera to mouse lymphocytes. Experimentally immunosuppressed mice did not show an increased propensity for spontaneous tumor formation. In addition, these mice also did not have a reduced latency time between initiation of carcinogenesis by chemical mutagens and actual progression to tumor formation [31, 32]. The final piece of evidence that casted doubt on the cancer immunosurveillance

hypothesis came from the seminal work of Osias Stutman, who extensively studied chemically-induced and spontaneous tumor formation in immunodeficient mice with the athymic *nude* mutation. After administering 3-methylcholanthrene (MCA) to nu/nu mice, Stutman observed no noticeable difference in the latency period or the incidence of sarcoma formation when compared to heterozygous nu/+ that underwent the same treatment. His conclusions, he stated, argued against a role for thymic-dependent immunity in preventing tumor formation [33]. These studies performed from the late-1960s through to the mid-1970's overwhelming produced a body of evidence against the cancer immunosurveillance theory, where it was eventually supplanted by other alternative theories. The field of immunology slowly switched its focus away from its putative anti-tumor effects and redirected it towards the study of immunity against pathogens.

Interest in the cancer immunosurveillance theory did not revive until one critical immune cytokine became the subject of interest in tumor rejection experiments. Interferon-gamma (IFNγ) was shown to play an important role in mediating the rejection of tumors when it was shown that mice treated with a monoclonal neutralizing antibody against IFNγ were less able to reject tumor transplants from syngeneic mice [34]. Additionally, while LPS was able to induce rejection of Meth A (fibrosarcoma tumor model) cells that had intact IFNγ signaling when transplanted into Balb/c mice. Conversely, IFN-γ insensitive Meth A cells grew progressively when transplanted, even when they were given LPS at concentrations that were supposed to have induced tumor rejection [34]. It was

also demonstrated that IFN-γ insensitive Meth A cells were able to elicit stronger tumor protective responses to subsequent challenges by wildtype tumor, compared to mice given wild type Meth A cells that had intact IFN-γ signaling [34]. This clearly implicated IFN-γ and the immune response in control of tumor growth. As the role of effector functions of CD8+ T cells were being elucidated, it was also discovered that in mice lacking the perforin gene, the product of which is responsible for forming pores on cells targeted for cytolysis by CTLs and NK cells, were more susceptible to chemically-induced tumor formation compared to wildtype mice [35]. As a follow-up, the same group also found that perforin-deficient mice exhibited a significantly higher incidence of spontaneous tumor formation compared to wildtype mice [36]. Observations that brought critical components of the immunological system in the anti-tumor response, once again stirred renewed interest in the immunosurveillance theory.

The evidence that undeniably brought back and mechanistically proved the existence of cancer immunosurveillance involved the use of Rag2 knockout mice. Deficiency of Rag2 specifically prevents recombination events in lymphoid cells that are critical to the generation of antigen receptors. Shankaran et al. showed that when treated with MCA, Rag2-/- had an increased frequency of tumor development compared to wildtype mice [37]. Also, Rag2-/- mice housed in sterile, pathogen-free environments develop more spontaneous tumors of epithelial origin compared to wildtype mice [30, 37]. These observations were, however, in direct conflict with the previously described experiments that utilized immunodeficient nu/nu mice. As it turns out, the *nude* phenotype is a result of a

spontaneous mutation in the *Foxn1* gene, a forkhead transcription factor that was critical for the differentiation and maintenance of thymic epithelial cells (TECs) [38]. Without TECs, no functional thymus is formed, and this athymia resulted in a lack of T cells. However, several groups have shown that this mutation is 'leaky' and that T cells may be able to form and reach the periphery [39, 40]. The Rag2-/- mice used in the latter experiments, had immune cell-specific targeted ablation of a protein that is typically only expressed in lymphocytes. The Rag2-/- mouse models, thus provided data that was more specifically focused on how ablation of lymphocytes affected tumor growth. In light of these new observations that showed how critical cellular and signaling components of the immune system were required to mount an effective anti-tumor response, a new era of research to dissect the mechanisms of these responses began in the field of tumor immunology.

1.1.2. Anti-Tumor Effector Functions of the Immune System

At the very outset, a tumor presents a perplexing puzzle to the immune response. Evolutionarily, the immune system has been designed to differentiate self and non-self, and once this recognition is established, to purge the elements of non-self (i.e. pathogens, cells infected with pathogens, etc.) from the body. A tumor presents an interesting combination of both self and non-self; as it arises from a single mutated cell *de novo* from within the body, it has all the characteristics of self. Yet, as a tumor grows and continues to mutate, it may acquire new antigenic determinants that are foreign or novel to the immune response, thus becoming partially non-self. The immunogenicity of a tumor is dependent on the balance of

these qualities, and whether a successful anti-tumor response can be mounted is critically dependent on the immune system's ability to distinguish tumors from surrounding normal tissues. The shaping of the immunogenicity of a tumor, and a tumor's ability to shape the immune response is known as a 'cancer immunoediting', an adaptation and extension to the 'cancer immunosurveillance' principle. Whereas the immunosurveillance theory ascribes the ability of the immune system to recognize and eliminate tumors, the immunoediting concept provides an additional framework to incorporate an increasing body of evidence supporting the immune system's shaping of tumor growth (which does not always necessarily result in eradication of the tumor), and conversely, how tumors may alter the effector functions of the immune response. In this section, the critical components and mechanisms behind the immune system's anti-tumor effects will be explored.

While many initial experiments testing the cancer immunosurveillance focused exclusively on the role of T cells in the anti-tumor response, these are not the only immune cells that play a role in tumor clearance. An improved understanding of how the immune system is divided into innate and adaptive compartments has provided a better picture of how the immune system first detects, then targets, and finally attempts to eliminate a tumor that has become a threat to the host. The first cells that are able to recognize and effectively target tumors appear to be natural killer (NK) cells [41]. NK cells are innate lymphocytes capable of differentiating self- vs. non-self and are armed with an assortment of cytolytic effector molecules that are meant to destroy cells infiltrated by

pathogens or have otherwise undergone transformation to a point where they become foreign to the host. One important mechanism by which these NK cells differentiate self- vs. non-self is by detection of major histocompatibility complex (MHC) class-I surface proteins. Basal expression of MHC-I is present on almost all cells of the body, where they are complexed with peptides derived from proteins that are recognized as self. Under circumstances where cells become infected with intracellular pathogens or when they undergo transformation, MHC-I molecules may become complexed with non-self peptides (of pathogenic or oncogenic origin), which provides a signal for the NK cells surveying these molecules to identity and destroy the infected or transformed cell [42]. Indeed, this was found to be a key mechanism for clearance of tumor cells in multiple studies [43]. Infected or transformed cells may also downregulate cell surface expression of MHC-I molecules. The absence of MHC-I molecules may also trigger NK cell-mediated cytolysis [41, 44-46]. Infected cells may also upregulate expression of surface proteins, MIC-A, MIC-B, ULBP-1, that act as ligands for activating cytotoxicity receptors present on the surface of NK cells. Tumor cells undergoing transformation may also be programmed to alert NK cells of this process by upregulating such cell surface markers, labeling themselves for destruction [47]. In order to counteract these activating receptors, normal, untransformed cells also express a compliment of surface inhibitory receptors that prevent non-specific NK cell killing. The question of whether NK cells act on tumor cells at all depends on an intricate balance of tumor cell surface expression of these NK cell activating and inhibitory receptors.

As NK cells survey tissues, they are frequently the first to be alerted to the presence of tumor formation and act to eliminate tumors while they are small enough, preventing them from becoming clinically apparent. Empirically, it has been shown that chronic depletion of NK cells by using a neutralizing antibody against NK1.1 (a NK cell-specific antigen) increases the frequency at which mice exposed to MCA form sarcomas [48]. Kim et al. showed that mice transgenically manipulated to have impaired NK cell function, were less able to reject tumors and control metastases *in vivo* and were less able to mediate tumor regression when adoptively transferred into tumor-bearing mice [49]. In another study, mice treated with a neutralizing depleting antibody against NKG2D, a cytotoxicity activating receptor on the surface of NK cells (as well as NKT and $CD8^+$ T cells), also increased the incidence of MCA-induced sarcoma formation [50]. It is interesting to note, however, than NKG2D-knockout mice exhibited comparable levels of tumor formation when exposed to MCA compared to wildtype mice, suggesting that the technical differences in the experimental approach to addressing NKG2D deficiency may very well impact the conclusions of the study [51].

NK cells mediate cytolysis by their complement of cytolytic granules containing perforin and granzyme. Upon polarized exocytosis from the NK cell, perforin mediates pore formation on the surface of the target cell, allowing granzymes to enter and cleave caspase proteins via their serine protease activity [52-55]. These cleaved caspases are then triggered to begin the apoptotic cascade within the target cell, eventually leading to cell death. Secondarily, NK cells may also

mediate target cell lysis by engagement of TNF receptor superfamily members, such as TNF-TNFR, Fas-FasL and TRAIL-TRAIL-R interactions [56]. NK cells express TNF, FasL, and TRAIL, which binds to TNFR, Fas, and TRAIL-R (uniformly known as death receptors) on the target cell and triggers the extrinsic apoptotic cascade, characterized by activation of executioner caspases 3, 6, and 7, and ultimately leading to cell death. The lysis and destruction of tumor cells by infiltrating NK cells begins a pro-inflammatory cascade that sets into motion an anti-tumor adaptive immune response.

The killing of tumor cells causes the release of pro-inflammatory cytokines, such as TNF-α, IFN-γ, and IL-12, which acts to recruit tissue-resident macrophages and dendritic cells (DCs) into the tumor microenvironment. NK cells are known to secrete very high levels of IFN-γ, which promote very important anti-tumor effects through two mechanisms, 1) to prime other immune cells and alert them to the presence of tumor, and 2) to act directly on the tumor cells by preventing proliferation, inducing apoptosis, or to upregulate expression of MHC-I molecules presenting oncogenic peptides that act as a red flag for tumor infiltrating NK and $CD8^+$ T cells [30, 37, 57-59]. Lesser studied, but a known mechanism by which IFN-γ prevents tumor proliferation includes its ability to cause tumor and stromal cell secretion of IP-10, and CXCL9-11, which are potently angiostatic, potentially preventing the tumor from gaining access to the host's blood supply [30, 60, 61]. Macrophages and DCs activated by the IL-12 and IFN-γ are now capable of acquiring, processing and presenting tumor antigens at the tumor-draining lymph nodes (TDLNs) to the cells of the adaptive immune response.

Within the TDLN, DCs that were primed in the tumor microenvironment by NK cell-secreted IFN-γ are stimulated to present tumor-associated antigens (TAAs) to $CD4^+$ and $CD8^+$ T cells that can recognize such antigens. $CD8^+$ T cells recognizing these TAAs in the context of MHC-I molecule have been observed to mediate anti-tumor responses in a variety of different cancer models. In an exciting pilot study in human patients with metastatic melanoma, it was shown that adoptive transfer of melanoma-specific $CD8^+$ T cells led to regression of the primary tumor and metastases [62]. In an unrelated, but similar study, melanoma biopsies from patients receiving autologous transfer of TAA-specific $CD8^+$ T cells showed that many of the tumor infiltrating lymphocytes were, indeed, melanoma-specific and could home to sites of tumor growth [63]. The ability of $CD8^+$ T cells to mediate anti-tumor responses have also been extensively studied in mice, where they are known to utilize mechanisms similar to NK cells (i.e. perforin, granzyme, and release of IFN-γ) in order to effectively clear tumors [64]. As a corollary to this, absence of $CD8^+$ T cells in mice (either by antibody neutralization or in transgenic knockout models) increases their likelihood of spontaneous tumor formation and the incidence of carcinogen-induced tumorigenesis when compared to wild-type mice [65, 66].

Though originally less appreciated, $CD4^+$ T cells and their role in tumor immunity have recently generated much interest. Mainly due to the ability of $CD8^+$ T cells to directly lyse tumor cells, these were the T cell subsets that were studied and tested the most in pre-clinical and clinical studies for their ability to induce tumor regression. $CD8^+$ T cells also recognize intracellular antigens in the context of

MHC-I, of which most oncogenic products would likely fall into, and so the scientific field logically focused on $CD8^+$ T cells and their MHC-I restricted tumor antigen specificities in the design of immunotherapeutic trials [67, 68]. It is now clear that $CD4^+$ T cells or T-helper cells have important anti-tumor functions [69]. $CD4^+$ T cells, like $CD8^+$ T cells, can become activated by DCs that have sampled tumor antigens and migrated back to the TDLN. DCs activated in the tumor microenvironment by the inflammatory cytokine milieu, can be stimulated to phagocytose, process, and present TAAs in the context of MHC-II, which $CD4^+$ T cells are specifically designed to respond to. Provided that the tumor itself also expresses MHC-II, tumor-infiltrating $CD4^+$ T cells with the appropriate TAA-specificity could also be activated *in situ*, although this is considered to be a less probable phenomenon [70]. Once activated, $CD4^+$ T cells are thought to aid the anti-tumor response in three ways: 1) to provide help to tumor-specific $CD8^+$ T cells, and 2) to enhance tumor-antigen presentation by activation of DCs, and 3) to directly induce tumor cell lysis. In many experimental studies, it has been shown that tumor specific $CD4^+$ T cells can directly provide help to $CD8^+$ T cells by secretion of cytokines (particularly IFN-γ and IL-2, T_H1-polarized cytokines) or by direct cell-cell contact and expression of co-stimulatory molecules on the surface of $CD4^+$ T cells [71-74]. $CD4^+$ T cells have also been shown to enhance the antigen-presenting capabilities of APCs, particularly DCs, through their secretion of IFN-γ. $CD4^+$ T cell-derived IFN-γ has also been demonstrated to increase DC expression of co-stimulatory molecules that act to more potently stimulate tumor-specific CD8+ T cells [74]. Much like $CD8^+$ T cells, $CD4^+$ T cells

have also demonstrated their ability to induce direct tumor cell lysis via their engagement of death receptors on tumor cells (i.e. Fas and TRAIL-R), and have even been suggested to utilize the perforin-granzyme pathway of eliciting tumor cell death [75-77]. Some groups have even demonstrated that $CD4^+$ T cells may be more efficient at inducing tumor regression than $CD8^+$ T cells, particularly when the tumor models used are resistant to CD8+ T cell-mediated rejection [78]. More empirically, it has been shown in a variety of tumor-vaccination studies that the depletion of $CD4^+$ T cells post-vaccination results in diminished tumor regression upon subsequent tumor re-challenge [79]. Additionally, it has been shown, at the very least, that $CD4^+$ T cells augment $CD8^+$ T cell responses against cancer, even if they are not acting as a the predominant cell subset responsible for direct tumor cell lysis [80].

While the majority of literature has focused on NK- and T cells as critical components of the immune system's anti-tumor response, the contribution of B cells and other immune cells to this function has also been a recent source of intense study. B cells have largely been considered to mediate anti-tumor responses only insofar as their ability to secrete tumor antigen-specific antibodies. Indeed, it has been shown that such antibodies have the ability to execute anti-tumor functions by either directly mediating complement fixation on tumor cells, antibody-dependent cellular cytotoxicity reactions, abrogation of aberrant oncogenic signaling or protein activity, and trapping of tumor-associated soluble molecules that generally aid in tumor progression [81]. In a more translational approach, new clinical studies have even used tumor antigen-specific antibodies

conjugated to chemotherapeutic drugs in order to deliver anti-cancer drugs in a more targeted manner [82]. What is less appreciated about B cells in their anti-tumor response is that in addition to their ability to differentiate into plasma cells and secrete antibodies, they may also act as a APCs and present tumor antigens [83, 84]. Under the appropriate conditions, B cells may be induced to become effective tumor antigen-presenting APCs [85]. Given their relative abundance over DCs, B cells manipulated in this manner make them an attractive target for immunotherapy. Recently, B cells have also gone a functional re-classification based on their cytokine secretory profile. Akin to T_H1 and T_H2 cells, B cell biologists are now proposing a dichotomous system of dividing B cells into B-effector 1 and 2 cell subtypes. Be-1 cells secrete T_H1-polarized cytokines such as IFN-γ, IL-12, and TNF-α, whereas Be-2 cells secrete IL-4, 6, 13, and other traditionally T_H2-associated cyotkines [86, 87]. Cells with this Be-1 profile have recently been implicated in a lung tumor model, where their ability to secrete IFN-γ in the local tumor microenvironment and activate tumor-infiltrating NK cells were demonstrated to be important [88]. More empirically, mice depleted of B cells prior to tumor challenge experience enhanced rates of tumor growth, suggesting that they do play a role in cancer immunosurveillance [89]. While this may be suggestive of B cells abilities to provide help to T cells, or to act as APCs, a new body of literature has shown that B cells can mediate cell lysis by the expression of cell surface death ligands, although direct B cell-mediated lysis of tumor cells have yet to be observed [90]. The role of B cells in the anti-tumor

response has only begun to be appreciated, and much more research still needs to be carried out to fully characterize their contribution to anti-tumor immunity.

Various other immune cells have also been shown to contribute to an effective anti-tumor response, although these studies have been few and far between. The role of macrophages in the anti-tumor response has been heavily debated, with more recent evidence suggesting that recruitment of these cells into the tumor is generally associated with negative prognoses, and enhanced tumorigenesis. However, depending on the cytokine milieu within the tumor microenvironment, tumor-associated macrophages may very well mediate anti-tumor responses, by acting as APCs and secreting pro-inflammatory cytokines that serve to heighten the response of other immune cells against the tumor [91, 92]. In addition to cytokine secretion, macrophages have also been demonstrated to mediate tumor cell death though the release of toxic reactive oxygen species and nitric oxide, which serves to make the make the tumor microenvironment inhospitable [93]. Tumor-associated macrophages have also been shown to secrete important angiostatic factors that prevent the recruitment of nearby blood supplies to support tumor growth [94]. Neutrophils, much better known for their antimicrobial properties and pro-inflammatory response to pathogens, have also been recently demonstrated to mediate anti-tumor effects, mostly via their ability to secrete many cytokines, chemokines, and otherwise pro-inflammatory molecules (i.e. proteases, reactive chlorinated oxidants and oxidative intermediates) that might adversely affect tumor growth [95]. As with macrophages, the local tumor microenvironment's cytokine profile affects the ability of tumor-associated

neutrophils (TANs) from engaging in anti-tumor functions [96]. Even eosinophils, a class of immune cells for which roles outside of tumor immunity have not been well-defined, have demonstrated involvement in anti-tumor processes. Eosinophil infiltration into mice have been shown to mediate objective tumor regression in tumor models transgenically manipulated to express IL-4 [97]. Mice transgenic for overexpression of IL-5 (a cytokine critical for eosinophil development), thus resulting in increased circulatory numbers of eosinophils, showed resistance to MCA-induced sarcoma formation [98]. Conversely, mutations that prevent eosinophils from trafficking to sites of inflammation, and eosinophil-deficient mice all showed increased susceptibility to sarcoma formation when exposed to MCA compared to wildtype mice [98]. Even less well characterized for their role in anti-tumor responses are mast cells, which have been shown to alternatively enhance and inhibit tumor growth [99]. Mast cells and their granulocytic contents have been shown to mediate a number of anti-tumor functions. Notably, masT cell-mediated release of proteases, such as tryptase has been shown to modulate the metastatic potential of tumor cells [100].

Once thought of as a theory with scant supporting evidence, cancer immunosurveillance and its reincarnation as the cancer immunoediting hypothesis has been well verified and tested by many groups. Given the evidence presented in this previous section, there is no doubt that the immune system exerts a variety of anti-tumor functions. The aim of the immune response, once it determines that a tumor is present and a threat to the host, is to eradicate it. While NK-, NKT, and T cells are the main cellular components involved in this

response, the role of other immune cells, such as DCs and their ability to trigger an adaptive, lasting response against tumors cannot be understated. Furthermore, immune cells that have had no previous known relation to anti-tumor immunity have now been observed to play supplementary roles in the overall ability of the immune system to mount an effective anti-tumor response. Despite the capable armaments of immune anti-tumor response, full eradication of tumor is not always possible, and for this reason, tumors become clinically apparent in immunologically competent hosts. In the following section, attention is turned towards how tumors are able to evade, suppress, or otherwise manipulate the immune system and its effector functions to work towards its advantage. The mechanisms by which tumors are able to do this vary greatly depending on the histological origin, stage and type of cancer in question.

1.1.3 Tumor immune-evasion strategies

Intrinsically, tumors are genetically unstable allowing mutations and traits in tumor cells to accumulate over time, such that they become well adapted to the environment that occupy. During tumorigenesis, the nascent tumor is under extreme selective pressure to evade the immune response. Based on anti-tumor functions described in the previous section, it is of no surprise that the immune system inadvertently selects for the most aggressive tumor cell variants. The cells that are not initially eliminated from the primary tumor by the immune response are selected and allowed to continue growing. This process is known as ‘immunosculpting’, where those tumor cells that are immunogenic enough to be detected by the immune response are eliminated, leaving a population behind

that becomes increasingly invisible to the immune response over time. A clonal population of immunologically silent tumor cells can then grow in an unrestricted manner and spread beyond the confines of the primary tumor microenvironment. If tumor cells cannot shed their antigenically identifiable elements, tumors may also acquire the ability to actively suppress the immune response via the secretion of tumor-derived soluble factors that could dampen the effector functions of the immune response. Tumors may also induce tolerance of the immune response, and wear it down to the point of exhaustion. In many cases, tumors will even recruit immune cells into the microenvironment, in order to utilize their effector functions for the purposes of promoting tumor growth.

Tumors employ several mechanisms to remain immunologically silent, and thus evade the anti-tumor functions of the immune response. Tumors may downregulate critical components of intracellular machinery that are required for antigen processing. By decreasing cell surface expression of MHC-I, and its associated β2m-microgloublin molecule, NK and $CD8^+$ T cells that survey for TAAs in the context of MHC-I will not be able to detect oncogenic proteins associated with the transformation process [101-103]. Other components of antigen processing such as the transporter associated with antigen-processing (TAP), tapasin and LMP2/7 have also been shown to be downregulated in several cancers [101, 104, 105]. Since IFN-γ signaling is critical for somatic cells to upregulate expression of MHC-I and the associated components of antigen processing, it is no surprise that many tumors have also evolved to become entirely insensitive to IFN-γ signaling [106]. Tumors may also decrease cell

surface expression of ligands for NK cell activating cytotoxicity receptors, such as ULBP-2 and MIC-B [107]. Moreover, it has been shown that some cancers may actually overexpress non-classical MHC-I-like molecules, such as HLA-G, or secrete soluble MIC antigens, which serve as inhibitory ligands or decoys for cytotoxicity receptors on NK and $CD8^+$ T cells [108, 109]. If the tumor cannot escape detection by surveying NK and $CD8^+$ T cells, they may prevent cytolysis by downregulating their surface expression of death receptors, such as Fas and TRAIL-R that are used to trigger the apoptotic cascade [110, 111]. Furthermore, it has been demonstrated that many cancers overexpress cytoplasmic anti-apoptotic proteins, such as Bcl-XL, that allow them to survive despite the ligation of death ligands to receptors on immune cells [112].

In addition to becoming immunologically silent, many tumors may also evolve to take on role in actively suppressing the immune response. Tumors may do so either directly upregulating expression of cell surface markers that inhibit immune cell effector functions. More commonly, tumors may elaborate a variety of soluble factors, known as tumor-derived soluble factors (TDSF), which create a generally immunosuppressive environment. As immune effector cells infiltrate into this environment in order to engage and eradicate tumors, the cytokine milieu within the tumor microenvironment may adversely affect their ability to induce anti-tumor responses. Due to the fact that tumor cell lysis mainly relies on the ability of NK and $CD8^+$ T cell to make direct cell-cell contact with tumor cells, tumors can be found to upregulate expression of inhibitory proteins on their cell surface, which serve to prevent or dampen the potential cytotoxic effects of tumor-specific

immune cells. B7-H1 (or PD-L1), is a well-characterized transmembrane protein known for its ability to bind PD-1 on the surface of activated lymphocytes. PD-1-PD-L1 ligation typically results in attenuation of T cell activation and proliferation. PD-L1 overexpression has been found in a variety of tumors, including renal cell carcinoma in humans [113]. Tumor cells have also been observed to usurp cytotoxic pathways that are typically used by NK and $CD8^+$ T cells. By upregulating expression of FasL, tumor cells have been shown to trigger apoptosis of Fas-expressing immune effector cells [114]. In addition to surface-bound FasL, human colon cancers have also been shown to secrete soluble FasL, which may also act to induce the death of TILs expressing Fas [115]. Many studies have shown that immune cells cultured *in vitro* in media conditioned by tumor cells have aberrant effector functions, suggesting that tumors may secrete soluble factors that affect immune responsiveness. Two of the most well-characterized TDSFs include IL-10 and TGF-β, two cytokines well known for their immunosuppressive functions [101]. The effects of TGF-β on the immune response, particularly as it pertains to anti-tumor immunity will be reviewed in a later section. IL-10 secretion is increased in a variety of human and mouse tumors, likely due to its ability to suppress secretion of pro-inflammatory cytokines by $CD4^+$ T cells (i.e. TNF-α, IL-6, and IL-1β), downregulate cell surface expression of MHC-I molecules, and inhibit the expression of other co-stimulatory markers that are required for triggering TILs from engaging in effective anti-tumor responses [116]. IL-10 is also known to suppress CD8+ T cell function in secondary responses by and has been observed to skew $CD4^+$ T cell responses

towards a TH2 phenotype, which is less effective at tumor clearance [117, 118]. Beyond altering expression of immune-modulating cell-surface markers, tumors may also enhance expression of enzymes that catalyze the formation of immunosuppressive metabolites and products. Indoleamine-2,3-dioxygenase (IDO) is an enzyme that catabolizes the amino acid tryptophan into kynurenine and is found to be upregulated by several different cancers [119]. By increasing IDO expression and activity, tumors can effectively deplete the microenvironment of tryptophan and its metabolites, which are important for T cell function [120]. In addition, kynurenine itself may act to suppress T cell function and induce apoptosis [121, 122]. Consistent with these observations, several *in* vitro studies have confirmed that constitutive expression of IDO by tumor cells results in diminished T cell function, and that inhibition of IDO activity in this setting allowed for partial reversal of the immunosuppression [123]. Furthermore, tumor cells genetically modified to overexpress IDO have been shown to resist rejection in *in* vivo tumor vaccine trials [123]. Beyond upregulating enzyme activity, tumors may also elaborate such factors as galectin-1 and gangliosides. Galectin-1, a carbohydrate-binding protein is overexpressed in certain mouse models of lung cancer. Its expression and binding to T cells is known to induce apoptosis, as well as inhibit T cell proliferation and secretion of IL-2 [124]. The inhibition of this molecule in experimental settings results in enhanced T cell-mediated rejection of tumors compared to tumors that express normal levels of galectin-1 [125]. Glycospingolipids, such as ganglioside GM1b, are secreted by FBL-3 erythroleukemia tumor cells, and have been shown to inhibit both *in vitro* and *in*

vivo T cell-specific responses against these tumors [126]. These are only a fraction of the TDSFs, cytokines, and other chemical mediators that tumors have evolved to secrete into their microenvironment for the sake of persisting in the host, and to inhibit the immune forces that seek to eradicate them. All these mechanisms work together in unison towards the end goal of inducing peripheral tolerance of immune cells to the presence of a growing tumor. Indeed, this is of no surprise given the strong immune system-driven pressure in the sculpting of a tumor's phenotype, and the inherent genetic and epigenetic instability of the tumor itself.

Although the tumor microenvironment generally fosters an environment that is immunosuppressive, many studies have demonstrated that increased infiltration of immune cells into the tumor is generally a good prognostic indicator. However, not all infiltrating immune cells have anti-tumor function, and in many cases, recruitment of such cells into the microenvironment may actually promote tumorigenesis. This phenomenon may seem paradoxical provided all of the anti-tumor effector functions of immune cells covered in the previous section. However, it is important to understand that the immune system, much like any other physiological system in the body, is governed by a series of regulatory checks and balances. The counter balance to the pro-inflammatory effects exerted by the immune response includes populations of immune cells that serve explicitly to regulate its function, thus preventing an overactive response that may prove detrimental to the host. Tumors have evolved to exploit these regulatory

cell populations for the purposes of dampening the effects of the anti-tumor functions reviewed above.

CD4+ regulatory T cells (T_{regs}), that are CD25hi and FoxP3+, have been described to play a major role in the suppression and regulation of immune-mediated responses [127]. Their main purpose is to prevent a runaway, pro-inflammatory immune response from overwhelming the host. While the mechanisms by which T_{regs} have been hotly debated, it is now generally accepted that T_{regs} are able to inhibit immune responses by physically interacting with other immune cells, and through the secretion of immunosuppressive cytokines that aim to dampen the response of activated immune cells [128]. T_{regs} are found in a variety of different tumors, and have also been reported to be active in TDLNs [129, 130]. It is believed that intratumoral T_{regs} downregulate immune effector functions by secreting TGF-β and IL-10 into the tumor microenvironment [131, 132]. Moreover, T_{regs} express on their cell surface PD-L1 and CTLA-4, potent ligands for inhibition of T cell responses [133]. Anti-tumor T cell responses ranging from activation, proliferation, and degranulation have all been described to be inhibited in some way by the presence of T_{regs}. In addition to secreting immunosuppressive cytokines and directly modulating T cell effector responses, T_{regs} are also thought to act as an IL-2 sink, owing to their high expression of CD25 (IL-2 α-chain receptor) [134]. By sequestering this critical T cell growth factor, anti-tumor TILs are prevented from becoming activated. The etiology of T_{regs} within the tumor is another source of contention amongst tumor immunologists. T_{regs} localized in TLDNs are also thought to dampen the anti-

tumor response by interacting with TAA-presenting APCs and potential effector T cells, and prevent proper activation and conversion of naïve T cells to TAA-specific effector T cells [135, 136]. By suppressing DC activation and inhibiting T cell priming, T_{regs} in the TDLN can effectively decrease both the number and efficacy of tumor-specific T cells generated in secondary lymphoid compartments, resulting in further peripheral T cell tolerance to tumor growth. Few studies have been able to track the development of tumor-specific T_{regs}, which are then hypothesized to return to the site of tumor growth to suppress immune responses locally. Some reports suggest that T_{regs} are converted *in situ* from infiltrating CD4+ effector T cells under the influence of tumor-derived TGF-β [137]. Regardless of their etiology, many *in vivo* studies have provided ample evidence that tumor-associated T_{regs} negatively affects anti-tumor responses. Their depletion from mice before tumor challenge (of different types) have resulted in enhanced anti-tumor immunity, and increased frequencies of T_{regs} in both tumor and sera of cancer patients are generally correlated to poor outcome [138-140].

In addition to T_{regs}, tumors may also recruit a variety of cells of the myeloid lineage into the microenvironment. Immunosuppressive macrophages found within the tumor microenvironment are known as tumor-associated macrophages (TAMs), and have been extensively studied for their dual ability to suppress the immune response and to promote tumorigenesis and metastases [141]. Macrophages have been described to undergo two types of activation. The pro-inflammatory macrophage phenotype, which infiltrates tissues and signals the presence of invading pathogens in a wound, is known as the classical activation,

resulting in a M1 macrophage. Conversely, monocytes under the influence of IL-4, 10, and 13 in immunosuppressive environments become 'alternatively' activated M2 macrophages. It is these M2 macrophages that are the predominant TAMs found within tumors [141]. TAMs are known to inhibit the immune response via production of TGF-β and IL-10, and have been demonstrated to effectively inhibit M1 macrophage pro-inflammatory responses that are otherwise more effective at eradicating tumors [91, 142]. CCL-8, 17 and 22 secreted by TAMs are also demonstrated to recruit either naïve CD4+ T cells or T cells with no cytotoxic functions into the tumor, causing them to become anergized or polarized towards a T_H2 response, again preventing an effective anti-tumor response [141]. TAMs are capable of all these immunosuppressive functions while simultaneously playing a critical role in tumor microenvironment remodeling to allow for angiogenesis and subsequent invasion of the primary tumor beyond its original tissue location [143]. A more recently described set of myeloid cells that have generated a lot of interest due to their immune suppression capabilities are the 'myeloid-derived suppressor cells' (MDSCs). MDSCs represent a heterogeneous group of immature myeloid cells (defined as CD11b+Gr-1+) that are widely found in tumors of different histologic origin [144]. As a group, it has been found that these cells secrete immunosuppressive TGF-β, and have been found to inhibit intratumoral T cell activation and secretion of IL-2 in a cell contact-dependent fashion [144, 145]. MDSCs have also been shown to induce Treg formation within tumors, and deplete the amino acid arginine, which is critical to T cell function, by upregulating the expression of the enzyme

arginase [146]. Finally, depletion of putative MDSCs (CD11b+Gr-1+ cells) with pharmaceutical agents (such as gemcitabine) has resulted in enhanced anti-tumor activity [147].

1.1.4 γc cytokines in cancer immunotherapy

Although IL-2 was discovered in the late 1970s, it was not until nearly a decade later that clinical trials testing its efficacy against cancer were initiated [148]. The ability of IL-2 to expand most T cell subsets, NK and NKT cells, as well as mediating the activation and expansion of B cells (although this was not appreciated until later) appeared to make it a suitable pharmacological agent against tumors. Rosenberg and colleagues in the early 1980s began experimenting with the use of IL-2 in pre-clinical models of cancer in mice and human. It was found that the use of IL-2 alone or in combination with lymphokine activated killer cells (LAKs; lymphocytes cultured in the presence of IL-2) were able to dramatically reduce the number of liver and lung metastases from a number of different mouse tumor models [149, 150]. In these early pre-clinical experiments, a sustained, high-dose of IL-2 was required to see therapeutic effects, as low and/or single doses of IL-2 showed no apparent anti-tumor effect. Initial phase II clinical trials utilizing high dose IL-2 (>600,000 IU/kg) in 255 patients with metastatic melanoma and renal cell carcinoma showed overall response rates of 15%, with 7% of patients achieving complete responses [151]. Given that few alternatives existed at the time for patients with metastatic disease, these phase II clinical trial results opened the door to a myriad of other clinical trials testing IL-2 in combination with other forms of existing therapies.

Since then, IL-2 has been the most tested γc cytokine in cancer immunotherapy. Trials combining IL-2 with standard chemotherapeutic regimens (e.g. decarbazine and cisplatin), radiation therapy, peptide-based tumor vaccines, antibodies/immune checkpoint blockade inhibitors, targeted therapies (e.g. drugs that specifically and preferentially inhibit mutated gene products in tumor cells) and adoptive transfer of TILs [152]. Results consistently show that IL-2 boosts the therapeutic response of the agent that it is tested with. Perhaps the most promising results utilizing IL-2 has come from trials combining high dose IL-2 infusion with adoptive transfer of IL-2 expanded TILs that were isolated the patients' tumors. Researchers at the NCI saw a 50% objective response rate, with 13% showing complete responses [153].

The effects of IL-2 alone or in combination with other treatment modalities seem to cause responses in only a subset of patients, producing a ceiling effect on its efficacy. Further, it appears IL-2 therapy may only be beneficial for certain types of cancer [154]. Work identifying biomarkers of IL-2 responders versus non-responders, as well as investigation into which cancers respond better to IL-2 treatment is ongoing [155]. The immunogenicity of individual types of tumors, as well as the patient's individual immune cell repertoire and constitution are likely to play the biggest factors, as it has been shown that IL-2 does not act directly to inhibit tumor growth, but rather operates strictly through augmenting immune responses for its observed anti-tumor effect [152]. An additional caveat to the use of IL-2 in cancer immunotherapy, which was not initially appreciated during the early clinical trials, is this cytokine's effect on the induction CD4+ T_{regs}. IL-2

administration is now known to preferentially expand peripheral T_{regs} compared to effector T cells, which are the subset researchers seek to expand for their antitumor effects [156]. The high affinity IL-2Rαβγ is expressed at a much higher degree on the surface of T_{regs} compared to effector T cell (due mainly to enhanced IL-2Rα/CD25 expression on T_{regs}), making them more sensitive to the effects of IL-2, in addition to their ability to sequester IL-2 from effector T cells [157]. CD4+ regulatory T cells are preferentially recruited and enriched in the tumor microenvionment of many different types of cancer. Many studies have shown that IL-2 administered in immunotherapeutic regimens can enhance Treg proliferation and aggregation in the tumor microenvironment, as well as in TDLNs [158]. Tumor-associated T_{regs} suppress anti-tumor immune responses by secreting IL-10, other immunosuppressive cytokines (e.g. IL-35), and their expression of high-affinity co-inhibitory ligands that compete against effector T cell co-stimulatory ligand binding to molecules such as CD80/86 on the surface of antigen presenting cells (APCs), which are critical for the induction of effective anti-tumor T cell responses [127]. Another serious drawback of IL-2 immunotherapy lies in its toxicity profile. Systemic IL-2 administration must take place in an in-patient setting as it causes severe hypotension and pulmonary edema, secondary to an increase in capillary permeability (also known as vascular leak syndrome). These symptoms may also be exacerbated by a storm of pro-inflammatory cytokines and acute phase reactants, such as TNF-α, IL-1β, and IL-6, which are released as a result of IL-2 administration [159, 160]. More long-term, IL-2 is known to cause lymphopenia and is also cardiotoxic [161].

Current strategies to maximize the therapeutic effects of IL-2 include targeted tumor delivery, to diminish the negative side effects of systemic delivery. Use of immunostimulatory bio-scaffolds, nano/microparticle delivery systems, complexing IL-2 with antibodies, or transduction of IL-2 cDNA into the tumor microenvironment have been attempted with varying degrees of success. Other groups, including our own, have tried to enhance the efficacy of IL-2-based cancer immunotherapy by modifying the structure of IL-2 [162]. Stagg and Penafuerte et al. showed that fusion of IL-2 to GMCSF (GIFT2, discussed in 1.2.1) enhances NK cell-mediated anti-tumor immunity beyond the effects of the two cytokines used independently [163]. Further, Penafuerte et al. was able to show that fusion of IL-2 to a TGF-β antagonist (FIST2) was able to overcome immunosuppression from tumor-derived TGF-β and enhance the pro-inflammatory effects of the IL-2 moiety of the fusion protein. Other groups have created IL-2 “superkines” with targeted mutations in IL-2 peptide sequence, such that they display higher affinity for the IL-2 receptor complex, resulting in enhanced IL-2 bioactivity (including greater T cell proliferation and *in vivo* anti-tumor activity), while apparently diminishing IL-2 related side-effects, such as flash pulmonary edema [162, 164]. The future of IL-2 as a immunotherapeutic agent will likely rest on more targeted delivery approaches, structural modification, and its use in combination with other immune modulating agents.

Pre-clinical studies utilizing IL-15 have been a promising alternative to IL-2 based approaches, due to many of their shared biological activities, with the key difference being that IL-15 seems to have a much safer toxicity profile [165]. IL-

15 also differs from IL-2 in their biological effect on CD4+ T_{regs}. Whereas IL-2 potently induces Treg formation, IL-15 has no such effect [166]. This difference makes IL-15 a more favorable candidate in cancer immunotherapy, since T_{regs} can potently suppress any intended anti-tumor effector T cell responses. A further difference in IL-15 cytokine immunotherapy is the biological response elicited from different cellular subsets. IL-15 stimulation of naïve T cells favors differentiation and proliferation of memory T cell subsets, as well as proliferation and activation of NK cells [167]. On the other hand, IL-2 tends to drive the terminal differentiation of naïve T cells into effector T cells [168]. Interestingly, unlike IL-15, IL-2 is mainly produced by activated T cells, whereas IL-15 is hardly produced by T cells and is typically expressed by DCs, macrophages, monocytes, stromal, and endothelial cells [169-171]. Although both IL-2 and IL-15 seem to share overlapping roles in NK cell proliferation and activation, mice deficient in IL-15 or IL-15Rα lack NK cells, but normal NK cell numbers are seen in IL-2 deficient animals [172, 173]. This would suggest that IL-15 is critical in the development of NK cells, whereas IL-2 may play more of an accessory role and come to importance in later stages of maturation or acquisition of effector functions. A number of groups have demonstrated that IL-15 may prove superior to IL-2 in both direct administration to tumor-bearing subjects or in adoptive cellular therapy approaches using either cytokines to expand cells prior to transfer [174, 175]. Similar to IL-2, IL-15 has been shown to be efficacious in a variety of pre-clinical mouse primary and metastatic tumor models (e.g. melanoma and colon carcinoma). In phase I clinical trials utilizing IL-15, it was

found that at maximum tolerated doses, IL-15 administration resulted in transient 10-fold expansion of NK cells, memory $CD8^+$ T cells, and γδ T cells [176]. Although trials involving the use of IL-15 are recent, and therefore still in the process of data collection, early efficacy data has shown that IL-15 can indeed cause tumor regression, and boost the effect of TILs in adoptive cell therapy approaches [166].

A key difference in the signaling infrastructure of IL-15 and IL-2 is likely the cause of differences seen in the biological effects of these respective cytokines. IL-2 is typically secreted as a soluble protein that binds to all three components of its receptor on the surface of a responding cell. IL-15, on the other hand, is almost always bound to the IL-15Rα chain when it is translated and chaperoned to the surface of the cell. From there, it is presented in *trans* to cells expressing the γc and βc [177]. Due to this difference, the use of monomeric, soluble IL-15 likely does not reflect the true potential efficacy of this cytokine, since its bioactivity may very well be limited by the amount of IL-15Rα chain-expressing cells). Modification of IL-15 by complexing it with a soluble isoform of the IL-15Rα chain (discussed in Chapter 2) or stabilization with antibodies could enhance its biological effects [178]. Similar to IL-2, use of IL-15 in the clinical setting is hampered by its short half-life and quick renal clearance. Indeed, several groups have shown that soluble IL-15Rα/IL-15 complexes improve the half-life and anti-tumor effect of IL-15 [179, 180]. A clinical trial involving the use of an IL-15Rα/IL-15-Fc heterodimeric fusion protein by Altor Biosciences was initiated in 2015 (NCT01885897). While the effects of IL-15 has proved promising, its future

development will likely involve its use in conjunction with other anti-tumor therapeutic platforms, as is currently being tested with conventional chemotherapeutics, biologics (e.g. Rituximab), and adoptive cell therapy utilizing autologous NK cells.

IL-21 is the next most studied γc cytokine in the context of cancer immunotherapy. IL-21 does not appear to be as potent a mitogen for T cells or NK cells as either IL-2 or IL-15. Expansion of TILs for adoptive transfers show that compared to IL-2 and IL-15, the use of IL-21 alone yields 50-fold lower T cell yield [181]. However, qualitatively, it appears that IL-21 may be important for maturation and acquisition of effector functions in T cells and NK cells, especially in conjunction with IL-2 and IL-15 [152]. IL-21 signaling seems to modulate expression of a great many number of transcription factors that are involved in effector differentiation, including Bcl-6 (memory T cell formation), Blimp-1 (effector $CD8^+$ T cell), and T-bet (T_H1 $CD4^+$ T cells, and effector $CD8^+$ T cells) [182, 183]. IL-21 is mainly produced by CD4+ T follicular helper (T_{FH}) cells, which promote the development of cytotoxic $CD8^+$ effector T cells through upregulation of genes such as granzyme B and perforin. Pre-clinical studies utilizing IL-21 have largely shown that *in vivo*, IL-21 acts as an immunostimulatory agent to promote the rejection of tumors. In B16 melanoma and fibrosarcoma models, delivery of IL-21 containing plasmids to tumor-bearing animals resulted in significant inhibition of tumor growth and increased survival [184]. The authors noted that this effect was dependent on NK cells, as their depletion abolished IL-21's therapeutic effect. IL-21, like IL-2 and IL-15 can stimulate NK cells to

produce IFN-γ, and also acts to enhance the cytotoxicity of NK cells [185]. TILs cultured in the presence of IL-21 persisted longer in tumor-bearing hosts and exhibited greater anti-tumor effect than TILs cultured in IL-2 in a mouse model of B16 melanoma [182]. However, IL-2 was required to maintain adoptively transferred TILs in the tumor-bearing host for the observed effect. Clinical trials involving the use of IL-21 are all relatively recent, owing to the fact that it was last of the γc cytokines to be discovered. In phase I clinical trials assessing safety, IL-21 seems to have a safer toxicity profile than IL-2, even at higher doses that could not be achieved due to dose-limiting toxicities with IL-2. Early phase 2 trial data shows that IL-21 monotherapy may have modest anti-tumor effects [186]. However, these early reports are based on very low numbers of patients and whether effects are durable will remain to be seen. As with IL-2 and IL-15, IL-21 monotherapy will not be able to induce regression of all cancer types in all patients. Current clinical trials are now focusing on the use of IL-21 with targeted therapies and biologics in an attempt to elicit synergistic anti-tumor effects.

Although IL-2, IL-15, and IL-21 are the three most studied γc cytokines for potential use in the setting of cancer immunotherapy, the other γc cytokines have also been tested and studied at least in pre-clinical models. IL-7 is well known for its effects on T cell development and thymopoiesis, its ability to promote the survival and maintenance of both naïve and memory T cell pools, and its ability in reconstituting adaptive immunity after lymphocyte depletion (e.g. by radiation, chemotherapy, etc.) [187]. In early experiments looking at the efficacy of IL-2, IL-4, or IL-7 monotherapies, isolation of $CD8^+$ T cells from the draining lymph nodes

of fibrosarcoma tumors were treated with each of the above cytokines and analyzed for their ability to lyse target tumor cells. IL-7 was shown to be the most effective cytokine at inducing $CD8^+$ T cell proliferation, and was shown to be 4-fold more effective at generating CTLs capable of eliminating established tumors compared to $CD8^+$ T cells cultured in media alone [188]. Adoptive transfer of T cells cultured in the presence of IL-7 and IL-15 were superior at inducing regression of established 4T1 mammary and B16 melanoma tumors compared T cells cultured in IL-2 alone [189, 190]. Due to its immune reconstitution effects and its known biology on T cells, IL-7 has also been tested in a variety of tumor vaccine studies, with the hope that it may act in an adjuvant-like capacity to spur more effective adaptive immune responses [191]. Initial studies showed that adjuvant IL-7 in the setting of tumor vaccines enhances their efficacy, both in whole-cell tumor vaccines and in viral-vectors expressing tumor-associated antigens. Whole-cell RM-9 prostate tumor cell vaccines transduced to express IL-7 also prolonged the survival of mice challenged with RM-9 tumor cells, compared to mice receiving the non-transduced RM-9 vaccine [192]. While the pre-clinical data was encouraging, early clinical trials involving the use of IL-7 showed that it could be safely administered with few dose-limiting toxicities, and that it could effectively expand both $CD4^+$ and $CD8^+$ T cell subsets with increased TCR repertoire diversity [193]. However, these effects were not correlated to any significant anti-tumor effects [194].

The role of IL-4 in anti-tumor therapy is the most contentious, with some data suggesting that it induces pro-inflammatory anti-tumor effects and contrary

evidence suggesting that it may actually promote tumor growth [195]. Initial experiments utilizing J558L plasmacytoma and K485 mammary adenocarcinoma cell lines expressing IL-4 showed that they were potently rejected by both immunocompetent mice and even in mice lacking functional T cells [97]. Similarly, in rats, use of an IL-4 expressing 9L glioma vaccine, showed greater efficacy on challenge with wildtype 9L gliomas compared to GMCSF, IFNα, and IL-12 expressing vaccines [196]. However, in a series of experiments showing that tumors in mice grew slower or were rejected completely in IL-4 knockout animals, the role of IL-4 in tumor immunity became more perplexing [197]. Further, it was shown that administration of IL-4 could actually promote metastases of B16 melanoma, and that a variant of highly metastatic B16 melanoma (B16F10) selectively induced CD4+ T cells that expressed high levels of IL-4 [198]. While phase I clinical trials show that IL-4 may be safely administered, small phase II clinical trials offer little evidence that IL-4 monotherapy provides clinical benefits to cancer patients [199]. Further research has shown that tumors upregulate expression of IL-4 receptor, where signaling could upregulate expression of pro-survival molecules, such as Bcl-XL and cFLIP, preventing apoptosis [200]. It has also been shown that IL-4 can inhibit $CD8^+$ T cell production of anti-tumor effector molecules, rendering them poorly cytolytic [201].

The least studied of the γc cytokines is IL-9 and its role in cancer is even less well-understood. IL-9 is produced mainly by T cells and was discovered as a T cell growth factor for long term T cell cultures [18]. It has been shown that treatment of $CD4^+$ T cells in the presence of IL-4 and TGF-β enhances the

formation of T cells expressing IL-9 (T_H9 cells) [202]. Physiologically, it appears that IL-9 promotes mast cell growth and activation, but also has effects on the immune system by skewing of $CD4^+$ T cell responses towards a T_H2 phenotype, and enhancing the suppressive effects of T_{regs} [18, 203]. Despite the net immunosuppressive effects of IL-9, it is important in mast cell-mediated isolation and expulsion of certain parasitic pathogens (e.g. *Trichuris muris* and *Schistosoma spp.*) [204]. Sporadic pre-clinical reports have shown that IL-9 can mediate anti-tumor immunity. Lu et al. showed that IL-9 skewed (primed in T_H9-polarizing conditions) $CD8^+$ T cells were more effective at inducing B16 tumor regression in an antigen-specific manner when adoptively transferred into tumor bearing hosts compared to conventionally IL-2 expanded $CD8^+$ T cells [205]. Purwar and colleagues showed that adoptive transfer of tumor antigen-specific $CD4^+$ T_H9 cells suppressed growth of B16F10 melanoma in mice. Further, they showed that this effect was specific to IL-9 as administration of an IL-9 neutralizing antibody abrogated the anti-tumor effects seen with T_H9 adoptive transfer [206]. Despite these observations, no clinical trials utilizing IL-9 have been undetrtaken. In fact, IL-9 neutralizing antibodies have reached the clinical trial stage, owing to its known role in promoting airway reactive disease and asthma, by encouraging infiltration of eosinophils and mast cells into the airway [207].

While the majority of γc cytokines' abilities to enhance the effector functions of immune cells may mediate tumor regression and eventually lead to the eradication of tumor, their ability to do so is exquisitely context-dependent.

Immune cells polarized in an immunosuppressive microenvironment generally are unable to mount effective anti-tumor responses, and in many cases, may even be co-opted by tumors to enhance tumorigenesis and suppress active anti-tumor immunity. Tumors and the microenvironment in which they elaborate and thrive are critical in predicting the outcome and efficacy of any immune response.

1.1.4 TGF-β and the anti-tumor immune response

As reviewed in the above sections, transforming growth factor-beta (TGF-β) plays a pleiotropic and very important role in both tumorigenesis and the immune response. There are three isoforms of TGF-β in humans (TGF-β1, 2, and 3), although TGF-β1 has been reported by many to be the most abundant isoform in the body, and the most relevant to tumor growth and immune suppression [208]. TGF-β is produced in the cell as a propeptide homodimer (in a small latency complex, associated with the latent associated peptide, LAP), before being secreted to the ECM in a large latent complex (LLC), where the LAP and TGF-β becomes associated with a latent TGF-β binding protein (LTBP). Release of active, soluble TGF-β is typically regulated by the control of proteases, such as plasmin and matrix metalloproteases (MMPs), or by changes in LLC conformation mediated by ECM glycoproteins [208]. TGF-β is important for many developmental and regulatory processes, by modulating transcription of genes that trigger apoptosis, cell cycle arrest, and tissue differentiation [209]. TGF-β is known to play a dual role in the growth of tumors. At the onset of tumorigenesis, TGF-β acts predominately as a tumor suppressor by inhibiting tumor cell growth, preventing the production of tumor stroma-derived mitogenic factors, and

suppression of pro-tumorigenic inflammation [210]. However, as tumor growth progresses, many cancers have mutated (or downregulate) TGF-β signaling components, and become insensitive the growth-inhibitory effects of this cytokine, they may secrete it to remodel the stroma for tumor invasion and to suppress the anti-tumor effector functions of the immune response [211].

Almost all human cells are capable of responding to TGF-β, and immune cells are no different. In fact, TGF-β is critical for the normal, homeostatic regulation of the immune system. Indeed, genetic deletion of TGF-β1 results in overwhelming inflammatory disease in mice, ultimately resulting in death within 3-5 weeks of age [212]. TGF-β has been shown to suppress the anti-tumor effects of NK cells by a variety of mechanisms [213]. *In vitro*, TGF-β1 inhibits NK cells responsiveness to IL-2 and its ability to proliferate in response to stimuli (such as IL-15) [214, 215]. TGF-β has also been shown to inhibit IFN-γ secretion, which is vital to the anti-tumor response [216]. Without being able to induce IFN-γ signaling, other infiltrating NK cells cannot become activated and tumor cells may not be stimulated to increase presentation of intracellular (and possibly oncogenic) antigens that could be recognized by infiltrating NK cells. TGF-β may disrupt IFN-γ production through the inhibition of T-bet, a well-known transcription factor for IFN-γ secretion and maintenance of T_H1 cell fate [217]. Moreover, TGF-β has been shown to actively suppress the cytolytic abilities of NK cells in *in vitro* and *in vivo* settings [214, 218]. Activating cytotoxicity receptors, which are the means by which NK cells identify target cells for lysis are also

apparently downregulated in the presence of TGF-β, specifically NKG2D and NKp30 [219].

$CD8^+$ T cells, which form another major anti-tumor effector cell population, are also profoundly suppressed by TGF-β. Much like its effects on NK cells, TGF-β inhibits $CD8^+$ T cell expression and secretion of IFN-γ. This is especially detrimental to $CD8^+$ T cell-mediated immunity, as these cells are designed to recognize antigen in the context of IFN-γ inducible MHC-I molecules. Lack of an IFN-γ response results in less effective cytotoxic responses. $CD8^+$ T cell effector molecules, such as perforin, granzyme, and FasL expression, are also all inhibited in the presence of TGF-β [220]. Interesting mechanistic studies have been done to dissect the relative contribution of TGF-β signaling *in vivo* on immune cells, as opposed to tumor cells. $CD8^+$ T cells genetically engineered to harbor a dominant negative TGF-β receptor (type II), which renders them insensitive to TGF-β, were shown to penetrate tumors with higher frequencies and displayed enhanced anti-tumor functions (i.e. cytokine secretion, tumor cell lysis, etc.) compared to wildtype $CD8^+$ T cells. More recent studies have also shown that TGF-β is also a key cytokine responsible for the apoptotic, contraction phase of $CD8^+$ T cells following their activation to effector status [221]. Although this has only been shown in models of bacterial or viral infections, it is speculated that TGF-β within the tumor microenvironment induces apoptosis of TAA-specific $CD8^+$ T cells, which were previously activated to become anti-tumor effector cells [222].

Beyond these two cell populations, the effects of TGF-β have been reported to be immunosuppressive in a variety of other cell types, as reviewed in [223]. Any meaningful immunotherapeutic strategies against cancer must take into account the innate anti-tumor effector responses of the immune system, the ways in which tumors have evolved to overcome these responses, and how to tip this balance in favor of tumor eradication and the formation of robust and long-lasting anti-tumor immunity.

1.2.0 Overview of granulocyte colony-stimulating factor (GMCSF) and γc interleukin fusion proteins (GIFTs) [224]

Cytokines hold an important place within the field of immunology; soluble substances secreted from white blood cells that could alter the behavior of other cells were predicted in the early-20th century, but was not confirmed until 1957 with the discovery of interferon, and later on, with the identification of a certain T cell derived “lymphocyte activating factor”, which was ultimately demonstrated to be IL-2 in 1976 [1, 10, 11]. Since then, the field of cytokine biology has expanded rapidly with the advancement of molecular cloning techniques, and more recently with genome-wide screens. Cytokines control everything from the maintenance of homeostasis to activation, proliferation, and even the programmed cell death of immune cells [225]. This made cytokines a natural target for manipulation and clinical translation. With all the important functions ascribed to cytokines, and in particular the interleukins, it was hoped that they would behave as a sort panacea for all the ailments of the immune system. However, despite the large numbers of clinical trials established over the past several decades, IL-2 remains the only FDA-approved common gamma-chain (γc) interleukin for clinical use,

others having been abandoned for reasons such as low therapeutic efficacy or harmful side effects.

In an attempt to improve upon the efficacy of endogenous cytokines, our group and others have explored the fusion of cytokines, or fusokines, as an experimental way to augment the effector functions of the immune response [226, 227]. We have discovered that fusokines can pharmacologically impel the clustering of unrelated, but activated cytokine receptors together, transducing unique and supraphysiological signals that ultimately confer novel biological effects in responsive cellular subsets [228]. Whereas monomeric cytokine therapy may elicit only physiological immune responses owing to the natural co-evolution of regulatory mechanisms to limit cytokine-mediated effects, fusokines are not bound by the same regulatory constraints [226]. Fusokines also allow for two different bioactive ligands to act in the same time and space, an important synergy that cannot be guaranteed even in combinatorial cytokine treatment. Moreover, fusokines direct their immunomodulating effects specifically to cellular subsets that express receptors for both moieties of the fusion. In this way, fusokines may be rationally designed to target only the cells from which we wish to elicit an effect. This important consideration has obvious implications for the therapeutic index of fusokines in the clinical setting, a problem that has posed a significant roadblock for the widespread use of endogenous cytokines, which have significant off-target effects [229-231]. With this in mind, we turn our attention to the ways in which **GMCSF** **I**nterleukin **F**usion **T**ransgene (**GIFT**)

fusokines are able to modulate the immune response, particularly in cancer and autoimmune conditions.

GMCSF is a FDA-approved recombinant molecule predominately utilized in bone marrow transplant settings to aid patients in the reconstitution of their granulocytic and monocytic hematopoietic compartments [232]. More experimentally, it has been repeatedly shown to be an effective cytokine at inducing anti-tumor responses in cancer vaccine models [233, 234]. This has been attributed to its ability to drive the maturation of dendritic cells (DCs), which present tumor-associated antigens to T cells, thereby triggering an adaptive immune response against the tumor. With a relatively safe toxicity profile, GMCSF became the parental cytokine from which the GIFTs were derived [235].

The γc family of cytokines consists of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. The unifying characteristic of all these cytokine members is that they utilize the γ-c chain (also known as CD132) as a part of their receptor complexes. These cytokine receptor complexes also include an alpha chain (α-chain), which confers ligand specificity to each individual cytokine. IL-2 and IL-15 additionally utilize a shared beta chain (β-chain, CD122) as a part of their receptor complexes.

The γc cytokines were a logical target for fusion to GMCSF because of their well-studied and potent pro-inflammatory effects. It is worth noting that interspecies differences in cytokine signaling exist to varying degrees, depending on the cytokine in question [236]. However, the major functions of individual cytokines are generally well conserved in mammalian species (e.g. mice, non-human

primates, and humans), from which all GIFT preclinical data discussed in this review have been derived. These cytokines have pleiotropic functions that are critical for both the homeostasis and effector functions of the immune cellular repertoire [237, 238]. While these cytokines use shared receptor components for ligand binding and signal transduction, the repertoire of receptors expressed are tightly regulated and vary amongst different immune cell types, ensuring fine-tuned specificity in response to cytokine stimulation.

1.2.1 GIFT-2

IL-2 was the first candidate to be fused to GMCSF due to its potent effects on T cell proliferation and activation. As one of the few cytokines approved by the FDA for the treatment of metastatic disease, IL-2 (Aldesleukin) is also known to be one of the most effective cytokine at promoting the loco-regional rejection of live tumor cells [239, 240]. GMCSF, on the other hand, has proven to be superior at inducing long-term anti-tumor immunity in irradiated tumor vaccine studies [79]. The hope was to create a fusokine capable of recruiting the complementary arms of the anti-cancer response in both the lymphoid and myeloid components of the immune system. Priming of tumor antigen presentation and maturation of dendritic cells enhanced by GMCSF can spur an adaptive anti-tumor response that may be subsequently amplified by IL-2.

GIFT-2 was created by cloning the cDNA of IL-2 in frame directly 3' to GMCSF [241]. Given the primary amino acid sequence of GIFT-2, computer-based molecular modeling predicted that both moieties of the protein would be able to

properly fold without major structural changes to the domains responsible for receptor binding. This was confirmed with bioactivity assays, assessing the proliferative capacity of CTLL-2 and JAWS-II cells when cultured in the presence of GIFT2, two cell lines that are dependent on IL-2 and GMCSF, respectively. With *in vitro* confirmation that both moieties of the fusion protein were bioactive, we next moved *in vivo* to ascertain the therapeutic effects of GIFT-2 in a mouse model of melanoma. GIFT-2 was just as effective as IL-2 at inducing loco-regional tumor rejection, and outperformed GMCSF in an irradiated tumor vaccine model where mice that received irradiated B16 melanoma cells secreting GIFT2 (B16-GIFT2) were protected from subsequent tumor challenge. Furthermore, in a therapeutic cancer vaccine model, more mice with pre-established B16 tumors were able to progress to a tumor-free state when given irradiated B16-GIFT2 cells compared to mice receiving irradiated B16 cells that secreted GMCSF and IL-2.

One interesting observation made in the GIFT-2 studies was that B16 tumors secreting GIFT-2 recruited significantly higher numbers of NK cells into the tumor microenvironment compared to B16 cells secreting GMCSF. Although GMCSF has been observed to impair NK cell function, which may explain why GMCSF cannot durably induce rejection of live tumor cells, the addition of B16 cells secreting IL-2 to these B16-GMCSF tumors could not rescue NK cell infiltration into the tumor [163]. This observation illustrates an important point when considering combinatorial cytokine therapy; use of individual cytokines together may elicit conflicting biological responses [242]. Such antagonistic responses

may be overcome with the use of a single, fused molecule, as is the case with GIFT-2. This observation also showcases how fusokines may act in a synergistic manner to confer a gain-of-function above and beyond simply administering two individual cytokines in the same time and space. Indeed, in follow-up studies using the human ortholog of GIFT-2 (hGIFT-2), Penafuerte et al. was able to show that compared to treatment with GMCSF and IL-2, hGIFT-2 could differentially activate human NK cells by increasing their expression of surface markers associated with enhanced cytotoxicity, allowing them to more effectively lyse target cells [163].

1.2.2 GIFT-15

IL-15 was next to be fused to GMCSF due to its similarity to IL-2. IL-15 utilizes a very similar receptor complex to IL-2, with the exception of an IL-15Rα-chain, which specifically allows for IL-15 to associate with the shared IL-2/15Rβ- and γ-c chains [243]. Like IL-2, IL-15 has been shown to stimulate T cell proliferation and NK cell activation. However, IL-15 is distinct in that it can prevent activation-induced cell death (AICD) of T cells and stimulate the proliferation of memory phenotype $CD8^+$ T cells, both processes that are actually inhibited in the presence of IL-2 [244, 245]. It was thought that GIFT-15 would behave similarly to GIFT-2, acting as a pro-inflammatory molecule. It was therefore an unexpected surprise when GIFT-15 behaved as a potent immunosuppressive agent. Rafei et al. first noticed this intriguing phenomenon when B16 cells transduced to express GIFT-15 grew at a significantly faster rate compared to wildtype B16 cells when implanted into mice [246]. Moreover, human U87 glioma

cells expressing GIFT-15 could be successfully xenografted into immunocompetent mice without subsequent rejection, pointing to this fusokine's profoundly immunosuppressive properties.

GIFT-15's surprises did not end there. When unfractionated splenocytes from mice were treated with this fusokine, a population of naïve MHC-II+ B cells was enriched. While B cells express both GMCSF and IL-15 receptors, they were not predicted to be the most responsive subset of immune cells to GIFT-15, especially given IL-15's well-characterized effects on NK- and T cells [247, 248]. Moreover, GIFT-15 converted these naïve B cells into a regulatory phenotype, characterized by high surface expression of CD1d and the secretion of IL-10 [249]. IL-10-secreting regulatory B cells (B_{regs}) have become a topic of intense interest in the field of clinical immunology, owing to their ability to negatively modulate the immune response in autoimmune conditions [250]. Their presence and dysregulation have been noted in conditions ranging from rheumatoid arthritis to systemic lupus erythematous in both human patients and experimental mouse models. Akin to their T cell counterparts, B_{regs} are able to suppress overt inflammatory processes by secretory factors, such as IL-10 and transforming growth factor-β (TGF-β), which inhibit IFN-γ production by T_H1 cells, as well as the *in vitro* differentiation of T_H17 cells [251, 252]. While naturally occurring B_{regs} exist in a variety of phenotypes in humans, they occur in very small numbers and are difficult to propagate *ex vivo*. GIFT-15 provided an obvious remedy to this problem by converting large numbers of naïve B cells into B_{regs}. With this insight, Rafei et al. found that B_{regs} derived from GIFT-15 treatment (GIFT-15 B_{regs}) could

durably induce remission of disease when adoptively transferred into mice with experimental autoimmune encephalomyelitis (EAE), a murine model of the autoimmune demyelinating human disease, multiple sclerosis (MS).

The transfer of two million GIFT-15 B_{regs} ameliorated severe neurological symptoms, such as hind-limb paralysis, in mice with EAE. These findings also correlated with histopathological data showing significantly lower numbers of proinflammatory cell infiltrates in the central nervous system of GIFT-15 Breg treated mice. Interestingly, GIFT-15 B_{regs} derived from IL-$10^{-/-}$ mice were unable to suppress neuroinflammation, suggesting that the secretion of IL-10 was necessary for their immunosuppressive effects. However, while IL-10 is necessary for suppression, it is not sufficient as GIFT-15 B_{regs} derived from MHC-$II^{-/-}$ mice were also incapable of dampening neuroinflammation. This latter observation suggests that interaction with CD4 T cells plays a critical role in the therapeutic efficacy of GIFT-15 B_{regs} in EAE. In addition to their ability to stop the progression of autoimmune reactions, GIFT-15 B_{regs} may also be effective in suppressing exuberant allogeneic responses. This has exciting clinical implications in the realm of solid organ or allo-bone marrow transplant where such allogeneic responses are detrimental.

GIFT-15's narrative serves to remind us of two very important points in the development of the fusokine platform. First, while it is possible to rationally design fusion proteins with a desired biological phenomenon in mind, the resulting fusokine may have novel, unpredictable, and unanticipated effects. Second, although fusokines may be used as a protein biologic for direct

administration to patients with autoimmune disease or cancer, their utility as agents to augment immune cells *ex vivo* may prove just as valuable. Indeed, the B_{regs} generated by GIFT-15 *ex vivo* becomes the therapeutic product that may be used to treat patients, as opposed to GIFT-15 itself. The dichotomy between creating a pharmaceutical molecule versus a cellular product for clinical use may seem like an arbitrary distinction but has meaningful implications related to regulatory approval.

1.2.3 GIFT-21

IL-21 is the most recently identified member of the γ-c family of cytokines and predominately acts to promote the function of mature effector cells in the immune system [253]. IL-21 differentiates CD4+ T- cells down the T_H17 pathway, activates NK cells, and stimulates $CD8^+$ T cells to mount anti-tumor responses [254-256]. We hypothesized that fusing GMCSF and IL-21 (GIFT-21) would lead to synergistic anti-cancer effects because of each cytokine's respective role in mediating inflammation. The GIFT-21 fusokine had unanticipated hypermorphic effects on the monocyte lineage of cells, inducing their maturation into a distinct dendritic cell (DC) population with tumoricidal properties [257]. GIFT-21 induced DCs (GIFT21 DCs) display enhanced antigen presentation properties and secrete substantially more pro-inflammatory cytokines that ultimately drive a T_H1-polarized response [258]. When adoptively transferred into B16 melanoma or D2F2 breast cancer bearing mice, GIFT-21 DCs were able to inhibit tumor growth even without prior antigen priming. Analysis of tumor explants revealed that GIFT-21 DCs could readily migrate into the tumor microenvironment within

24 hours post-transfer to sample antigen, while conventional DCs (cDCs, monocytes matured into DCs with GMCSF and IL-4), were absent. DCs have been the subject of many immunotherapeutic studies in clinical oncology, since they act as gatekeepers to an effective adaptive immune response [259]. Unfortunately, DCs are relatively rare, difficult to isolate, and even more challenging to propagate *ex vivo* to numbers that result in meaningful clinical outcomes when administered to patients. Furthermore, many clinical trials focus on priming DCs by exposing them to a single (or a few pre-selected) antigen(s) before transfusion, limiting the repertoire of antigenic determinants presented, and the efficacy of the ensuing adaptive immune response [260]. GIFT-21 provides an attractive alternative to DC-based immunotherapy by converting readily available and abundant monocytes into hyperactivated DCs, capable of inducing effective anti-tumor responses even without prior exposure to tumor antigens.

1.2.4 GIFT-4

GIFT-4, borne of the linkage of GMCSF and IL-4, triggers an anti-tumor response that is B cell dependent [261]. Mice with pre-established B16 or melan-a $GNAQ^{Q209L}$ melanoma tumors treated with GIFT-4 displayed significant inhibition of tumor growth compared to control mice receiving GMCSF and IL-4 treatments. B16 tumors engineered to express GIFT-4 (B16-GIFT4) were significantly attenuated when implanted into wildtype C57BL/6J mice, but this effect was lost when the same cells were implanted into B cell deficient μMT mice. The latter result provides evidence that GIFT-4's ability to suppress melanoma growth is

dependent on B cells. Deng et al. were further able to show that the human ortholog of GIFT-4 could stimulate the proliferation and activation of B cells derived from the peripheral blood of healthy human subjects. These GIFT-4 treated B cells (GIFT4 B cells) expressed substantially higher co-stimulatory and antigen-presentation markers, in addition to secreting significantly higher concentrations of IL-2 and IL-6, amongst other pro-inflammatory cytokines. GIFT4 B cells robustly promoted T cell proliferation in *in vitro* co-cultures and primed them to become anti-tumor cytotoxic effectors by inducing T cell production of granzyme B, granulysin and IFN-γ. These GIFT-4 B cell-primed T cells specifically lysed A375 human melanoma cells *in vitro* and *in vivo* when adoptively transferred into NOD *scid* gamma (NSG) mice lacking T-, B-, and NK-cells. Interestingly, GIFT-4 has very little direct effect on purified T cells, but will only license T cells to become anti-tumor effectors in the presence of GIFT4 activated B cell mediators. In addition to its effect in melanoma, GIFT-4 has more recently been shown to directly alter chronic lymphocytic leukemic (CLL) cells. GIFT-4 expressing CLL cells upregulated co-stimulatory markers and expanded anti-tumor T cells capable of lysing autologous CLL tumor [262]. GIFT-4 opens the exciting possibility of developing B cells as effectors for cancer immunotherapy.

1.2.5 GIFT-7

The fusion of IL-7 to GMCSF resulted in GIFT-7, a fusokine that preferentially affects the T cell compartment. This was not surprising, given that lymphopoeisis and many other facets of T cell development are critically dependent upon the

function of IL-7 [263]. Indeed, IL-7$^{-/-}$ mice are severely lymphopenic due to an arrest of T cell development at the pro- to pre-T cell transition in the thymus [264]. Further, IL-7 is required for the homeostatic proliferation and survival of naïve T cells [265]. GIFT-7 acted as a potent mitogen for IL-7 receptor (IL-7R) high T cell precursors derived from the thymus. The earliest of these T cell precursors, the double-negative (CD4^{-}, CD8^{-}) thymocytes, were the most responsive, with further subset analysis revealing that a CD44-intermediate expressing population of DN thymocytes expanded by more than 4-fold over GMCSF and IL-7 treated thymocytes. *In vivo* administration of GIFT-7 to young mice resulted in transient hyperplasia of the thymic cortex, an important site for T cell selection and maturation. This effect was even more pronounced in aged mice (10-15 months old), where GIFT-7 treatment led to hypercellularity of thymic cortical tissue and enhanced output of T cells into the periphery. The potential clinical significance of this observation was not lost on Hsieh and colleagues, who found that aged mice pre-treated with GIFT-7 and subsequently challenged with murine cytomegalovirus (MCMV) were superior at inducing anti-MCMV specific T cell responses compared to mice pre-treated with GMCSF and IL-7 [266]. Thymic atrophy and involution occurs as a natural process of ageing in mice, as well as humans. As we age, thymic output decreases, reducing the number of T cells in the peripheral circulation. This is one of the reasons we become more susceptible to infections as we age [267]. The ability of GIFT-7 to reverse the effects of thymic atrophy makes it an attractive molecule for clinical translation, particularly in conditions where immune senescence and exhaustion are

contributors. Chronic viral infections, cancer, and age-associated immune deficiencies are just some of the conditions that could immensely benefit from a molecule that enhances both the number and the repertoire of circulating T cells.

1.2.6 GIFT-9

IL-9 is perhaps the least studied of the γ-c cytokines, and the last of this family to be fused into a GIFT fusion protein. Best known for its affect on mast cell growth and function, IL-9 has also been ascribed a protective role against parasitic infections and implicated as a critical mediator of allergic inflammation [204, 268, 269]. Akin to IL-9, GIFT-9's predominant biological effect was seen on mast cells. GIFT-9 behaved as a hyperagonist of the IL-9 receptor (IL-9R) and was better able to induce the growth of bone-marrow mast cells (BMMCs) compared to equimolar concentrations of GMCSF and IL-9 [228].

1.2.7 GIFT Summary

With GMCSF fusions to all of the known γ-c cytokines completed, we have summarized the major findings of our GIFT fusokine research program in this article. The GIFT-family of fusokines demonstrates that the marriage of two bioactive leukines results in the formation of a novel fusion protein endowed with the ability to significantly alter lymphomyeloid cell physiology. This is due to the GIFTs ability to cluster activated GMCSF and interleukin receptors together, an unnatural interaction that results in the transduction of a unique signal, ultimately conferring responsive cells with unheralded phenotypes and effector functions; functions that may be exploited for therapeutic use in a clinical setting.

Our study of GIFTs has yielded novel insights into cytokine biology and cellular signal transduction. More importantly, they have established the fusion of cytokines as a viable biopharmaceutical platform to elicit a gain-of-function from specific cellular subsets based on their receptor expression patterns. In addition to clinical trials currently underway utilizing γc cytokines (Table 1), it is our hope that the GIFT family of fusokines will open the door to hypothesis-driven cytokine coupling strategies and meaningfully add to the growing armament of immunotherapeutic biologics.

1.2.8 Table Legends and Table

Table 1. Summary of GIFT-mediated biological effects and potential clinical applications.

The responder cell subsets, the species from which these cells were derived, and the known cellular signaling events activated by each member of the GIFT family are listed. Hyper/hypo-phosphorylation effects (Hyper/Hypo pSTAT) are based on the strength of GIFT-induced phosphorylation of STAT substrates relative to phosphorylation by GMCSF and/or the derivative monomeric common gamma-chain (γ-c) cytokine. The clinical applications outlined for each GIFT are based on *in vitro* effects observed and *in vivo* data derived from experimental animal models. H: human, M: mouse, NHP: non-human primate, MS: multiple sclerosis, SLE: systemic lupus erythematous, RA: rheumatoid arthritis, allo-BMT: allogeneic bone marrow transplant.

Fusokine	Responder Cell Subsets	Biological effect and phenotype	Potential clinical application	Signal transduction	Refs.
GIFT-2	Macrophages, NK cells	Expansion and hyperactivation of NK cells.	Cancer immunotherapy, viral infections	Hyper pSTAT-1/3/5	163, 241
GIFT-4	Naïve B cells	Expansion and conversion of naïve B cells to B effector cells. GIFT-4 B cells may also license naïve T-cells to become anti-tumor CTLs.	Cancer immunotherapy	Hyper pSTAT-1/3/5/6	261, 262
GIFT-7	Thymocytes, peripheral T-cells	Expansion of peripheral CD4+ T cells and double-negative (DN) thymocytes.	Age-associated thymic insufficiency, chronic viral infections, cancer immunotherapy.	Hyper pSTAT-5	265
GIFT-9	Mast cells	Expansion of bone-marrow derived mast cells		Hyper pSTAT-1	228
GIFT-15	Naïve B cells	Conversion of naïve B cells into immunosuppressive regulatory B cells.	Autoimmune disease (e.g. SLE, RA, MS), chronic inflammatory conditions, solid organ transplant, allo-BMT.	Hyper pSTAT-3 Hypo pSTAT-5	246, 249
GIFT-21	Monocytes, dendritic cells (DCs)	Conversion and maturation of monocytes into hypermorphic DCs.	Cancer immunotherapy	Hyper pSTAT-3	257, 258

Table 1. Summary of GIFT-mediated biological effects and potential clinical applications. Adapted from [224].

Chapter 2

Fusion of interleukin-15 (IL-15) to the *sushi* domain of the IL-15 receptor-alpha and dimeric TGF-β receptor (FIST-15) in cancer immunotherapy

Portions of this work was published in [270]

2.1.0 Abstract

The clinical efficacy of immune cytokines used for cancer therapy is hampered by elements of the immunosuppressive tumor microenvironment such as TGF-ß. Here we demonstrate that FIST15, a recombinant chimeric protein composed of the T cell stimulatory cytokine IL-15, the sushi domain of IL-15Rα and a TGF-β ligand trap, can overcome immunosuppressive TGF-β to effectively stimulate the proliferation and activation of natural killer (NK) and $CD8^+$ T cells with potent antitumor properties. FIST15-treated NK and $CD8^+$ T cells produced more IFNγ and TNFα compared to treatment with IL-15 and a commercially available TGF-β receptor-Fc fusion protein (sTβRII) in the presence of TGF-β. Murine B16 melanoma cells which overproduce TGF-β were lysed by FIST15-treated NK cells in vitro at doses ~10-fold lower than NK cells treated with IL-15 and sTβRII. Melanoma cells transduced to express FIST15 failed to establish tumors in vivo in immunocompetent murine hosts and could only form tumors in beige mice lacking NK cells. Mice injected with the same cells were also protected from subsequent challenge by unmodified B16 melanoma cells. Lastly, mice with pre-established B16 melanoma tumors responded to FIST15 treatment more strongly compared to tumors treated with control cytokines. Taken together, our results offer a preclinical proof of concept for the use of FIST15 as a new class of biological therapeutics that can coordinately neutralize the effects of immunosuppressive TGF-β in the tumor microvironment while empowering tumor immunity.

2.2.0 Introduction

Extensive studies documenting the use and efficacy of cytokine-based immunotherapy for cancer in the pre-clinical setting have largely failed to materialize into significant improvements in clinical therapy for cancer patients. To date, interleukin 2 (IL-2) remains the only FDA-approved cytokine monotherapy for the treatment of cancer [271]. Cytokines, in particular those belonging to the common gamma-chain (γc) family, IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, have been the most studied for their potential anti-tumor properties [272]. These cytokines typically signal via a heterodimeric receptor complex, sharing the use of a common gamma-chain, CD132, for ligand binding and signaling, in addition to a ligand-specific receptor alpha-chain [4]. In the case of IL-2 and IL-15, the receptor complex is heterotrimeric, owing to the use of an additional IL-2/15 receptor beta-chain (CD122) [273]. These γc cytokines are thought to mediate their anti-tumor effects through the activation of innate and adaptive arms of the immune system [272].

Indeed, IL-2 has been shown to potently activate tumor infiltrating $CD8^+$ T cells and natural killer (NK) cells, enhancing their ability to induce tumor cytolysis and clearance [148]. However, its serious and potentially life-threatening toxicity profile combined with its low objective responsive rate has made it a last line treatment in patients with metastatic disease [274]. Similar to IL-2, IL-15 has also been shown to potently suppress tumor growth in a variety of pre-clinical models

[275]. In contrast, however, IL-15 has less systemic toxicity than IL-2 and has been shown to be well tolerated in non-human primate models and preliminary human trials [165-167]. Whereas IL-2 can actually promote tumor growth by inducing regulatory $CD4^+$ T cell (T_{reg}) formation and activation-induced cell death (AICD) of $CD8^+$ T cells exposed to high concentrations of cognate antigen [276, 277], IL-15 has no discernible effect on T_{reg} formation and resists AICD by inducing expression of anti-apoptotic proteins [244, 278]. Moreover, IL-15 has a non-redundant, but critical, role in the formation and maintenance of memory $CD8^+$ T cells; an immunological effect particularly desired in cancer immunotherapy because it hints at durable, long-lasting protection against future tumor formation [174, 279].

Despite the beneficial effects of IL-15 and other γc cytokines, we and others have demonstrated that tumor-derived immunosuppressive factors severely abrogate the efficacy of cytokine and cell-based immunotherapies [280-283]. Transforming growth factor-beta (TGF-β) is one such immunosuppressive factor overexpressed by the vast majority of solid tumors [210]. TGF-β is a pleotropic cytokine involved in cell growth and differentiation, acting as a tumor suppressor early in tumorigenesis, but takes on oncogene functions late in tumorigenesis, as the tumor becomes insensitive to its growth inhibitory effects. TGF-β secreted by tumors promotes angiogenesis, potentiates the ability of tumors to metastasize from its primary site, and inhibits tumor infiltrating lymphocytes from activating and engaging in their effector functions within the tumor microenvironment [284].

$CD8^+$ T cells and NK cells are particularly sensitive to these inhibitory effects, being unable to efficiently proliferate, produce proinflammatory cytokines, and activate cytolytic pathways in the presence of TGF-β [214, 220]. Beyond directly inhibiting these subsets to evade immune responses, TGF-β can convert and recruit immune cells to promote tumor growth, such as tumor associated macrophages, myeloid tumor derived suppressor cells (MDSCs), $CD4^+$ T_{reg}, and tolerogenic dendritic cells (DCs) [285].

To enhance the efficacy of pro-inflammatory IL-15 for use in cancer immunotherapy, we here describe a novel protein therapeutic consisting of IL-15 and the *sushi* subunit of the IL-15Rα chain fused to a TGF-β ligand trap. Termed FIST-15 (Fusion of Interleukin 15 with IL-15Rα-*sushi* and TGF-β receptor), we detail the design and use of this protein as a bifunctional biopharmaceutical for use in cancer immunotherapy. We found that FIST-15 can functionally couple the immunostimulatory bioactivity of IL-15 with inhibition of TGF-β, resulting in a synergistic anti-tumor response. We found that FIST-15 predominately acts through NK cells to mediate control and clearance of tumor *in vivo*, and that FIST-15 treatment of immune competent mice bearing established tumors resulted in significant delay in tumor outgrowth and improvement in overall survival.

2.3.0 Materials and Methods

Reagents and antibodies

Antiphosphorylated and total STAT3 and STAT5 and tubulin antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-mouse FcR III/II, CD3, CD4, CD8, CD19, NK1.1, CD25, IFNγ, TNFα, IL-2, CD49b, CD95L, TRAIL, CD314, STAT5 (pY694) and their isotype control antibodies for flow cytometry were obtained from BD Biosciences (San Jose, CA). Perforin and granzyme B antibody was obtained from eBioscience (San Diego, CA).

FIST-15 protein generation

The mouse IL-15 cDNA with the mouse IL-15Rα-*sushi* domain (Genscript, Piscataway, NJ) was modified by removing the 3′ nucleotide encoding the STOP codon, and subsequently cloned in frame with two tandem repeats of mouse TGFβ receptor II ectodomain cDNA (Invivogen, San Diego, CA) to generate the cDNA for mouse IL-15/IL-15Rα-*sushi*/sTβRII fusion protein (also known as Fusion of Interleukin 15 with Sushi to TGF-β receptor; FIST-15). N'-terminally, the construct also contained a VEGF signal peptide (MNFLLSWVHWSLALLLYLHHAKWSQA), a Tobacco etch virus protease cleavage site (ENLYFQS), and an 8X His-Tag for protein purification. A stop codon was also added to the C' terminus of the construct. The vectors encoding FIST-15 were used to transfect HEK293T cells. The supernatants of transfected cells were collected after 48h, concentrated with Centricon Plus-70 (Millipore, Billerica, MA), and the molar concentration of FIST and IL-15 were quantified by IL-15/IL-15Rα complex ELISA (eBioscience, San Diego, CA). For all experiments, the mock or RPMI 1640 media contain equal volume of

concentrated media conditioned by non-transfected HEK293T cells. Infectious retroparticles encoding FIST-15 were generated with 293-GP2 packaging cells (Clontech, Mountain View, CA) and used to modify genetically C57BL/6-derived B16-F0 melanoma and pancreatic cancer MC-38 (colon adenocarcinoma) cell lines. Primary splenocytes and purified NK cells were maintained in RPMI 1640 supplemented with L-glutamate, HEPES, β-mercaptoethanol, 50U/ml penicillin and streptomycin, and 10% FBS. B16-F0 (ATCC, Manassas, VA) and MC-38 cell lines (gift from Dr. Pnina Brodt, McGill University, QC) were maintained in DMEM medium supplemented with 10% FBS and 50 U/ml penicillin and streptomycin (Wisent Technologies).

Intracellular signaling, cytokine profile, and cell proliferation analysis

NK and $CD8^+$ T cells were isolated from splenocytes of immune competent C57BL/6 mouse spleens by magnetic separation with the EasySep Mouse NK and $CD8^+$ T Cell Isolation Kits (Stemcell Technologies) according to the manufacturer's recommendations. Population purity assessed by flow cytometry was >95%. FIST-15 or control-stimulated $CD8^+$ T cells and NK cells were generated by culturing cells with 500 pM of FIST or controls (IL-15, sTβRII, and IL-15 with sTβRII) for 3 to 4 days at 37°C. For cell surface marker staining, cells were resuspended in PBS with 2% FBS, incubated with anti-mouse FcR III/II for 15 minutes and labeled with conjugated antibodies specific for CD3, CD4, CD8, CD11b, CD19, CD25, CD27, CD44, CD45, CD62L, CD49b, CD95L, CD127, CD314, Granzyme B, KLRG1, IFNγ, IL-2, NK1.1, TNFα, TRAIL. The expression

of these cell surface markers was determined by FACS Canto cytometers (BD Biosciences) and analyzed by Flow Jo. Leukocyte Activation Cocktail (BD Biosciences) was used to treat FIST-15 or control-stimulated $CD8^+$ T cells and NK cells for 4-6 hours at 37°C before cell surface staining, fixation/permeabilization with BD Cytofix/Cytoperm (BD Biosciences), and intracellular staining for cytokines and effector molecules. Proliferation was assessed utilizing CFSE CellTrace (Life Technologies) pre-labeled lymphocytes stimulated with FIST-15 or control cytokines and analyzed by flow cytometry.

In vitro cytotoxicity assays

B16-F0 melanoma cells expressing GFP were allowed to adhere overnight before purified NK cells from C57BL/6 mice were added at an Effector:Target ratio of 20:1. Increasing doses of FIST-15 or control cytokines were added to the co-culture for 48 hours. Floating cells and debris were washed away and adherent cells were trypsinized, washed, and analyzed for GFP positivity. Event counts were normalized to AccuCheck Counting Beads (ThermoFisher). Granzyme B and caspase 6 serine protease activity was assayed by PanToxiLux cytotoxicity assay kit (OncoImmunin). Co-cultures of NK cells and B16-F0 with FIST-15 or controls were set up as described above. 24 hours post-culture, the fluorogenic substrate was added to the culture for 2 hours, after which cells were trypsinized, washed, and stained with CD45. Cells were then assayed by flow cytometry to determine substrate cleavage (FL-1 fluorescence) on CD45

negative, B16-F0 cells.

In vivo experiments

All experimental C57BL/6 and knockout mice ($CD4^+$ T cell, $CD8^+$ T cell, μMT, and Beige) were females of 6 to 8 weeks old from The Jackson Laboratory (Bar Harbor, ME). Mice were implanted subcutaneously with 1×10^6 FIST-15 transduced B16-F0 (B16-FIST-15) melanoma cells. Tumor volume was determined over time. Two weeks after implantation, mice were rechallenged with wildtype B16-F0 tumors, and monitored for changes in tumor volume and survival. In a therapeutic setting, 1×10^6 B16-F0 tumor cells were subcutaneously implanted into C57BL/6 immune competent mice. Once mice developed palpable tumors, they were treated with 4 intraperitoneal doses of FIST-15 (~3ug/dose). Tumor volume and percentage of survival were determined over time. All animal experiments were performed under approved protocols of the institutional animal use and care committee (Emory University).

2.4.0 Results

Generation and characterization of murine IL-15/IL-15Rα/sTβRII fusion protein: FIST-15

We generated a plasmid construct encoding the fusion of murine IL-15 and the sushi domain of the IL-15 receptor-alpha (IL-15Rα-*sushi*; Thr 34 to Pro 109) to the C'-terminus of two tandem TGF-β traps consisting of portions of the TGF-β receptor ectodomain (TβRII-ECD) containing the conserved, structured regions required for TGF-β binding (Gln 74 to Thr 180) flanked by amino acid linkers derived from the unstructured region of the TβRII-ECD. Cloned in frame N'-terminal to these three domains were a VEGF signal peptide to direct for protein secretion to the extracellular space, an 8x-His tag, and a Tobacco etch virus (TEV) protease cleavage site for downstream protein purification (Fig. 2.1A and B). The mature, secreted FIST-15 protein is 506 amino aids in length and migrates as a ~100 kDa protein under reducing conditions on SDS-PAGE (Fig. 2.1C). A variant plasmid of this construct excluding the IL-15Rα-*sushi* domain (FIST-15Δsushi) was also generated, creating a protein of approximately 75 kDa under reducing conditions in size (Fig. 2.1C). Both constructs were able to inhibit TGF-β signaling by inhibiting Smad2 phosphorylation in unfractionated murine splenocytes treated with recombinant TGF-β1 (Fig. 2.2A and C). To analyze the effect of these constructs on the IL-15 signaling pathway, STAT3 and STAT5 phosphorylation status of unfractionated splenocytes was interrogated after FIST-15 and FIST-15Δsushi treatment. STAT5 phosphorylation was significantly diminished with FIST-15Δsushi treatment compared to IL-15. Addition of the IL-

15Rα-*sushi* domain in FIST-15 rescued STAT5 phosphorylation and induced STAT3 phosphorylation to levels comparable to equimolar IL-15 treatment as observed by immunoblot (Fig. 2.2B). Intracellular flow cytometric analysis of $CD8^+$ T cells also showed that addition of IL-15Rα-*sushi* domain significantly enhanced pSTAT5 signaling compared to FIST-15Δsushi to levels, bringing STAT5 activation to levels comparable to equimolar IL-15 treatment (Fig. 2.2D).

FIST-15 induces NK and $CD8^+$ T cell proliferation and activation in TGF-β rich environments

In order to determine the physiological effects of FIST-15 treatment on lymphomyeloid cells, we cultured splenocytes with FIST-15 or equimolar control cytokines (IL-15+sTβRII) for 72 hours and assessed the proliferation and phenotype of major cellular subsets. An increased proportion of NK and $CD8^+$ T cells were noted after three days of FIST-15 culture when compared to splenocytes that were untreated (Supplementary Fig. S2.1A). CFSE-labeling of these subsets revealed that the proportional increases seen were due to FIST-15 driven proliferation (Fig. 2.3A). FIST-15 was superior to equimolar treatment with IL-15 alone and IL-15+sTβRII at inducing $CD8^+$ T cell proliferation; while FIST-15 treated NK cells proliferated at a comparable rate to control cytokine treatment (Fig. 2.3B). $CD4^+$ T cells and B cells did not proliferate in response to FIST-15 (Supplementary Fig. S2.1B). In the presence of TGF-β1, FIST-15 significantly enhanced NK and $CD8^+$ T cell proliferation compared to control treated cells (Fig.

2.3A and B). Upon PMA/ionomycin stimulation, FIST-15 significantly augmented the ability of $CD8^+$ T cells to produce TNFα and IFNγ, but not IL-2, compared to control treated cells in the presence of exogenous TGF-β1 (Fig. 2.4A and B). FIST-15 significantly enhanced the proportion of $TNF\alpha^+IFN\gamma^+$ double positive $CD8^+$ T cells compared to IL-15+sTβRII treated $CD8^+$ T cells (Fig. 2.4C). While IL-15+sTβRII treatment enhanced the proportion of $CD8^+$ T cells that were $TNF\alpha^+IL\text{-}2^+$ and $IFN\gamma^+IL\text{-}2^+$, the relative contributions of these two subsets to the entire population are low (<15%). FIST-15 treatment also significantly decreased the proportion of triple negative $CD8^+$ T cells.

FIST-15 enhances NK cell effector molecule expression and augments *in vitro* cytolysis of B16-F0 melanoma and MC-38 colon adenocarcinoma cells by NK cells

Similar to $CD8^+$ T cells, FIST-15 treated NK cells also displayed significantly increased production compared of IFNγ compared to control treated NK cells under TGF-β rich conditions, when stimulated with PMA/ionomycin (Fig. 2.5A). Absolute numbers of NK cells secreting IFNγ, TNFα, or IL-2 were also significantly increased compared to control cytokine treatment (Fig. 5A). NK cells treated with FIST-15 also exhibited enhanced cytokine polyfunctionality, by significantly increasing the proportion of $TNF\alpha^+IFN\gamma^+$ double positive NK cells, and decreasing the proportion of NK cells that fail to produce any cytokine upon stimulation (Fig. 2.5B). To test whether FIST-15 stimulated NK cells could inhibit

tumor growth *in vitro*, we utilized the murine B16-F0 melanoma cell line transduced to express GFP (B16-GFP) in a cytotoxicity assay. B16-GFP cells were allowed to adhere overnight before being placed in co-culture with murine splenic NK cells for 48 hours at increasing concentrations of FIST-15. Adherent B16-GFP cells were then trypsinized and analyzed by flow cytometry for GFP^+ events. While FIST-15 had no direct effect on B16-GFP growth (data not shown), NK cells in the presence of FIST-15 significantly diminished B16-GFP growth (Fig. 2.6A). Using non-linear regression, a concentration of FIST-15 or IL-15 + sTβRII required to inhibit 50% of B16-GFP growth (IC_{50}) could be determined. Compared to treatment with equimolar IL-15 and sTβRII treated NK cells, FIST-15 achieved an IC_{50} approximately 10.5-fold lower (2.0 pM, FIST-15 *vs* 21.0 pM, IL-15 + sTβRII). Low or lack of MHC-I expression on target tumor cells, such as in B16-F0 melanoma has been known to spontaneously induce NK cell-mediated cytolysis (Supplementary Fig. S2.2). In order to test whether FIST-15 could stimulate NK cells to lyse MHC-I expressing cells, we utilized syngeneic MC-38 colon adenocarcinoma cells (Supplementary Fig. S.2.2). MC-38 cells labeled with CFSE were allowed to adhere overnight before co-culture with NK cells and FIST-15 or control cytokines. MC-38 cells were also susceptible to NK cell-mediated lysing in the presence of FIST-15, despite their MHC-I expression (Fig. 2.6B). However, increased concentrations of FIST-15 were required to induce comparable lysis to B16-F0 cells. FIST-15 was more potent compared to IL-15+sTβRII at inducing MC-38 cytolysis, achieving an IC_{50} approximately 14.7-fold lower (10.5 pM, FIST-15 *vs* 155.1 pM, IL-15 + sTβRII). We next investigated the

effect of FIST-15 treatment on the expression of effector molecules associated with NK cell cytotoxicity. We found significantly higher surface expression of death receptor ligands, such as Fas ligand, and the NK cell activating receptor, NKG2D, on the surface of FIST-15 treated NK cells, compared to controls (Fig. 2.6C). Intracellularly, FIST-15 treated NK cells produced significantly higher amounts of granzyme B, a serine protease released from cytotoxic granules, which activate caspases in target cells to initiate apoptosis. To determine if this was the mechanism by which FIST-15 treated NK cells induced B16-F0 cell death; we utilized a fluorochrome-based cytotoxicity assay to measure the activity of granzyme B and caspase 6 in B16-F0 cells co-cultured with NK cells and FIST-15 or control cytokines (Fig. 2.6D). After 24 hours of co-culture, higher serine protease activity was found within B16-F0 cells cultured with NK cells and FIST-15 compared to controls.

FIST-15 antitumor effect in immunodeficient mice

In order to test the anti-tumor effects of FIST-15 expression *in vivo*, we first sought to determine the effects of locoregional FIST-15 expression in the tumor microenvironment. To do this, we stably transduced B16-F0 cells to produce FIST-15. These cells, B16-FIST-15, displayed similar *in vitro* growth kinetics to mock GFP-transduced B16-F0 cells (B16-GFP), but failed to form tumor *in vivo* in immunocompetent C57Bl/6J mice (Fig. 2.7A). Mice receiving B16-FIST-15 tumor cells were also protected against subsequent rechallenge by wildtype B16-F0

melanoma cells (Fig. 2.7B). Mechanistically, we determined the immune subsets that mediated anti-tumor effect of FIST-15 through the use genetic knockout mouse models lacking individual lymphomyeloid subsets. Consistent with our *in vitro* studies, we found that a lack of functional NK cells allowed for the establishment of tumors by B16-FIST-15 cells (Fig. 2.7C). In contrast, lack of $CD4^+$, $CD8^+$ T cells, or B cells did not affect the ability of mice to mount anti-tumor responses against B16-FIST-15 cells. We rechallenged these genetic knockout strains that had received B16-FIST-15 cells with wildtype B16-F0 tumor and found that a lack of B cells and $CD4^+$ T cells significantly correlated with increases in tumor volume (Fig. 2.7D).

FIST-15 treatment significantly inhibits growth of pre-established tumors

To test the efficacy of FIST-15 as a therapeutic agent in the setting of pre-established tumor, we implanted 1×10^6 wildtype B16-F0 cells subcutaneously into the flank of immunocompetent C57Bl/6 mice and waited seven days for tumor to establish. We then treated tumor-bearing mice with intraperitoneal administration of FIST-15, IL-15 and sTβRII, or PBS every second day for 1 week (4 doses total) and monitored the mice for tumor progression and survival. FIST-15 treated mice displayed a significant delay in tumor growth compared to PBS and IL-15+sTβRII treated mice (Fig. 2.7E). We also observed a significant improvement in overall survival of FIST-15 treated mice compared to controls (Fig. 2.7F).

2.5.0 Discussion

IL-15 based monotherapy been met with moderate success in the pre-clinical arena utilizing experimental tumor models, with some groups reporting no effect, some effect, or significant effects on tumor growth [166, 275]. Consistent with other groups, we have shown that modification of the IL-15 domain of FIST-15 with the addition of an IL-15Rα-*sushi* domain increases its biological activity [286]. The reason for this, we believe, is primarily due to IL-15's unique signaling properties. IL-15 is physiologically expressed in a complex with IL-15Rα, which acts as a chaperone for IL-15 [177]. The complex is typically expressed on the surface of DCs, monocytes, and macrophages. This IL-15/IL-15Rα complex is then *trans* presented to cells expressing the γc (CD132) and the IL-2/15Rβc (CD122), resulting in phosphorylation and activation of JAK1/STAT3 and JAK3/STAT5 pathways in the cells bearing these receptors [287]. It is interesting to note that while other groups have shown that IL-15Rα results in superagonist activity, we have found that the addition of the IL-15Rα-*sushi* merely rescues STAT5 signaling to levels comparable to bacterially derived, mature IL-15, without inducing hyperphosphorylation of STAT5 (Fig. 2D) [286, 288]. This was also evident in *in vitro* cultures showing that primary NK cells expanded at similar rates with IL-15 or FIST-15, in the absence of TGF-β (Fig. 3B). The addition of the IL-15Rα-*sushi* domain to FIST-15 eliminates the need for *trans* presentation, thereby increasing its bioavailability to γc and IL-2/15Rβc expressing NK and $CD8^+$ T cells.

The neutralization of TGF-β by FIST-15 is also likely to play a role in its efficacy *in vitro* and *in vivo*. TGF-β is well documented for its ability to block $CD8^+$ T cell proliferation and activation [220]. Moreover, TGF-β has been shown to directly antagonize the pro-inflammatory effects of IL-15 in $CD8^+$ T cells [221]. In keeping with these reports, we have also observed that addition of TGF-β1 to primary cultures of NK and $CD8^+$ T cells significantly inhibited their proliferation, activation, and effector functions. However, FIST-15 treatment of these cells was able to overcome these deficits. At equimolar ratios, it appears that the tandem TβRII-ECD of FIST-15 is more effective at capturing soluble TGF-β1 than the commercially available sTβRII fusion protein. Compared to IL-15 and sTβRII, FIST-15 was superior at inducing IFNγ and TNFα production in $CD8^+$ T cells, two cytokines that are critical in mediating anti-tumor responses (Fig. 2.4A and 4B). We also assayed for the ability of FIST-15 stimulated cells to produce more than one cytokine at once (e.g. both TNFα and IFNγ, simultaneously) and found FIST-15 to be superior at inducing polyfunctional $CD8^+$ T cells (Fig. 2.4C). Such polyfunctionality has been correlated to the ability of $CD8^+$ T cells to mount robust anti-tumor responses [289]. The lack of significant differences in IL-2 production may have to do with the subset of $CD8^+$ T cells being expanded by FIST-15. $CD25^{hi}$ central memory phenotype $CD8^+$ T cells (in contrast to $CD25^{lo}$ memory $CD8^+$ T cells) are prone to terminal effector differentiation, characterized by high granzyme B and decreased IL-2 production [290]. Indeed, FIST-15 treated $CD8^+$ T cells retain all the markers of central memory phenotype T cells ($CD62L^+CD44^+$), but compared to IL-15 and sTβRII treated $CD8^+$ T cells, express

higher surface levels of CD25 (Supplementary Fig. S2.2) and intracellular granzyme B (Supplementary Fig. S2.3), without appreciable differences in IL-2 production. This would suggest that FIST-15 might preferentially expand memory $CD8^{+}CD25^{hi}$ T cells with a predilection for effector differentiation, an observation that agrees with studies demonstrating the superior polyfunctionality of $CD8^{+}CD25^{hi}$ T cells compared to $CD25^{lo}$ cells when adoptively transferred into tumor bearing mice [289]. TGF-β has been known to suppress formation of memory $CD8^{+}$ T cells, and tumor derived TGF-β has been proposed to do the same *in vivo*, resulting in inefficient priming of primary anti-tumor T cell responses and subpar memory recall responses [291, 292]. *In vitro*, we were able to demonstrate that FIST-15 can effectively expand central memory $CD8^{+}$ T cells (Supplementary Fig. S2.2).

Like $CD8^{+}$ T cells, *ex vivo* stimulation of NK cells with FIST-15 resulted in significantly higher expression of pro-inflammatory IFNγ, but not increase TNFα or IL-2, compared to IL-15 and sTβRII treatment. However, the absolute number of NK cells expressing these cytokines post-FIST-15 treatment became significant, owing to FIST-15's enhanced mitogenic response on NK cells in the presence of TGF-β1 (Fig. 2.5A). NK cells have been reported to be exquisitely sensitive to the inhibitory effects of TGF-β; arresting NK cells in immature states, downregulating their cytotoxicity receptors, and inhibiting their cytokine production and cytolytic capabilities [214]. To test the effect of TGF-β on NK cell cytotoxicity, we specifically chose B16-F0 and MC-38 tumor cells, known to

overexpress TGF-β or are insensitive to TGF-β growth-inhibitory effects, respectively, as targets in our *in vitro* killing assays. NK cells without the addition of exogenous cytokines, such as IL-15, failed to induce appreciable lysis of target tumor cells. FIST-15 proved to be a more potent stimulus of NK cell cytotoxicity compared to IL-15 and sTβRII, most likely due to its increased ability to neutralize tumor-derived TGF-β. B16-F0 cells were more susceptible to lysis than MC-38 by NK cells, perhaps owing to their poor immunogenicity. Lack of inhibitory killer-immunoglobulin like receptors (KIRs) ligand expression, such as MHC-I on target cells, lowers the threshold for NK mediated killing (Fig. 2.5B). MC-38 cells have been reported to express MHC-I basally, while B16-F0 expression of MHC-I has been reported to be low at baseline, but may be induced with IFNγ treatment [293, 294]. While increased recruitment of $CD8^+$ T cells into the tumor microenvironment has been well correlated to improved prognosis, the presence of tumor infiltrating NK cells and its effect on prognosis is more controversial. Recent studies have shown that tumor-infiltrating NK cells may convert into immunosuppressive myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment, spurring the growth of tumor [295]. Specifically, in EL-4 lymphoma and CT-26 colon adenocarcinoma models, Park et al. found that $CD11b^+CD27^+$ NK cells were prone to MDSC conversion when adoptively transferred into tumor-bearing mice, and that exposure of these NK cells to pro-inflammatory IL-2 prevented this conversion [295]. We found that FIST-15 treated NK cells expressed were predominately $CD11b^{lo}CD27^{lo}$ and $CD11b^{lo}CD27^{hi}$ (Supplementary Fig. S2.4).

The ability of FIST-15 treated NK cells to lyse target tumor cells is correlated with their expression of cytotoxic effector molecules. Intracellularly, we detected significantly higher amounts of granzyme B expression within FIST-15 treated NK cells, compared to controls, which resulted in more serine protease activity within target B16-F0 cells (Fig. 2.5C and 5D). We were unable to detect perforin expression across any of the treatment conditions by intracellular flow cytometry (data not shown). This is likely attributable to the short course of stimulation that NK cells were subjected to in this study. FIST-15 treated NK cells exhibited significantly higher surface expression of Fas ligand, a membrane protein capable of transducing pro-apoptotic signaling through CD95 Fas receptor. However, we did not detect any significant differences in TNF-related apoptosis-inducing ligand (TRAIL) expression in FIST-15 treated NK cells, another transmembrane surface protein capable of inducing apoptosis on target cells. Finally, we analyzed expression of NK cell activating receptor, NKG2D, which is known to be downregulated on NK cells by tumor-derived TGF-β [219]. NKG2D ligation with surface proteins upregulated in transformed and stressed cells promotes activation of NK cytolytic activity [296]. We found a significantly higher proportion of FIST-15 treated NK cells expressing NKG2D than control treated NK cells. Interestingly, NKG2D was also upregulated on the surface on FIST-15 treated $CD8^+$ T cells (Supplementary Fig. S2.3), where its role has been described as a co-stimulatory signal for activation [296].

The inability of B16-F0 tumors expressing FIST-15 to establish in immune competent mice was our first indication that FIST-15 was pro-inflammatory *in vivo* (Fig. 2.6A). This tumor rejection could have pointed to FIST-15 activation of either innate or adaptive immune cell subsets. To mechanistically ascertain the cellular subsets responsible for FIST-15 mediated tumor rejection, we utilized genetic knockout animals. *Beige* mice, lacking functional NK cells, were the only mice where B16-FIST-15 cells were able to form tumor (Fig. 2.6C). This would suggest that NK cells play a critical role in preventing tumor establishment in response to FIST-15 secretion locoregionally. This is unsurprising, given many reports suggesting that IL-15 mainly acts *in vivo* on NK cells to prevent tumor outgrowth [297, 298]. It should be noted that while 4 of 5 *Beige* mice developed tumors by day 7 post tumor-implantation, all of these tumors eventually regressed by day 14, likely pointing to the importance of NK cell mediated control of tumor establishment and early outgrowth. We were, however, surprised to see that B16-FIST-15 tumors failed to grow in mice lacking $CD8^+$ T cells, given the *in vitro* effects we had observed. We surmise that FIST-15 may activate innate immune cells *in vivo*, in particular NK cells, to effectively clear tumor before the need for an adaptive response was required. However, FIST-15 may have acted as an adjuvant for the adaptive arm of the immune system as B16-FIST-15 implanted mice were protected against subsequent B16-F0 tumor rechallenge in wildtype mice (Fig. 2.6B). This result stands in contrast to a recent study of IL-15 therapy in a model of established murine liver cancer, where NK cells were shown to be dispensable, but depletion of $CD8^+$ T cells resulted in uncontrolled

tumor growth [299]. The liver represents a microenvironment rich in TGF-β, thereby potentially inhibiting the cytotoxic capabilities of any infiltrating NK cells [300]. In settings such as these, use of FIST-15 may be beneficial to boost NK cell function.

When genetic knockout strains implanted with B16-FIST-15 were rechallenged with B16-F0 tumors, we observed that μMT mice lacking B cells uniformly developed tumors, and that these tumors grew at the fastest rate (Fig. 2.6D). This observation is in line with other studies suggesting an important role for antibody-mediated rejection of these tumors, most likely via antibody-dependent cell-mediated cytotoxicity (ADCC) [301]. Without the formation of tumor-specific antibodies, ADCC would be unable to occur, therefore resulting in enhanced tumor growth. Although NK cells are known to be the primary mediators of ADCC *in vivo*, it was interesting that B16-FIST-15 immunized *Beige* mice were protected from B16-F0 rechallenge. Opsonization of tumor cells by antibodies may also play a role in B16-F0 clearance by macrophages and monocytes in an Fcγ receptor-dependent manner, but we were unable to test this hypothesis within our experimental layout [301].

To our knowledge, this is the first report of immunotherapy combining IL-15 with TGF-β blockade. While other groups have shown that the use of IL-15 can elicit anti-tumor effects, especially when it is complexed with IL-15Rα or α-IL-15 antibodies, many of these studies failed to address the active mechanisms of

immunoediting and immunosuppression within the tumor microenvironment, such as the secretion of TGF-β [299, 302, 303]. In order fully realize the potential of IL-15-based immunotherapy; combinatorial strategies with other immune and tumor modulating agents will likely be employed. Indeed, reports combining IL-15 with chemotherapy, radiation, and other adjuvants have shown improved efficacy over the use of IL-15 alone [167]. Combinatorial therapy of IL-15 with checkpoint blockade inhibitors, such as α-PDL1 and α-CTLA4, has shown particular promise, due in part to the effect of these agents on tumor-associated CD4+ T_{regs}, which potently suppress anti-tumor effects of NK and $CD8^+$ T cells [285, 304]. Similarly, FIST-15 may act to inhibit tumor growth through sequestration of TGF-β and inhibition of $CD4^+$ T_{reg} formation. Derepression of tumor infiltrating NK and $CD8^+$ T cells from $CD4^+$ T_{regs} would further sensitize these cells to the activating effects of FIST-15.

In conclusion, this study provides evidence that FIST-15 can combine IL-15 agonism to TGF-β neutralization into a single immunotherapeutic agent. FIST-15 activates both innate and adaptive arms of the immune system, augmenting NK and $CD8^+$ T cell effector functions, even in TGF-β rich conditions, such as that found within the tumor microenvironment. More importantly, FIST-15 can inhibit tumor growth *in vitro* and *in vivo*, by enhancing NK cell cytolytic activity. Given the dearth of pharmacological agents available for NK cell expansion [305], we believe that FIST-15 holds great promise as a potential biologic to expand NK cells *ex vivo* for adoptive cell therapy or as a standalone immunotherapeutic

agent for use in cancer; driving pro-inflammatory IL-15 signaling on immune cells while attenuating an important axis of tumor immune evasion.

2.6.0 Figure Legends

Figure 2.1. Design and expression of murine FIST-15 and FIST-15Δsushi. FIST-15 peptide sequence is shown in (A) with a schematic of critical domains in FIST-15 and FIST-15Δsushi shown in (B). Immunoblot of murine IL-15 and murine TGF-β receptor (type II), on conditioned supernatant of human embryonic kidney (HEK293T; transduced with SV40 Large-T antigen) cells transfected with a plasmid containing cDNA of FIST-15 and FIST-15Δsushi. TβRII-ECD: sequences binding to TGF-β and linker amino acids derived from the unstructured regions of the TGF-β receptor ectodomain, sushi: interleukin-15 receptor alpha chain-*sushi* domain, IL-15: interleukin-15.

Figure 2.2. FIST-15 and FIST-15Δsushi signaling properties. Primary murine splenocytes were used as responder cells in immunoblots. FIST-15Δsushi is capable of neutralizing TGF-β1 mediated phosphorylation of Smad2 (pSmad2; Ser465/467), but was deficient at inducing phosphorylation of STAT5 (pSTAT5; Tyr694) (A). With the addition of the IL-15Rα-*sushi* domain to FIST-15Δsushi (FIST-15), STAT5 phosphorylation is rescued and STAT3 phosphorylation (pSTAT3; Tyr705) is found to be comparable to equimolar IL-15 stimulation (B). Addition of the IL-15Rα-*sushi* domain to FIST-15Δsushi did not alter its ability to neutralize TGF-β1 (C). Flow cytometric analysis of pSTAT5 (Tyr694) on primary splenic $CD8^+$ T cells upon IL-15, FIST-15Δsushi, and FIST-15 treatment over 2

hours (D). Mean fluorescence intensity (MFI) of the pSTAT5 signal ± SEM on primary splenic $CD8^+$ T cells was determined. Statistical significance was determined by Student *t* test comparing FIST-15 to FIST-15Δsushi. *, $P < 0.05$. No significant differences were detected between FIST-15 and IL-15 treatment at any time points.

Figure 2.3. FIST-15 induces NK and $CD8^+$ T cell proliferation in the presence of TGF-β. Murine splenocytes labeled with CFSE were cultured with IL-15, IL-15 + sTβRII, or FIST-15 (1000 pM) in the presence or absence of TGF-β1 (5 ng/ml) for 72 hours before flow cytometric analysis in (A). The replicative index, representing fold-expansion of cells that undergo division ± SEM of $CD8^+$ T cells and NK cells are shown in (B). Representative plots are shown in (A) and data in (B) are from three independent experiments. Statistical significance was determined by Student *t* tests. * $P<0.05$, ** $P < 0.01$, ****, $P < 0.0001$, $P > 0.05$ was considered to be not significant (ns).

Figure 2.4. FIST-15 treatment is superior at inducing $CD8^+$ T cell production of TNFα and IFNγ, but not IL-2, compared to IL-15 and sTβRII treatment. Primary splenic $CD8^+$ T cells treated with IL-15 + sTβRII or FIST-15 (500 pM) for 72 hours in the presence or absence of TGF-β1 (5 ng/ml), followed by a 6 hour stimulation with PMA/ionomycin/brefeldin A, were analyzed by flow cytometry for TNFα, IFNγ, and IL-2 production. Gating was determined by isotype controls. Representative plots are shown in (A). Histograms comparing the mean percentage of RPMI (untreated), IL-15 and sTβRII, and FIST-15 (500 pM) treated $CD8^+$ T cells for 72 hours in the presence of TGF-β1 (5 ng/ml) followed by a 6 hour

PMA/ionomycin/brefeldin A stimulation expressing TNFα, IFNγ, or IL-2 ± SEM (top panel) and the absolute number of cells expressing these cytokines (bottom panel) from three independent experiments are shown in (B). Representative pie charts displaying the proportion of $CD8^+$ T cells from the above treatment conditions producing each combination of TNFα, IFNγ, and IL-2 and histograms displaying the mean ± SEM for each combination are shown in (C). Data from three independent experiments are shown. Statistical significance was determined by Student *t* tests. * *P < 0.05*, ** *P < 0.01,* *** *P < 0.001*, ****, *P < 0.0001*.

Figure 2.5. FIST-15 augments NK cell cytokine production. Primary splenic NK cells cultured in RPMI media (untreated), treated with IL-15 + sTβRII, or FIST-15 (500 pM) for 72 hours in the presence of TGF-β1 (5 ng/ml), followed by a 6 hour stimulation with PMA/ionomycin/brefeldin A, were analyzed by flow cytometry for TNFα, IFNγ, and IL-2 production. Histograms present the mean of the percentage of NK cells ± SEM (left panel) and absolute number of NK cells ± SEM (right panel) expressing each cytokine (A). Representative pie charts displaying the proportion of NK cells from the above treatment conditions producing each combination of TNFα, IFNγ, and IL-2 and histograms displaying the mean ± SEM for each combination are shown in (B). Data from three independent experiments are shown. Statistical significance was determined by Student *t* tests * *P < 0.05*, ** *P < 0.01*, *** *P < 0.001*.

Figure 2.6. FIST-15 enhances NK cell cytotoxicity and cytolytic effector molecule expression. B16-GFP cells were co-cultured with purified splenic NK cells in the presence of IL-15 and sTβRII or FIST-15 (0.32 – 1000 pM) for 48 hours. %B16-GFP survival was calculated by dividing the number of GFP^+ events in each condition by the number of GFP^+ events in control wells containing B16-GFP cells without NK cells. Event counts were normalized to counting beads. Each point represents the mean percent survival ± SEM (A). CFSE-labeled MC-38 cell survival in the presence of NK cells with IL-15 and sTβRII or FIST-15 was similarly measured. %MC-38 survival was calculated by dividing the number of $CFSE^+$ events in each condition by the number of $CFSE^+$ events in control wells containing CFSE-labeled MC-38 cells without NK cells (B). The percentage of NK cells expressing NK activating receptor, NKG2D, Fas ligand (FasL), TNF-related apoptosis inducing ligand (TRAIL), and granzyme B (GrB) ± SEM after 72 hour treatment with FIST-15 or control cytokines, followed by a 6 hour stimulation with PMA/ionomycin/brefeldin A, is shown in (C). Serine protease (granzyme B and upstream caspase) activity using a fluorogenic substrate was measured by flow cytometry in B16-F0 target cells following 24 hour NK cell co-culture with IL-15 and sTβRII or FIST-15 treatment (0.32 – 200 pM). Percent positivity (% serine protease activity) ± SEM is shown in (D). Inset shows representative plots for co-cultures at the 40pM dose. Gating was determined using B16-F0 cells cultured in the presence of the fluorogenic substrate in the absence of NK cells as a negative control. Data from two (A, B, and D) and three (C) independent

experiments are shown. Statistical significance was determined by Student *t* tests * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2.7. FIST-15 inhibits B16-F0 tumor growth *in vivo* through NK cell activity and significantly delays growth of pre-established tumors. 1×10^6 B16-F0 transduced with FIST-15 (B16-FIST-15) or a vector containing GFP (B16-GFP) cells were implanted subcutaneously into the flank of immunocompetent C57Bl/6 mice (n=5, each) and monitored for tumor growth. Graph of tumor volumes at day 7 post-implantation ± SEM is shown in (A). Mice that had received B16-FIST-15 (B16-FIST-15 immunized, n=5) were rechallenged on day 14 post-implantation subcutaneously with 1×10^6 B16-F0 cells on the contralateral flank compared to naïve mice (B16 naïve; n=5) and monitored for tumor growth. Graph of tumor volumes from ± SEM is shown in (B). Syngeneic mouse strains lacking $CD4^+$ (*Cd4*$^{-/-}$), $CD8^+$ (*Cd8*$^{-/-}$) T cells, B cells (μMT), or functional NK cells (*Beige*) were implanted with 1×10^6 B16-FIST-15 cells and tumor volume ± SEM from two independent experiments was measured on day 7 post-implantation in (C). Genetic knockout strains receiving B16-FIST-15 were rechallenged 14 days post-implantation with 1×10^6 B16-F0 cells. Graph showing tumor volume ± SEM from two independent experiments at day 12 post-rechallenge is shown in (D). In the FIST-15 therapeutic model, wildtype C57Bl/6J mice were implanted with 1×10^6 B16-F0 cells subcutaneously. Day 7 post-implantation, once palpable tumor had formed, mice were randomized into treatment groups receiving: PBS (n=7), IL-15 + sTβRII (n=9), and FIST-15 (n=11). Mice were given intraperitoneal injections every second day for 1 week (4 doses total, indicated by arrows) and monitored

for tumor growth. Graph measuring tumor volume ± SEM is shown in (E). Survival of mice in the three groups depicted in a Kaplan-Meier plot is shown in (F). Representative data are shown from two (A, B, E, and F) independent experiments. Statistical significance was determined by Student *t* tests (A, B, and E), one-way ANOVA with Tukey multiple comparisons test in (C and D), and the log-rank test in (F). Statistical significance between IL-15 + sTβRII and FIST-15 conditions are denoted in (E and F). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ****, $P < 0.0001$.

Supplementary Figure Legends

Supplementary Figure S2.1. FIST15 treatment increases the proportion of $CD8^+$ T cells NK cells without inducing proliferation of $CD4^+$ T cells or B cells *in vitro*. Unfractionated splenocytes were cultured in media only (untreated), IL-15 + sTβRII, or FIST15 (500 pM) for 72 hours and analyzed by flow cytometry for $CD3^+CD8^+$ ($CD8^+$ T cells) and $CD3^-NK1.1^+$ (NK cells) events. Representative plots are shown in (A). CFSE-labeled $CD4^+$ T cells ($CD3^+CD4^+$) and B cells ($CD19^+$) cells were cultured with media only (untreated), IL-15, or FIST15 (500pM) for 72 hours. CFSE dilution plots are shown in (B).

Supplementary Figure S2.2. MHC-I Expression on B16 and MC-38 tumor cell lines. B16-F0 and MC-38 cell lines were stained with anti-H-2kb/MHC-I antibody (black line) or isotype control (shaded histogram) in (A).

Supplementary Figure S2.3. FIST15 treatment promotes $CD8^+$ memory phenotype cell expansion. Unfractionated splenocytes from C57Bl/6J mice were treated with media only (untreated), IL-15 + sTβRII, or FIST15 (500pM) in the presence of TGF-β1 (5ng/ml) for 72 hours and analyzed by flow cytometry for surface expression of CD44 and CD62L on $CD8^+$ T cells (A) and CD25 in (B). Representative plots are shown in (A). Representative histogram of CD25 expression in untreated (light gray line), IL-15 + sTβRII (dark gray line), FIST15 (black line), isotype control (shaded) are shown in (B). Graph representing the percentage of $CD8^+$ T cells expressing CD25 ± SEM is shown in (C) from two independent experiments. Percentage of CD122 expressing splenic $CD8^+$ T cells treated with FIST15 or IL-15 + sTβRII (500 pM) in the presence of TGF-β1 (5ng/ml) was also analyzed by flow cytometry at 3, 24, 48, and 72 hours post-stimulation. Graph showing the mean CD122 expression ± SEM is shown in (D) from two independent experiments. Statistical significance was determined by Student *t* test. ** $P < 0.01$ *** $P < 0.001$.

Supplementary Figure S2.4. NK cell response to FIST15 treatment. Unfractionated splenocytes from C57Bl/6J mice were treated with IL-15 + sTβRII or FIST15 (500pM) in the presence of TGF-β1 (5 ng/ml) for 72 hours and analyzed by flow cytometry for differences in the proportion of NK cells ($CD3^-$ $NK1.1^+$) (A) and the expression profile of CD11b and CD27 on these NK cells (B). Percentage of CD122 expressing splenic NK cells treated with FIST15 or IL-15 + sTβRII (500 pM) in the presence of TGF-β1 (5ng/ml) was also analyzed by

flow cytometry at 3, 24, 48, and 72 hours post-stimulation. Histogram showing the mean percentage of CD122 expression ± SEM is shown in (C) from two independent experiments.

Supplementary Figure S2.5. FIST15 augments $CD8^+$ T cell cytotoxic effector molecule production. Primary splenic $CD8^+$ T cells were cultured with media only (untreated), IL-15 + sTβRII, or FIST15 (500pM) in the presence of TGF-β1 (5 ng/ml) for 72 hours and stimulated with PMA/ionomycin/brefeldin A for 4-6 hours. The percentage of $CD8^+$ T cells expressing NK activating receptor, NKG2D, Fas ligand (FasL), TNF-related apoptosis inducing ligand (TRAIL), and granzyme B (GrB) ± SEM is shown in (A). Absolute number of $CD8^+$ T cells expressing effector molecules ± SEM are shown in (B). Data shown from three independent experiments are shown. Statistical significance was determined by Student *t* test. ** $P < 0.01$, *** $P < 0.001$. **** $P < 0.0001$.

A

MNFLLSWVHWSLALLLYLHHAKWSQAHHHHHHHHH
ENLYFQGSIPPHVPKSVNSDVMASDNGGAVKLPQL
CKFCDVRLSTCDNQKSCMSNCSITAICEKPHEVCV
AVWRKNDKNITLETVCHDPKLTYHGFTLEDAASPK
CVMKEKKRAGETFFMCACNMEECNDYIIFSEEYTT
SSPDIPPHVPKSVNSDVMASDNGGAVKLPQLCKFC
DVRLSTCDNQKSCMSNCSITAICEKPHEVCVAVWR
KNDKNITLETVCHDPKLTYHGFTLEDAASPKCVMK
EKKRAGETFFMCACNMEECNDYIIFSEEYTTSSPD
GTGGSSGGTTCPPPVSIEHADIRVKNYSVNSRERY
VCNSGFKRKAGTSTLIECVINKNTNVAHWTTPSLKC
IRDPSLAHYSPVPSGGSGGGGSGGGSGGGGSLQ
NWIDVRYDLEKIESLIQSIHIDTTLYTDSDFHPSCKVT
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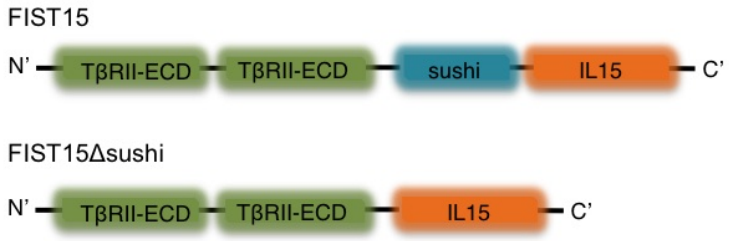
INTS

B



C

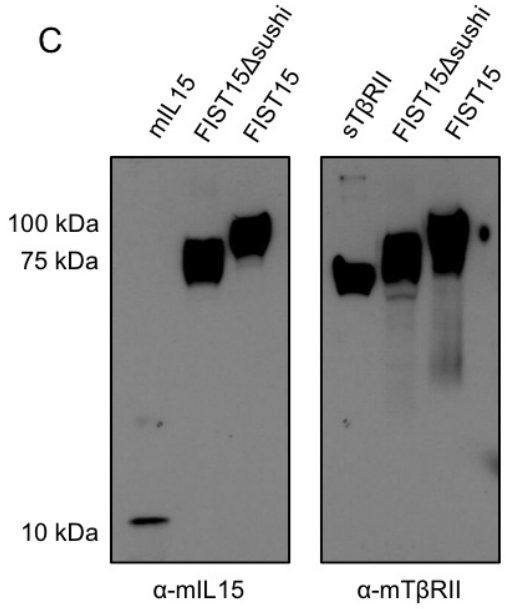


Figure 2.1

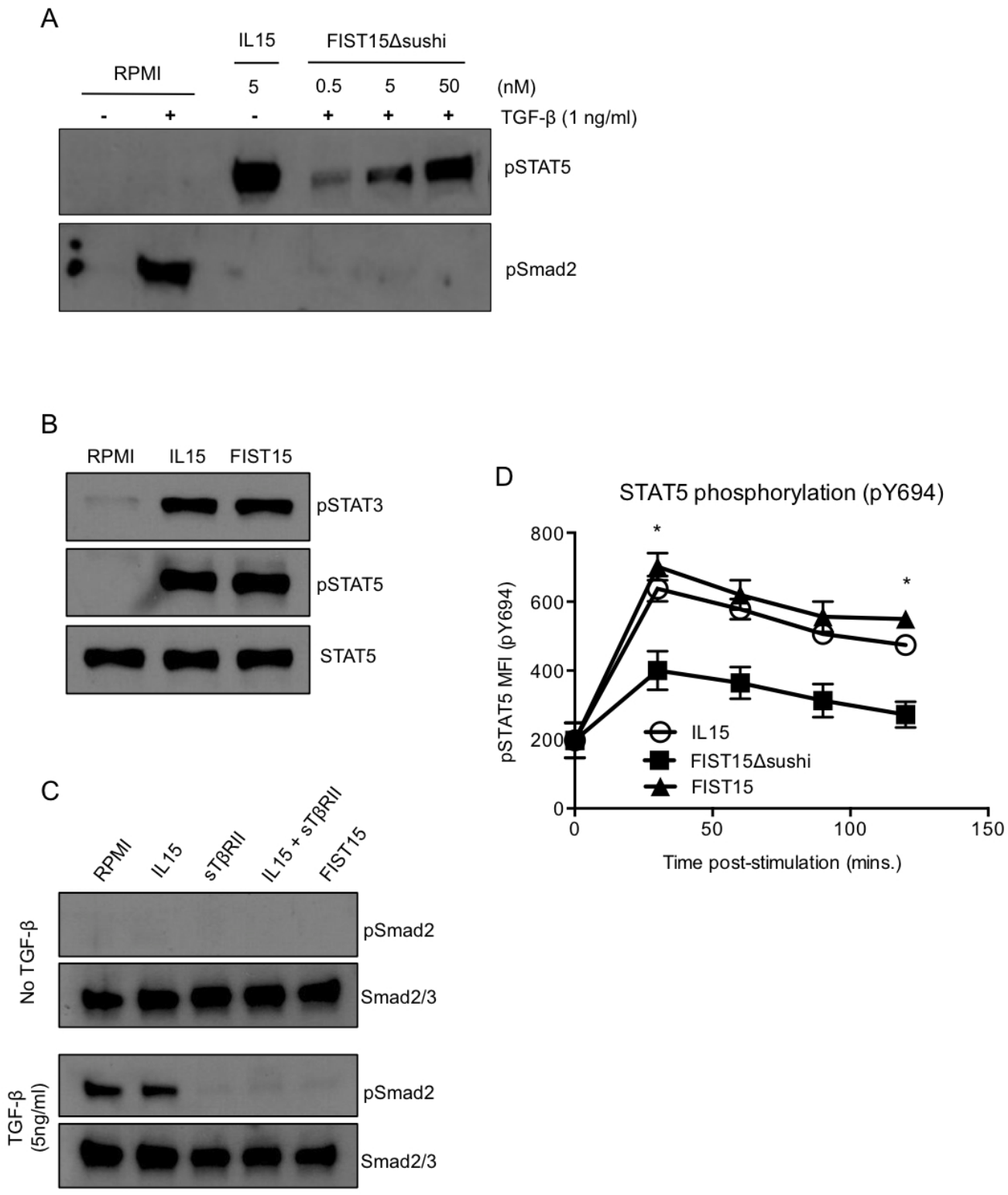

A
IL15
FIST15Δsushi
RPMI
5
0.5
5
50
(nM)
- + - + + +
TGF-β (1 ng/ml)
pSTAT5
pSmad2
B
RPMI
IL15
FIST15
pSTAT3
pSTAT5
STAT5
C
RPMI
IL15
sTβRII
IL15 + sTβRII
FIST15
No TGF-β
pSmad2
Smad2/3
TGF-β (5ng/ml)
pSmad2
Smad2/3
D
STAT5 phosphorylation (pY694)
pSTAT5 MFI (pY694)
800
600
400
200
0
0
50
100
150
Time post-stimulation (mins.)
IL15
FIST15Δsushi
FIST15
*
*

Figure 2.2

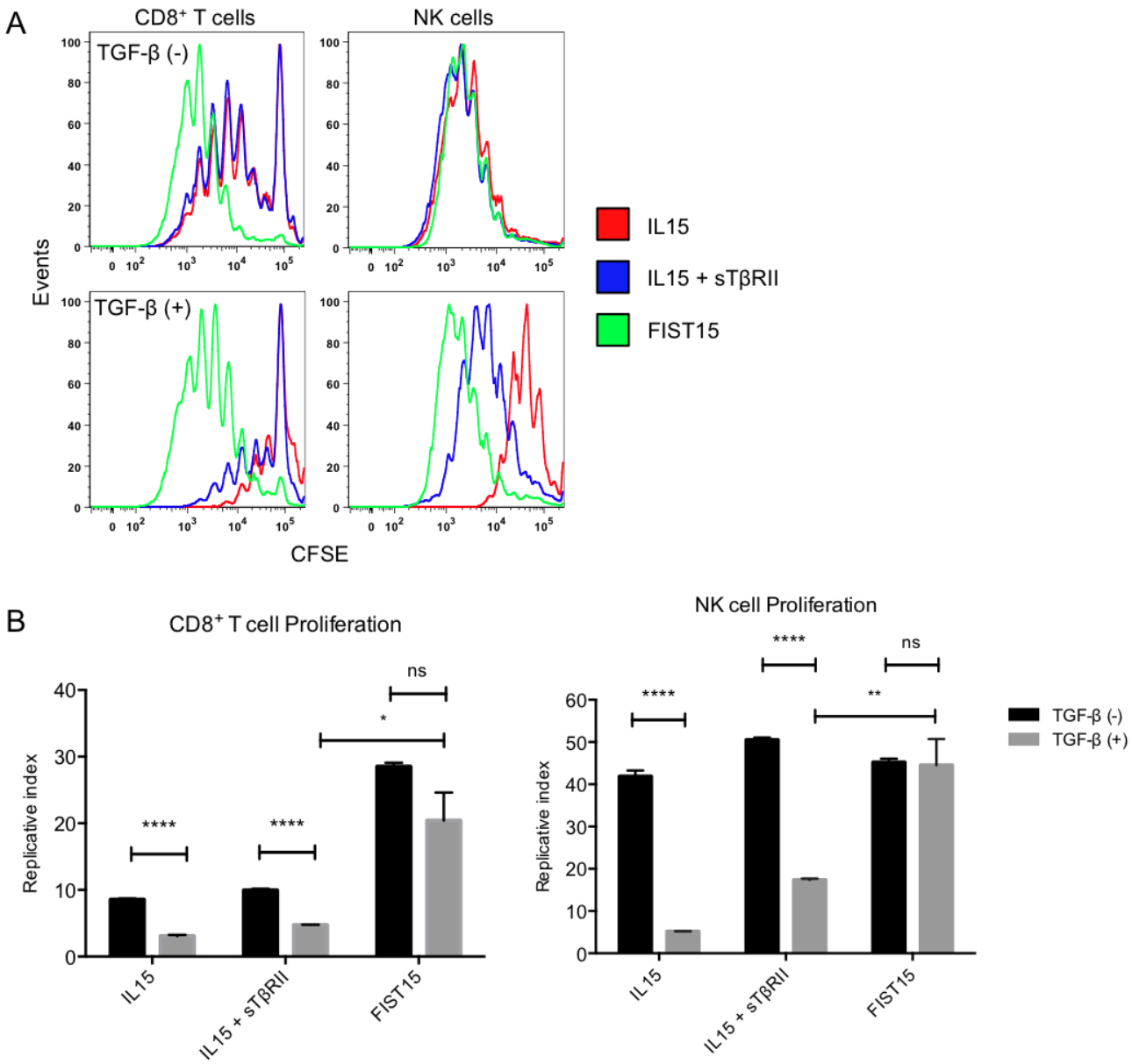

A
CD8+ T cells
NK cells
TGF-β (-)
TGF-β (+)
Events
CFSE
IL15
IL15 + sTβRII
FIST15
B
CD8+ T cell Proliferation
NK cell Proliferation
Replicative index
****
ns
*
**
TGF-β (-)
TGF-β (+)
IL15
IL15 + sTβRII
FIST15

Figure 2.3

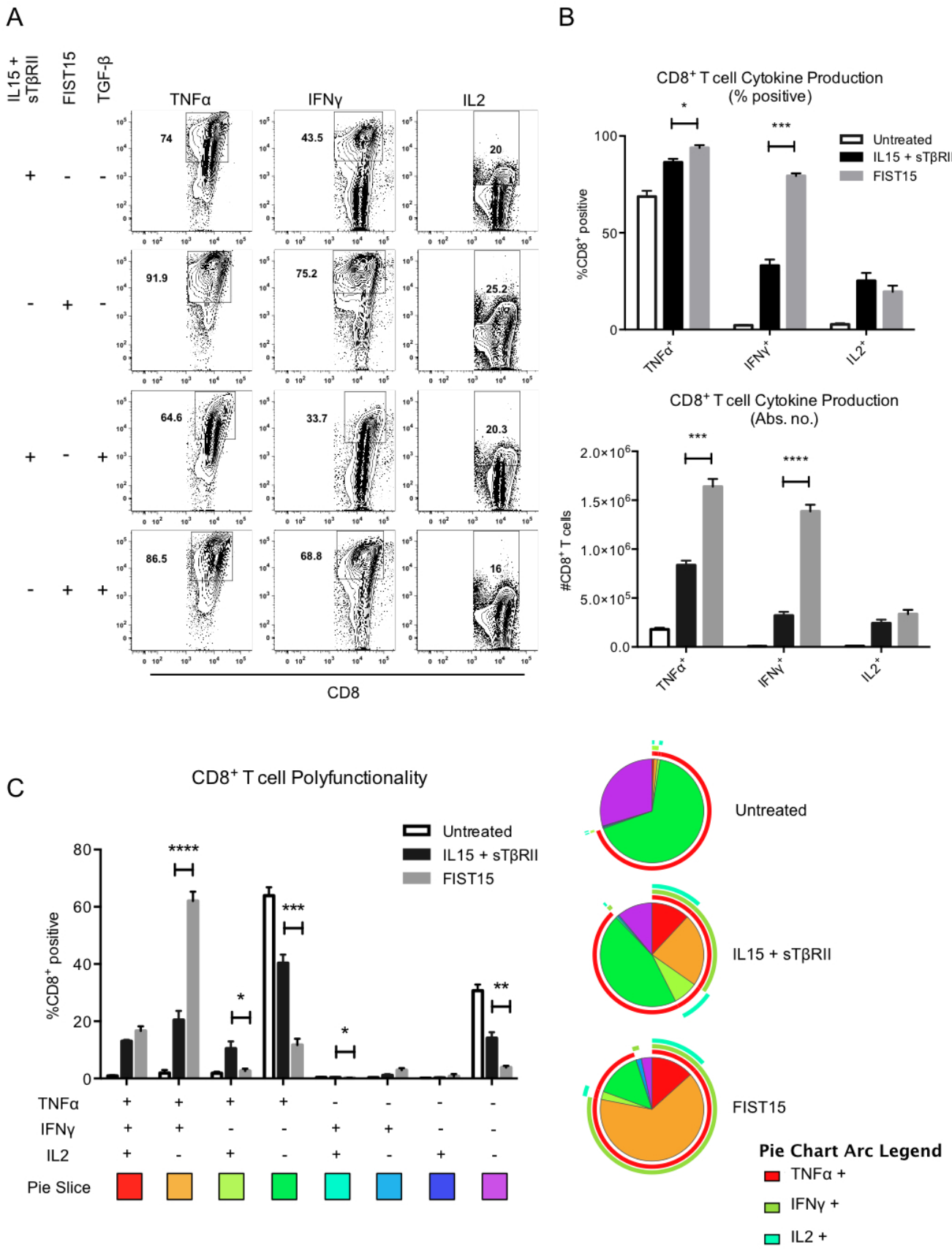

A
IL15 + sTβRII
FIST15
TGF-β
TNFα
IFNγ
IL2
74
43.5
20
91.9
75.2
25.2
64.6
33.7
20.3
86.5
68.8
16
CD8
B
CD8+ T cell Cytokine Production (% positive)
%CD8+ positive
Untreated
IL15 + sTβRII
FIST15
TNFα+
IFNγ+
IL2+
CD8+ T cell Cytokine Production (Abs. no.)
#CD8+ T cells
C
CD8+ T cell Polyfunctionality
%CD8+ positive
Pie Slice
Pie Chart Arc Legend
TNFα +
IFNγ +
IL2 +

Figure 2.4

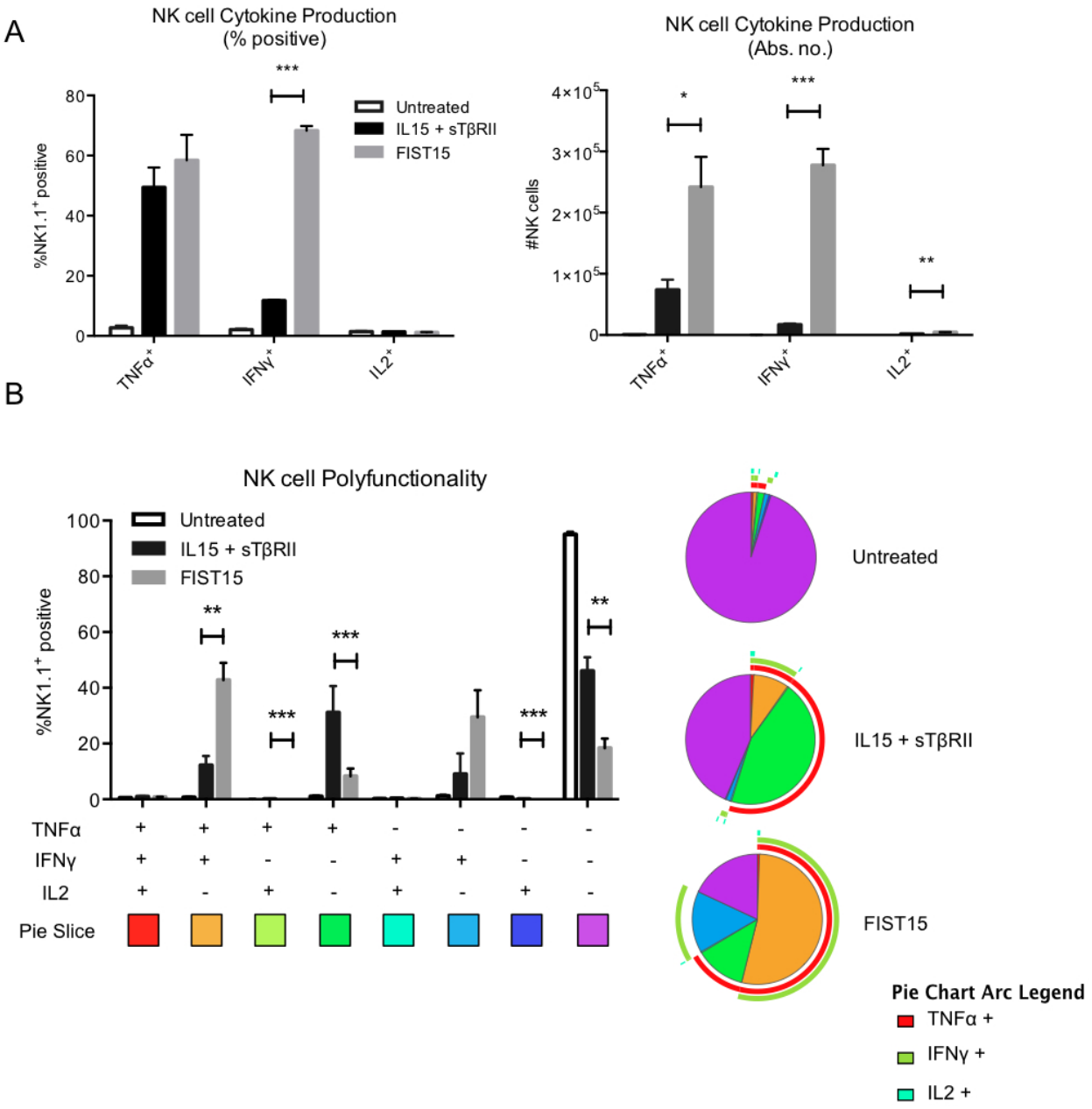

A
NK cell Cytokine Production
(% positive)
%NK1.1+ positive
Untreated
IL15 + sTβRII
FIST15
TNFα+
IFNγ+
IL2+
NK cell Cytokine Production
(Abs. no.)
#NK cells
B
NK cell Polyfunctionality
TNFα
IFNγ
IL2
Pie Slice
Untreated
IL15 + sTβRII
FIST15
Pie Chart Arc Legend
TNFα +
IFNγ +
IL2 +

Figure 2.5

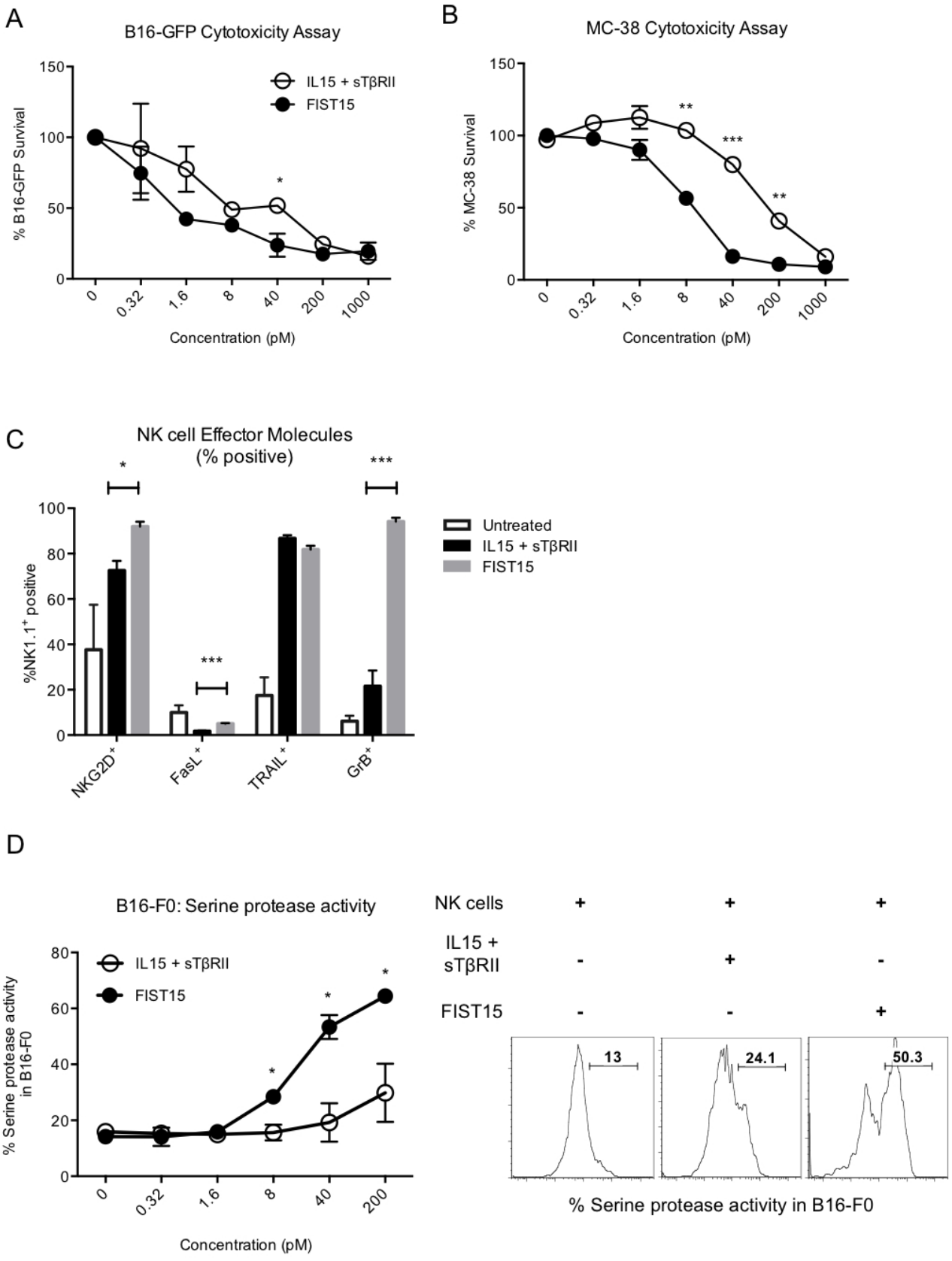

A
B16-GFP Cytotoxicity Assay
% B16-GFP Survival
IL15 + sTβRII
FIST15
Concentration (pM)
B
MC-38 Cytotoxicity Assay
% MC-38 Survival
Concentration (pM)
C
NK cell Effector Molecules
(% positive)
%NK1.1+ positive
Untreated
IL15 + sTβRII
FIST15
NKG2D+
FasL+
TRAIL+
GrB+
D
B16-F0: Serine protease activity
% Serine protease activity in B16-F0
Concentration (pM)
NK cells
IL15 + sTβRII
FIST15
13
24.1
50.3
% Serine protease activity in B16-F0

Figure 2.6

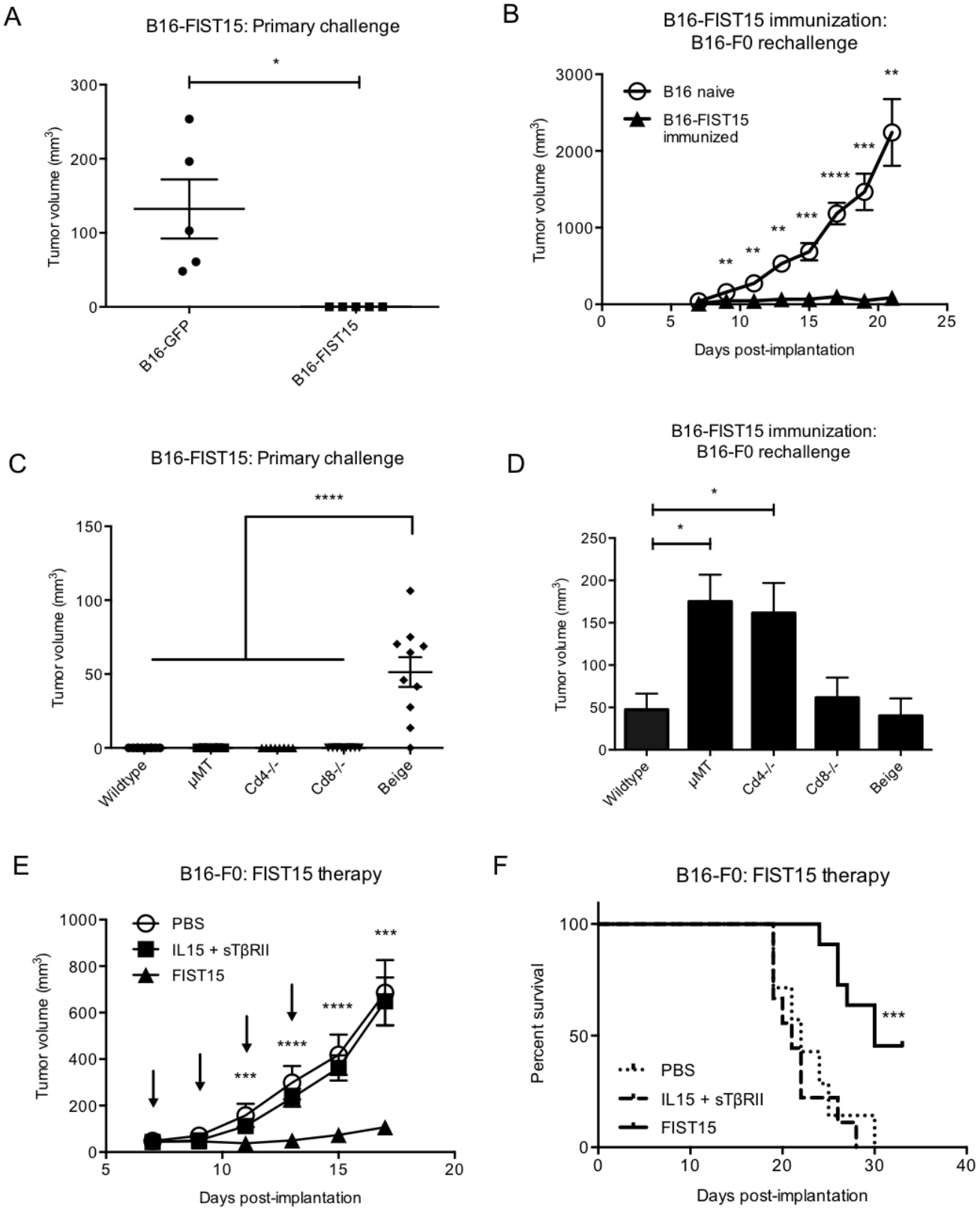
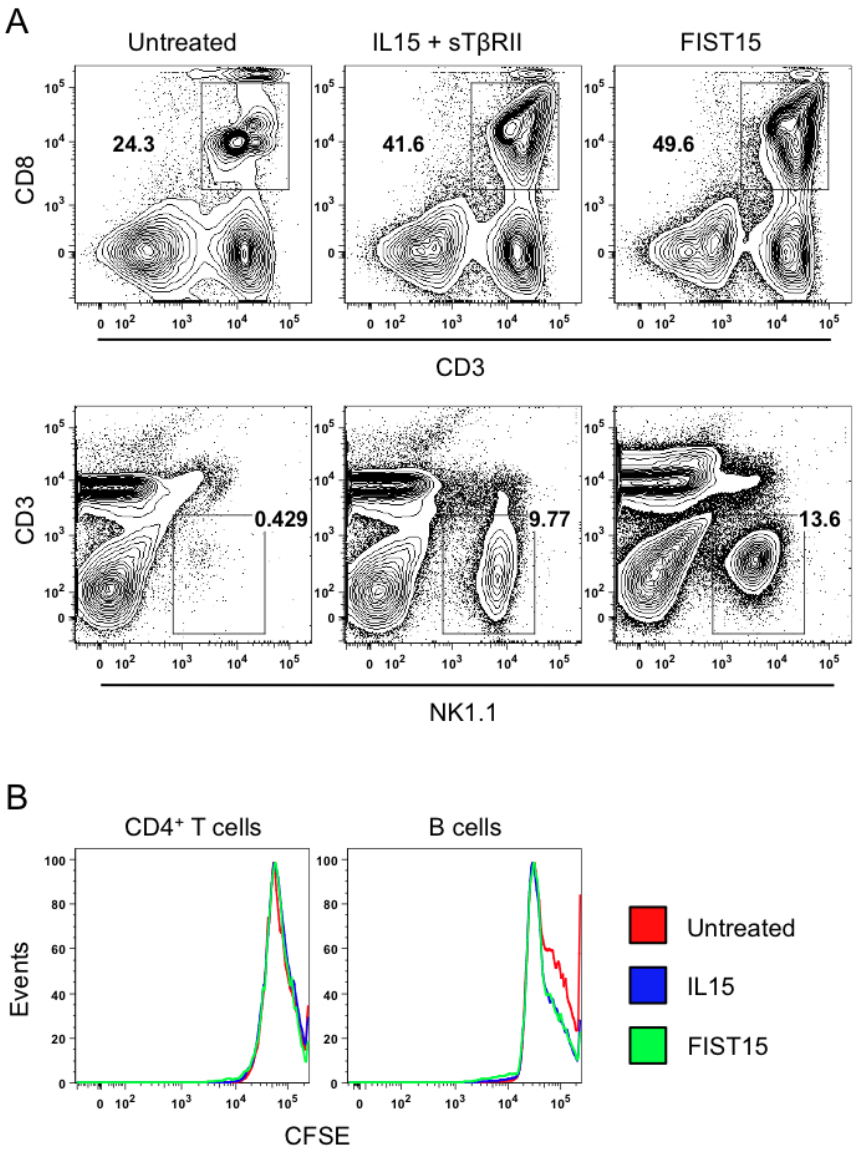
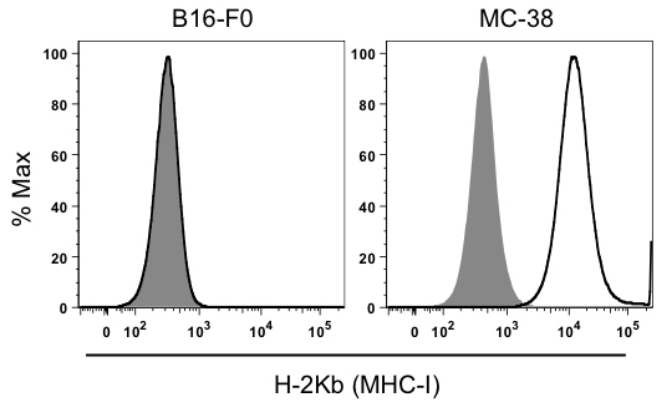

A
B16-FIST15: Primary challenge
Tumor volume (mm3)
B16-GFP
B16-FIST15
B
B16-FIST15 immunization:
B16-F0 rechallenge
B16 naive
B16-FIST15 immunized
Tumor volume (mm3)
Days post-implantation
C
B16-FIST15: Primary challenge
Tumor volume (mm3)
Wildtype
µMT
Cd4-/-
Cd8-/-
Beige
D
B16-FIST15 immunization:
B16-F0 rechallenge
Tumor volume (mm3)
Wildtype
µMT
Cd4-/-
Cd8-/-
Beige
E
B16-F0: FIST15 therapy
PBS
IL15 + sTβRII
FIST15
Tumor volume (mm3)
Days post-implantation
F
B16-F0: FIST15 therapy
Percent survival
PBS
IL15 + sTβRII
FIST15
Days post-implantation

Figure 2.7

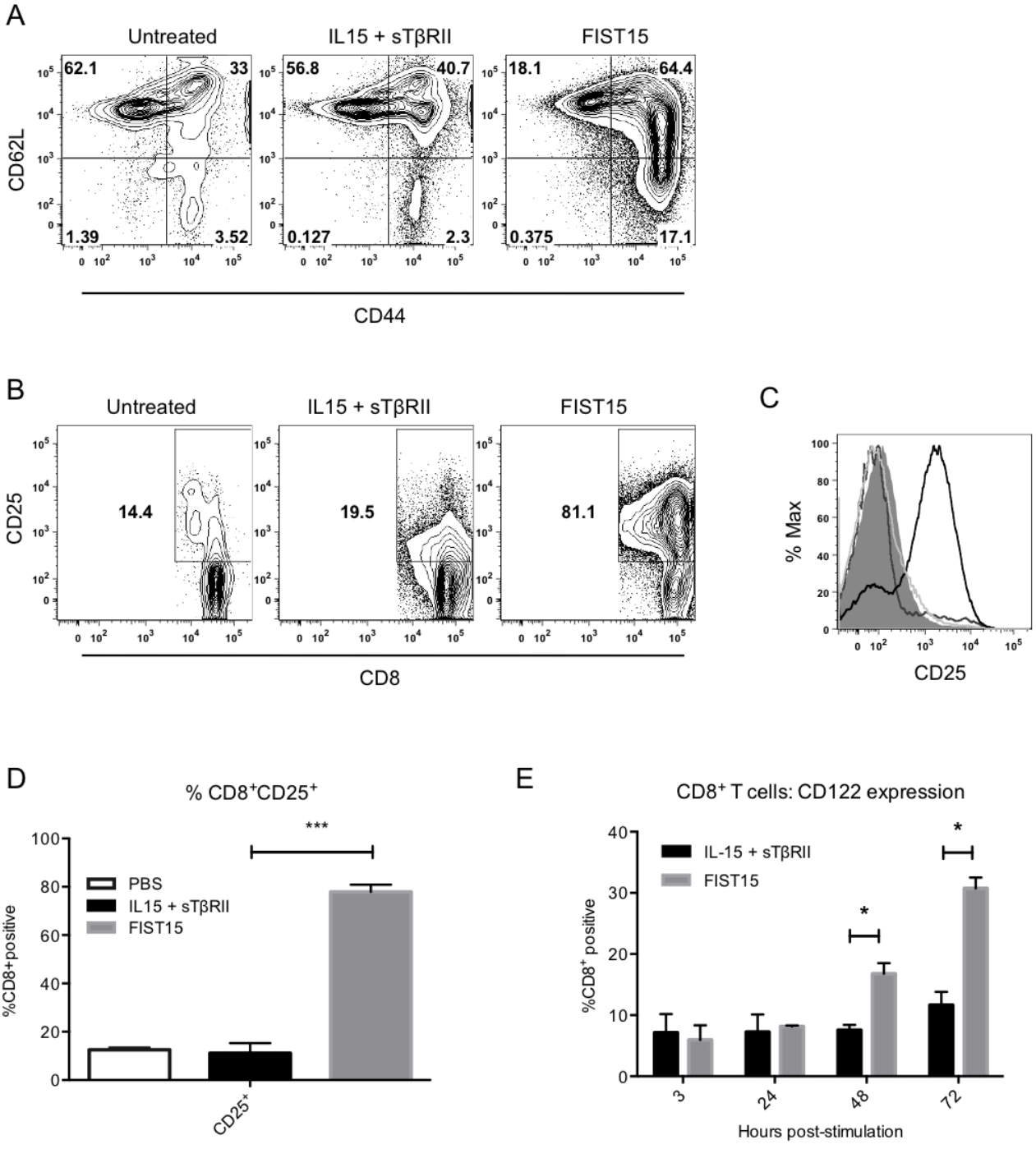


Supplementary Figure S2.1

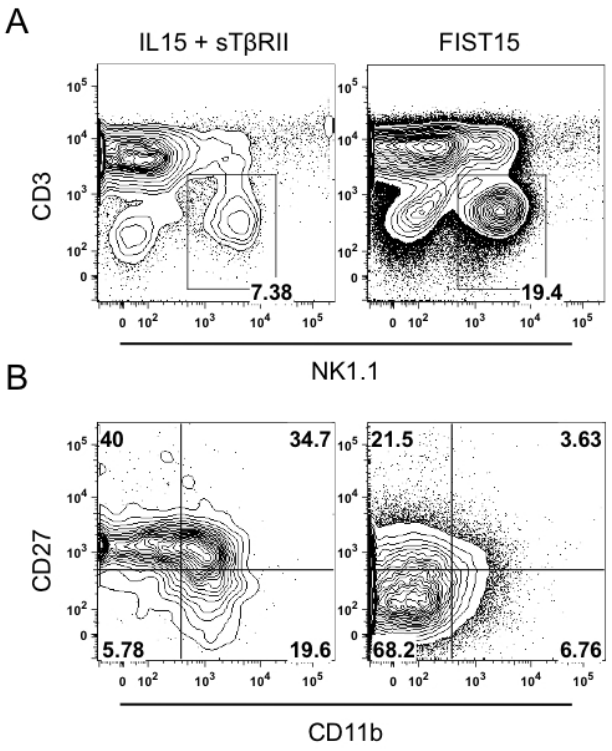
A



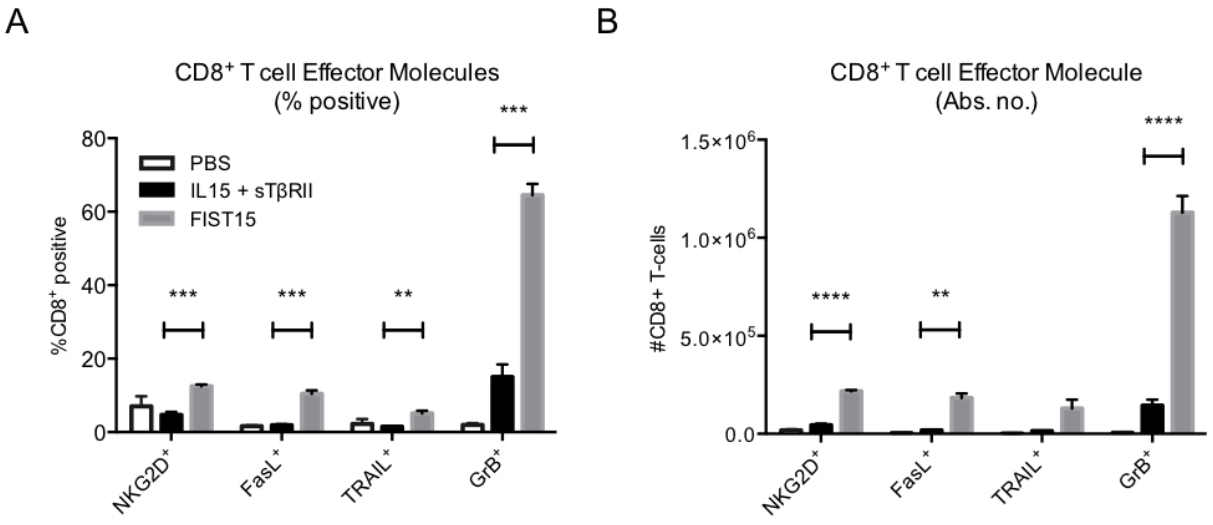
Supplementary Figure S2.2



Supplementary Figure S2.3



Supplementary Figure S2.4



Supplementary Figure S2.5

Chapter 3

Alternative immunotherapeutic applications of FIST-15

3.1.0 FIST-15 in MCMV infection

3.1.1 Introduction

Due to the ability of FIST-15 to expand NK cells and $CD8^+$ T cells, we hypothesized that FIST-15 might be beneficial in the context of a pathogenic viral infection. In order to test this hypothesis, we chose to utilize the well-characterized murine cytomegalovirus (MCMV). MCMV is a member of the beta-herpesvirus family; a double-stranded, enveloped DNA virus. Due to the fact that CMVs are species-specific, MCMV has been used in mice as a surrogate model to study how the immune system responds to HCMV infection in humans [306]. Beyond sharing similarities in viral structure, both MCMV and HCMV infect similar organs in their respective hosts and induce similar pathology [307]. Moreover, both viruses follow the same course of infection, which is generally divided into three phases: (1) a replicative phase (~1 week) where the virus spreads to multiple organs and is able to establish before the immune system can respond, (2) a persistent phase (~2 weeks) where viral replication begins to be controlled and is generally limited to one or two organs in an immunocompetent host, and (3) a latent or chronic phase where viral replication is suppressed and undetectable. Infected hosts tend to remain asymptomatic to infection, unless they become immunosuppressed, in which case viral replication may reactivate, resulting in life-threatening complications, such as pneumonia, gastrointestinal inflammation, central nervous system involvement, and development of carcinomas [308, 309].

Immunologically, the control of HCMV and MCMV infections require innate and adaptive arms of the immune system [310]. More specifically, early control of MCMV infection has been shown to critically depend on a functionally intact NK cell response [311]. In C57BL/6 mice, NK cells expressing the activating receptor, Ly49H, preferentially proliferate in response to MCMV infection by binding to the virally-encoded m157 protein [312]. Ly49H-m157 signaling results in perforin-mediated cytotoxicity of infected cells, resulting in diminished viral replication [313]. Mouse strains lacking Ly49H expression on NK cells, such as Balb/c mice, are much more susceptible to MCMV pathology due to their inability to control early viral replication [314, 315]. In these mouse strains, and in C57Bl/6 mice infected with MCMV that is missing the m157 protein (MCMVΔm157), the $CD8^+$ T cell response plays a vital role in viral control [316].

We found that administration of FIST-15 in MCMV-infected mice resulted in higher numbers of NK cells in the spleen. These NK cells were more activated by expression of effector molecules on their cell surface compared to control-treated animals. Interestingly, we found that administration of FIST-15 reduced the number of MCMV-specific $CD8^+$ T cells and was inversely correlated with NK cell expansion. However, $CD8^+$ T cells from FIST-15 treated mice produced more IFN-γ upon *ex vivo* stimulation compared to control animals.

3.1.2 Materials and Methods

MCMV infection, mice, and FIST-15 treatment

C57Bl/6J mice (Jackson Laboratories, ME) were infected with 1×10^5 pfu of murine cytomegalovirus (MCMV; Smith strain, ATCC, Manassas, VA). Mice were treated with PBS, FIST-15 (0.9μg/dose), or equimolar recombinant IL-15 and a soluble TGF-β receptor ectodomain-Fc chimeric protein (sTβRII). Treatment was administered on days 3, 5, 7, and 9 post-infection. Mice were sacrificed on day 10 post-infection.

Flow cytometry

Primary mouse splenocytes from MCMV-infected animals were harvested day 10 post-infection. Splenocytes were blocked with anti-mouse FcR III/II for 15 minutes at room temperature, before surface staining with CD3, CD8, NK1.1, CD25, CD44, CD62L, CD69, CD127, CD279, KLRG1, and TRAIL antibodies for flow cytometry were obtained from BD Biosciences (San Jose, CA). MCMV-specific CD8+ T cells were identified with HGIRNASFI-H-$2D^b$ tetramer (NIAID Tetramer Core Facility, GA). Cell staining was acquired on a FACSCanto II (BD Biosciences) and data analyzed with FlowJo software (Treestar Inc).

Intracellular cytokine staining

Splenocytes were isolated as above and plated at a density of 1×10^6 cell/ml overnight in RPMI1640 media RPMI 1640 supplemented with L-glutamate, HEPES, β-mercaptoethanol, 50U/ml penicillin and streptomycin, and 10% FBS. Cells were stimulated with a BD Leukocyte Activation Cocktail for 4-6 hours and stained for surface markers as indicated above and subjected to fixation and

permeabilization by BD CytoFix/CytoPerm buffers. Cells were then stained intracellularly for granzyme B (eBioscience), IFN-γ (BD Biosciences) or their respective isotype controls. Cell staining was acquired and analyzed as above.

3.1.3 Results

FIST-15 expands NK cells but not $CD8^+$ T cells in the spleen of MCMV infected mice

Mice were inoculated with $1x10^5$ pfu of MCMV intraperitoneally (i.p.) and left for 3 days before treatment with i.p. PBS, IL-15 and sTβRII, or FIST-15 was initiated (Fig. 3.1A). The number of splenocytes harvested between PBS, IL-15 and sTβRII treated, and FIST-15 treated mice were not significantly different, although there was a trend towards an increased number of splenocytes in the latter two groups (Fig. 3.2A). Further subset analysis found that while the number of CD8+ T cells remained roughly equal between control and FIST-15 treatment, the number of NK cells were significantly greater in both proportion and absolute numbers compared to control and untreated mice (Fig. 3.2B and C). FIST-15 treatment increased the absolute number of NK cells by approximately 4-fold (Fig. 3.2C).

FIST-15 treatment is associated with fewer MCMV-tetramer specific $CD8^+$ T cells, but increases the proportion of central memory phenotype $CD8^+$ T cells

We utilized a K(b)-restricted MHC-tetramer to stain for MCMV-specific $CD8^+$ T cells in the spleen of untreated, control treated, and FIST-15 treated animals.

Although there were no significant differences between the percentage and absolute number of MCMV-specific CD8+ T cells, FIST-15 treated mice tended towards being significantly less than untreated animals (Fig. 3.2A). On average, untreated animals had the highest percentage of MCMV-specific $CD8^+$ T cells, followed by control treated animals, and finally FIST-15 treated mice had the fewest numbers of MCMV-specific CD8+ T cells (Fig. 3.2A). When bulk $CD8^+$ T cells were analyzed further for differentiation, it was found that FIST-15 treated mice had significantly increased proportion of their CD8+ T cells taking on a central memory phenotype ($CD62L^+CD44^+$). On the other hand, untreated and control treated mice had CD8+ T cells that were predominately naïve or effectors (Fig. 3.2B). $CD8^+$ T cells from untreated mice expressed significantly greater levels of KLRG1 and exhibited lower surface levels of IL7Rα (CD127), providing additional evidence that $CD8^+$ T cells in untreated, MCMV-infected mice were effectors (Fig. 3.2C). Conversely, FIST-15 treated mice had higher proportions of $CD8^+$ T cells that expressed low KLRG1, but high CD127.

FIST-15 augments CD8+ T cell expression of IFN-γ

Upon *ex vivo* stimulation, CD8+ T cells from FIST-15 treated animals expressed significantly higher levels of IFN-γ compared to PBS and control treated animals (Fig. 3.3A). There were no differences in the expression of granzyme B by any of the treatment conditions. Further, there were no apparent differences in the state of activation of the $CD8^+$ T cells across treatment groups as determined by surface expression of CD69 and CD25. While differences in PD-1 expression on

$CD8^+$ T cells did not reach significance between treatment groups, $CD8^+$ T cells from FIST-15 treated animals trended towards less PD-1 expression compared to PBS and control groups (Fig. 3.3B). Additionally, no differences were detected between the expression of NKG2D activating receptor and TRAIL on the surface of $CD8^+$ T cells between treatment groups (data not shown).

FIST-15 augments NK cell expression of effector molecules in MCMV infected mice

FIST-15 significantly enhanced the proportion of NK cells expressing granzyme B (Fig. 3.4A and B). While the percentage of cells expressing NKG2D and TRAIL were not significantly different between treatment groups, the absolute number of NK cells expressing these markers found in the spleen of infected animals was (Fig. 3.4B). FIST-15 treated animals had significantly higher numbers of NK cells expressing TRAIL, NKG2D, as well as granzyme B. There were no significant differences in the number of $IFN\gamma^+$ NK cells between treatment groups. NK cells from FIST-15 treated mice expressed less KLRG1 on their surface, and the majority of these NK cells were of the $CD11b^-CD27^+$ intermediate maturity phenotype (Fig. 3.4C and D).

3.1.4 Discussion

The control of CMV in mice is known to critically depend on NK cells, especially early on in infection [317]. $CD8^+$ T cells, also play a much more critical role, albeit later in the infection and contribute to memory responses against subsequent

MCMV infection [318]. Our observation that FIST-15 did not expand the pool of splenic $CD8^+$ T cells was surprising to us. In fact, the absolute number of $CD8^+$ T cells was roughly equivalent across all three treatment conditions. Compared to our studies within tumor models (Chapter 2), we did not see an increase in the proliferation of $CD8^+$ T cells in response to FIST-15 treatment. However, doses of FIST-15 utilized in these experiments were 4-fold less than in the tumor challenge experiments. The most interesting observation was that FIST-15 treated mice had profoundly decreased numbers of MCMV-specific $CD8^+$ T cells compared to PBS and IL-15+sTβRII treated mice. It was originally hypothesized that given the mitogenic effects of FIST-15 on $CD8^+$ T cells, FIST-15 treatment would increase the number of MCMV-specific $CD8^+$ T cells, resulting in better viral control. This decrease in both the proportion and absolutely numbers of $CD8^+$ T cells that were antigen-specific signaled that FIST-15 have skewed the $CD8^+$ T cell response away from responding to MCMV or that FIST-15 treatment may have resulted in enhanced viral control through another means.

Although we could not definitively rule out the first hypothesis, the second hypothesis is consistent with reports by other groups showing that NK cells and early control of viral replication and infection modulates the ensuing $CD8^+$ T cell response. Particularly, Mitrovic et al found that the magnitude of the $CD8^+$ T cell response against MCMV was inversely correlated engagement of Ly49H on NK cells to m157 viral protein on the surface of infected cells [316]. The use of an MCMV strain lacking m157 protein expression and consequent lack of NK cell activation drives a stronger $CD8^+$ T cell response against the virus. Efficient NK

cell Ly49H engagement, however, diminished the need for robust $CD8^+$ T cell responses, since viral control could be established early on. It is plausible that FIST-15 driven NK cell proliferation and activation could result in enhanced early control of viral replication and account for the diminished MCMV-tetramer specific $CD8^+$ T cell responses seen at day 10 post-infection. Further, the magnitude of the $CD8^+$ T cell response to MCMV infection is known to be positively correlate with viral load, suggesting that viral titers may be higher in PBS and IL-15+sTβRII treatment conditions compared to FIST-15 treated animals. Additionally, we found that the majority of splenic $CD8^+$ T cells from FIST-15 treated mice were predominately naïve ($CD62L^+CD44^-$) or central memory phenotype ($CD62L^+CD44^+$), while in PBS and control treated mice, the majority of the $CD8^+$ T cells were phenotypically effector cells ($CD62L^-CD44^+$). This would suggest that in PBS and control treated conditions, $CD8^+$ T cells were in a state poised to leave the secondary lymphoid structures towards the periphery. However, FIST-15 treated mice had very few effector $CD8^+$ T cells, suggesting a lower state of overall activation. The expression of KLRG1 and CD27 on $CD8^+$ T cells also agrees with the hypothesis that viral control may be more optimal in settings of robust early NK cell activation by FIST-15. In FIST-15 treated mice, $CD8^+$ T cells expressed lower levels of KLRG1 and higher levels CD127, suggesting that these cells were poised to take on a long-lived memory fate. Conversely, PBS and control treated mice had $CD8^+$ T cells that were largely $KLRG1^{hi}$ and $CD127^{lo}$, suggesting that they are likely to be short-lived effector cells [319]. Finally, lower PD-1 expression observed in FIST-15 treated $CD8^+$ T cells, would

suggest that they were perhaps less prone to exhaustion and/or less activated, compared to control treated $CD8^+$ T cells [320]. Functionally, overactivated or exhausted $CD8^+$ T cells may be less likely to respond to antigenic stimuli. Indeed, we found that FIST-15 treated $CD8^+$ T cells that had lower PD-1 expression readily produced more IFNγ when polyclonally activated, compared to control or PBS treated mice whose $CD8^+$ T cells expressed higher levels of PD-1.

Beyond the increased number of NK cells observed in FIST-15 treated mice, their surface phenotype and effector molecule expression profile also indicate that they may be more effective at neutralizing viral infection. NK cells from FIST-15 treated mice expressed lower levels of $CD62L^+$, suggesting they were more likely to egress from secondary lymphoid organs into the periphery. Further, they expressed higher levels of NKG2D activating receptor, as well as TRAIL (compared to PBS treated mice, but not to IL-15+sTβRII treated mice), which may induce lysis of target cells expressing TRAIL-receptor (DR-5). Moreover, FIST-15 treated mice had NK cells expressed much higher levels of granzyme B compared to the other two treatment conditions, suggesting that they may be more cytotoxic in nature. When we interrogated the maturation phenotype of NK cells from the different treatment groups, the majority of NK cells from FIST-15 treated mice were of an intermediate maturity ($CD11b^-CD27^+$) compared to the other treatment conditions, where the NK cells were predominately mature ($CD11b^+CD27^-$). Although the increase proportion of mature NK cells in control and PBS treated condition may seem to be of potential benefit in the context of this viral infection, $CD11b^+CD27^-$ NK cells are in replicative senescent and are

less able to secrete cytokines compared to NK cells of intermediate maturity ($CD11b^{+/-}CD27^{+}$) [321]. NK cells from FIST-15 treated animals also expressed lower levels of KLRG1. This molecule is known to negatively regulate the function of NK cells by inhibiting Akt phosphorylation [322]. Its expression on NK cells is inversely correlated with their ability to proliferate, activate, and secrete IFNγ. Accordingly, in further subset analysis, the NK cells that express the highest levels of KLRG1 are of the mature $CD11b^{+}CD27^{-}$ subset, while intermediately mature NK cells express the lowest levels (data not shown). Although we surmise that NK cell activation and expansion by FIST-15 could result in earlier MCMV control, thus resulting in a decreased antigen-specific $CD8^{+}$ T cell response, we cannot definitively prove this as we were unable to collect viral titer from the spleen. Similarly, it was not possible for us to reach any particular conclusion on the efficacy of FIST-15 treatment, since we did not collect viral titer data. The interpretability of the results was also limited by the small sample sizes in this study. Despite this, we had intended for these studies to be a series of pilot experiments to determine the *in vivo* effect of FIST-15 on $CD8^{+}$ T cells and NK cells in the context of a well-established viral infection model. The results prove intriguing insofar as the data may suggest that innate immune responses to viral infection may prove critical in the modulation of adaptive immune responses. From an immunotherapeutic angle, this data may foreshadow the use of FIST-15 as an agent to boost NK cell activity against viral agents. Though augmentation of antigen-specific $CD8^{+}$ T cell responses or antibody production by B cells have often been the focus of antiviral

immunotherapy, the role that innate immune cells play in such infections is often overlooked. Immunotherapy targeting innate immune effectors may prove advantageous, especially in the setting of chronic viral infections, in which antigen-specific adaptive immune responses may succumb to exhaustion. It has been reported that while the neutralization of TGF-β only modestly increases antigen-specific $CD8^+$ T cell responses, it does not substantially promote viral clearance in a model of persistent lymphocytic choriomenigitis virus (LCMV-CL13) [323]. Similarly, the efficacy of IL-15 as an immunotherapeutic agent in viral infections entirely depends on the viral infection in question. Loss of IL-15 in vesicular stomatitis virus (VSV) or LCMV does not seem to alter the course of infection, but results in lethal and uncontrolled viral replication in vaccinia infection [324]. The strategy of coupling of TGF-β neutralization together with pro-inflammatory IL-15 signaling has not been tested in viral infection models. Our preliminary data from these studies suggest that TGF-β neutralization and IL-15 signaling together in the context of a viral infection may yield interesting insights into how these two pathways may affect the ensuing immune response. Further research into the potential therapeutic efficacy of this combinatorial strategy should also be considered.

3.1.5 Figure Legends

Figure 3.1. FIST-15 increases the number of NK cells in MCMV infection. Schematic of infection and treatment conditions are shown in (A). Absolute number of splenocytes from MCMV-infected mice treated with PBS, IL-15 +

sTβRII, and FIST-15 are shown in (B). Absolute $CD3^+CD8^+$ T cells from the three treatment groups are quantified in (C). Absolute number of $CD3^-NK1.1^+$ cells from the three treatment groups are quantified in (D). Histograms depict the mean ± SD. Statistical significance was determined by Student *t* test. ** *P < 0.01*.

Figure 3.2. FIST-15 treatment is associated with decreased MCMV antigen-specific $CD8^+$ T cell responses and enhanced memory formation. Representative flow cytometric plots of $CD3^+CD8^+$ T cells that are MCMV antigen specific (HGIRNASFI-H-$2D^b$-APC) (top panel) and quantification of the absolute number of these cells (bottom panel) are shown in (A). Representative flow cytometric plots of $CD3^+CD8^+$ T cells expressing memory and activation markers CD44 and CD62L are shown in (B). Representative flow cytometric plots of $CD3^+CD8^+$ T cells expressing memory and activation markers KLRG1 and IL-7Rα (CD127) are shown in (C). Histograms depict the mean ± SD. Statistical significance was determined by Student *t* test.

Figure 3.3. FIST-15 treatment is associated enhanced IFN-γ production and decreased PD-1 expression in $CD8^+$ T cells. Representative flow cytometric plots of $CD3^+CD8^+$ T cells that express IFN-γ after PMA/ionomycin stimulation (top panel) and quantification of the absolute number of these cells (bottom panel) are shown in (A). Representative flow cytometric plots of $CD3^+CD8^+$ T cells expressing PD-1 (left panel) and a representative histogram comparing PD-1 expression between the three treatment groups are shown in (B). Histograms

depict the mean ± SD. Statistical significance was determined by one-way ANOVA and Tukey's post-test for multiple comparisons. ** *P < 0.0001*

Figure 3.4. FIST-15 treatment increases the number of intermediate maturity NK cells capable of expressing granzyme B. Representative flow cytometric plots of $CD3^{-}NK1.1^{+}$ NK cells that express granzyme B after PMA/ionomycin stimulation (top panel) are shown in (A). The quantification of the proportion of NK cells expressing IFN-γ, granzyme B (GzmB), NKG2D activating receptor, and TRAIL (left panel) and absolute number of these cells (right panel) are shown in (B). Representative flow cytometric plots of all $CD3^{-}NK1.1^{+}$ NK cells expressing maturity markers CD11b and CD27 (left panel) and the absolute number of NK cells of intermediate maturity ($CD11b^{-}CD27^{+}$) from each treatment group is shown in (C). Representative flow cytometric plots of all $CD3^{-}NK1.1^{+}$ NK cells expressing maturity markers KLRG1 (left panel) and the absolute number of $KLRG1^{+}$ NK cells from each treatment group is shown in (D). Histograms depict the mean ± SD. Statistical significance was determined by Student's *T* test. ** *P < 0.0001*

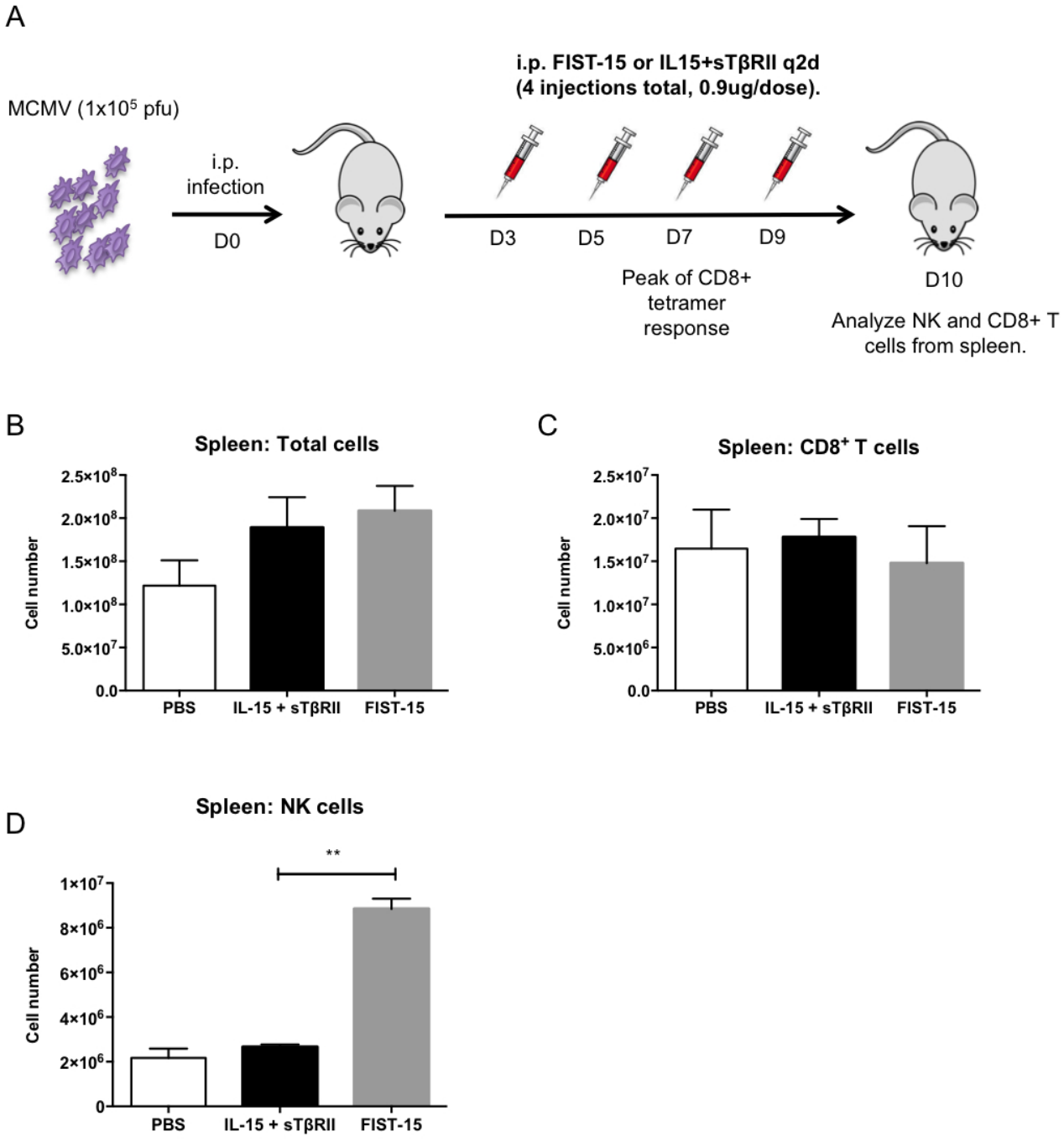

A
MCMV (1x10^5 pfu)
i.p. infection
D0
i.p. FIST-15 or IL15+sTβRII q2d
(4 injections total, 0.9ug/dose).
D3
D5
D7
D9
Peak of CD8+ tetramer response
D10
Analyze NK and CD8+ T cells from spleen.
B
Spleen: Total cells
Cell number
2.5×10^8
2.0×10^8
1.5×10^8
1.0×10^8
5.0×10^7
0.0
PBS
IL-15 + sTβRII
FIST-15
C
Spleen: CD8+ T cells
Cell number
2.5×10^7
2.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
0.0
PBS
IL-15 + sTβRII
FIST-15
D
Spleen: NK cells
**
Cell number
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
0
PBS
IL-15 + sTβRII
FIST-15

Figure 3.1

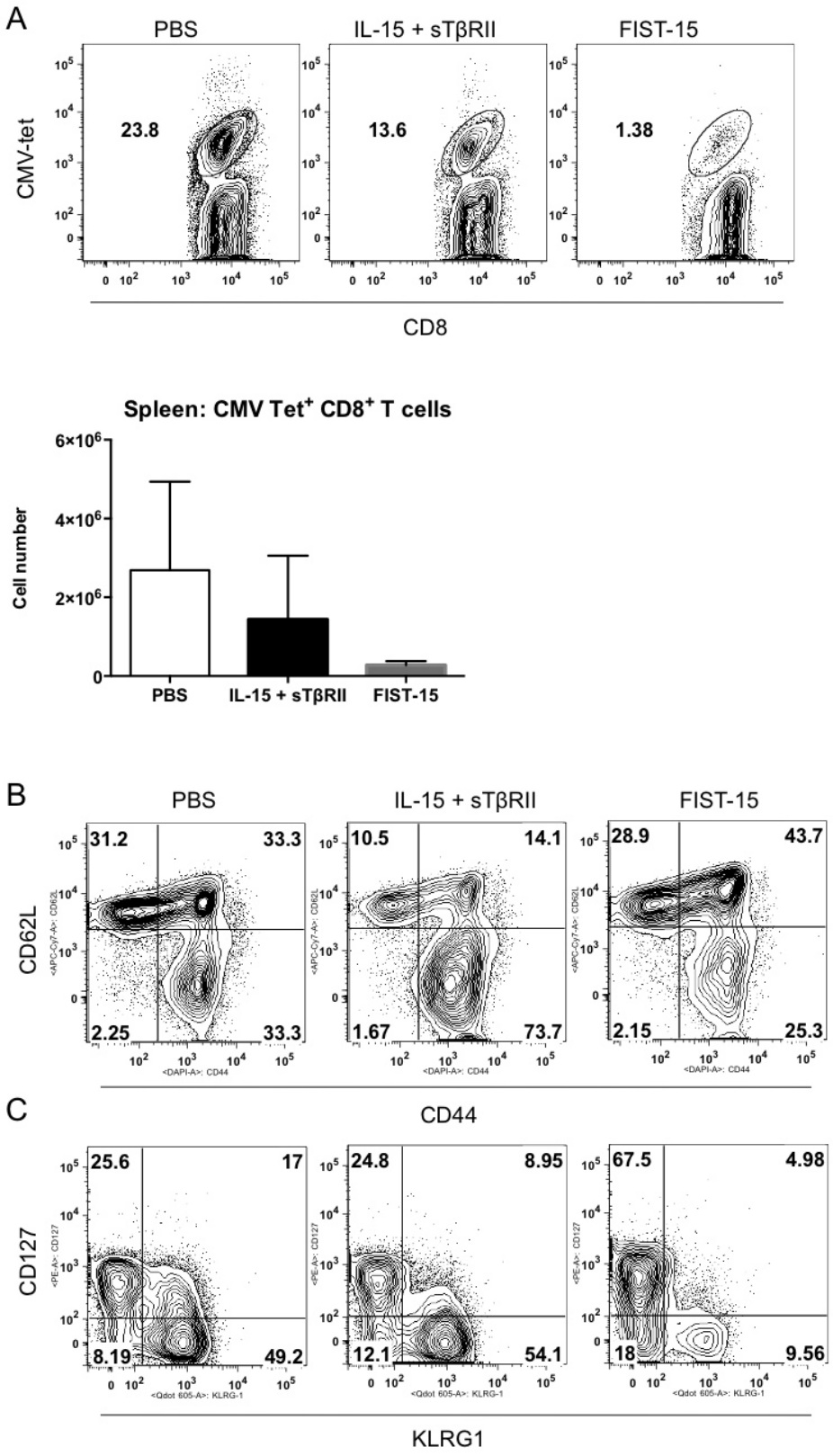

A
PBS
IL-15 + sTβRII
FIST-15
CMV-tet
23.8
13.6
1.38
CD8
Spleen: CMV Tet+ CD8+ T cells
Cell number
6×10^6
4×10^6
2×10^6
0
PBS
IL-15 + sTβRII
FIST-15
B
PBS
IL-15 + sTβRII
FIST-15
CD62L
31.2
33.3
2.25
33.3
10.5
14.1
1.67
73.7
28.9
43.7
2.15
25.3
CD44
C
CD127
25.6
17
8.19
49.2
24.8
8.95
12.1
54.1
67.5
4.98
18
9.56
KLRG1

Figure 3.2

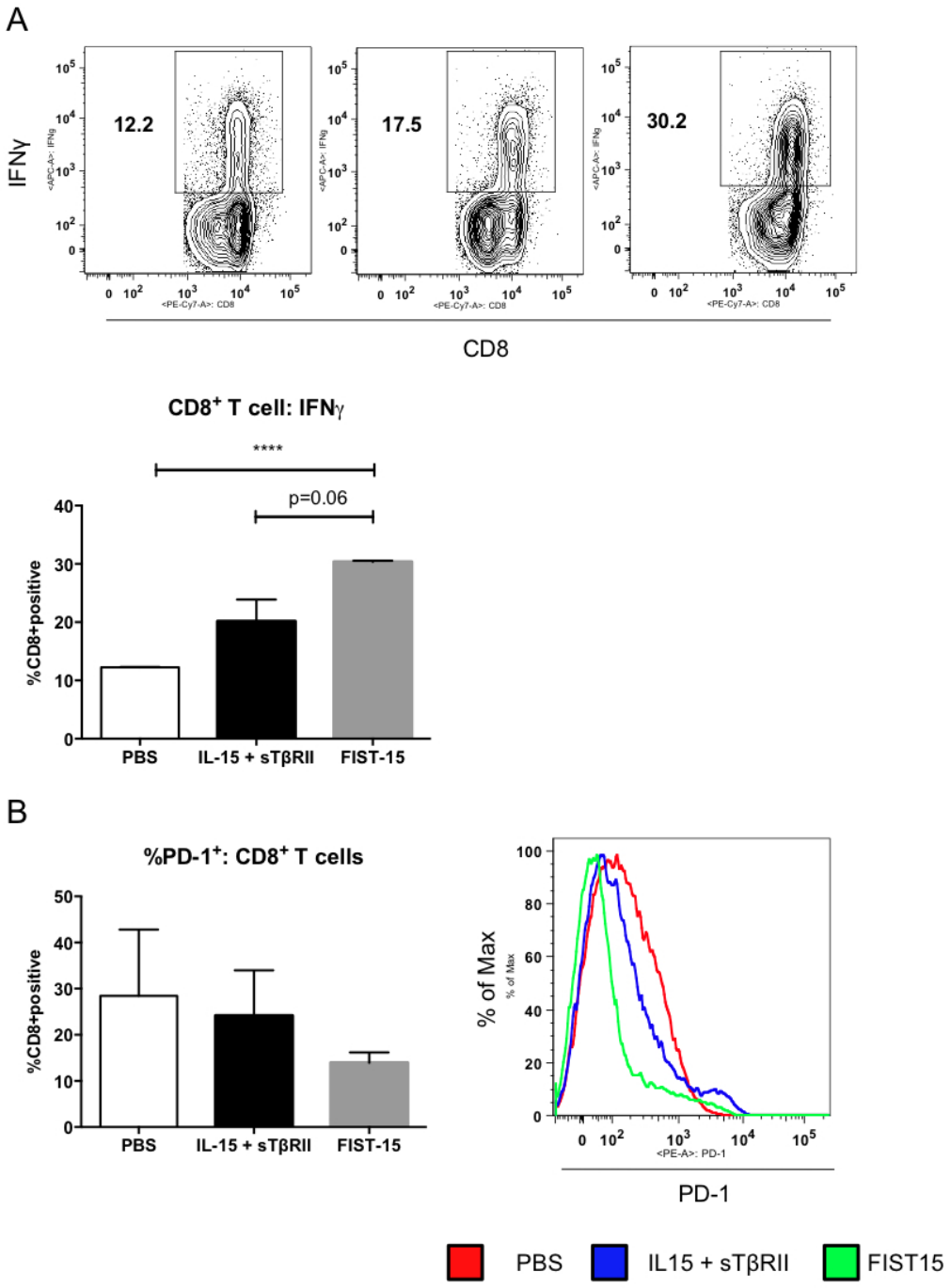

A
IFNγ
12.2
17.5
30.2
CD8
CD8+ T cell: IFNγ
****
p=0.06
%CD8+positive
PBS
IL-15 + sTβRII
FIST-15
B
%PD-1+: CD8+ T cells
%CD8+positive
PBS
IL-15 + sTβRII
FIST-15
% of Max
PD-1
PBS
IL15 + sTβRII
FIST15

Figure 3.3

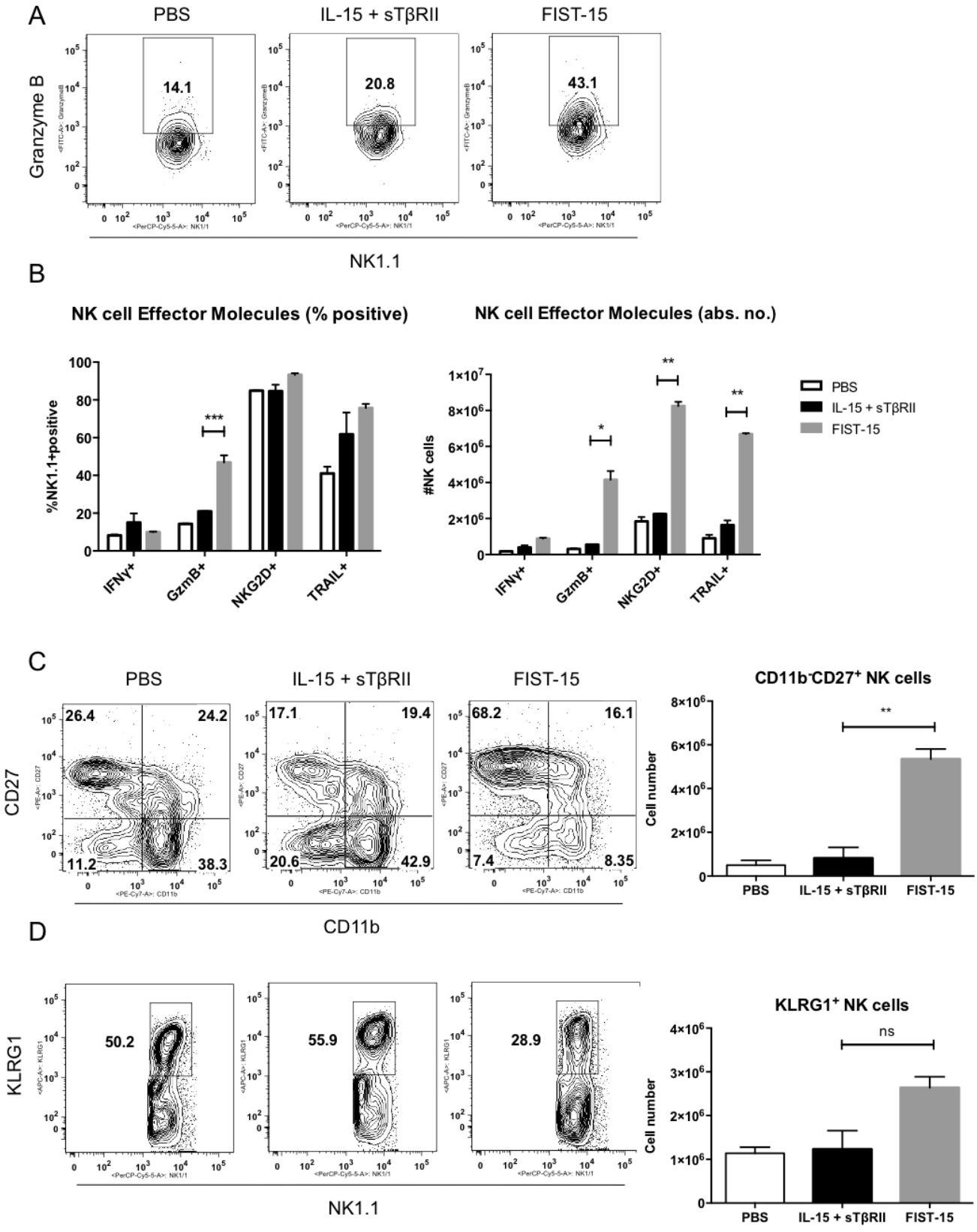

A
PBS
IL-15 + sTβRII
FIST-15
Granzyme B
14.1
20.8
43.1
NK1.1
B
NK cell Effector Molecules (% positive)
NK cell Effector Molecules (abs. no.)
%NK1.1+positive
#NK cells
IFNγ+
GzmB+
NKG2D+
TRAIL+
PBS
IL-15 + sTβRII
FIST-15
C
PBS
IL-15 + sTβRII
FIST-15
CD27
26.4
24.2
11.2
38.3
17.1
19.4
20.6
42.9
68.2
16.1
7.4
8.35
CD11b
CD11b-CD27+ NK cells
Cell number
D
KLRG1
50.2
55.9
28.9
NK1.1
KLRG1+ NK cells
ns

Figure 3.4

3.2.0 FIST-15 in CCl_4-induced liver fibrosis

3.2.1 Introduction

Liver fibrosis represents the end-stage of the vast majority of liver diseases that affects millions of individuals worldwide [325]. Despite the epidemiological impact of this phenomenon, the cellular and molecular mechanisms governing the process of fibrosis are only beginning to be elucidated. Further, with the exception of liver transplantation, no definitive cures exist for end stage liver disease and liver fibrosis. As we have a greater understanding of how chronic liver disease results in fibrosis, more interventions to treat liver fibrosis are being explored. Recent understanding of how the immune system plays a critical role in the process of liver inflammation, injury, and subsequent fibrotic process have created opportunities for potential intervention before and during the liver tissue remodeling in fibrosis [326].

Historically, liver fibrosis was thought to be a passive result of repeated insult and injury to the parenchyma of the liver. However, our current understanding of fibrosis is that it is an active process mediated by both by parenchymal cells of the liver, as well as resident and infiltrating immune cells [327]. In fibrosis, there is excessive production of extracellular cell matrix (ECM) proteins, such as type 1 and 3 collagens, elastin, and other glycoproteins, particularly fibronectin [328]. In addition to increased ECM production, there is also decreased degradation of this ECM. Matrix metalloproteinases (MMPs) that are responsible for ECM degradation are transiently upregulated during acute liver injury [329]. Over time in liver fibrosis, however, diminished MMP-mediated ECM degradation occurs as

a result of overexpression of tissue inhibitor of metalloproteinases (TIMPs) [330]. The decrease in ECM breakdown eventually results in chemical cross-linking of collagen fibrils occur, resulting in ‘bridging fibrosis’ and excessive scar formation, creating both a physical and functional barrier between the sinusoids of the liver and the hepatocytes.

The principal cell type implicated in the production of excess ECM is known as hepatic stellate cells (HSCs) [331]. In normal livers, HSCs reside in the perisinusoidal space in a quiescent state, where they store vitamin A and elaborate a physiologic basement membrane matrix of proteins to maintain the structure of the parenchyma of the liver. However, over the course repeated injury and inflammation, HSCs become activated, a process whereby HSCs lose their stores of vitamin A, undergo proliferation, and begin to synthesize an abnormal amount of type 1 collagen fibrils in the extracellular matrix. In the latter stages of this activation, HSCs can become myofibroblastic, exhibiting enhanced contractility and motility from the secretion of α-smooth muscle actin (αSMA) [332]. Transdifferentiation of HSCs to myofibroblast like cells is driven predominately by TGF-β and platelet derived growth factors (PDGF) [333, 334]. These myofibroblastic cells also secrete a milieu of proinflammatory cytokines, such as CCL2, resulting in the recruitment of monocytes and the continued remodeling and fibrosis of the liver parenchyma [335, 336].

HSCs therefore present an important target in liver fibrosis therapies because their activation and ensuing response to liver injury is what underlies the pathogenic process of fibrosis. When HSCs become activated, they are

susceptible to lysis by liver-resident NK cells [337]. During HSC activation, MHC-I is downregulated on their cell surface, providing a signal for NK cells to induce their lysis [338]. Further, the release of retinoic acid during liver damage upregulates the expression of cell stress markers Rae-1 (mouse) and MIC-A (human), ligands for the NKG2D activating receptor on NK cells [339]. Ligation of Rae-1 and NKG2D results in enhanced NK cell cytokine secretion and cytotoxicity. Depletion of NK cells in mouse models of liver fibrosis results in severely worsened fibrosis [340]. Conversely, activation of NK cells by agents, such as polyI:C and IFNα, have shown to improve outcomes in liver fibrosis models [341]. Additionally, NK cells can inhibit the liver fibrosis process by secreting IFNγ, which blocks the HSC transdifferentiation process to myofibroblasts [342].

NK cell therapy in liver fibrosis has been met with challenges. While the liver represents an organ enriched in NK cells, induction of proliferation and activation of intrahepatic NK cells are challenging. The use of agents such as polyI:C and IFNα have systemic and toxic side effects. Furthermore, the liver represents an extremely tolerogenic microenvironment. The secretion of immunoregulatory cytokines, such as TGF-β, especially during liver remodeling in fibrosis, poses a particular challenge in induction of NK cell activation and cytotoxicity against activated HSCs. The use of an immunotherapeutic agent such as FIST-15 could simultaneously address all of these concerns. FIST-15 can augment NK cell activity against activated HSCs by inducing their proliferation and activation. By neutralizing TGF-β, FIST-15 can also remove the inhibition of the tolerogenic

microenvironment on NK cells in the liver. Moreover, the neutralization of TGF-β would have the added effect of preventing collagen and αSMA deposition, as well as the process of HSC transdifferentiation, both of which depend on TGF-β.

3.2.2 Materials and Methods

Induction of liver fibrosis and cytokine treatment in mice

C57Bl/6 mice (Jackson Laboratories, ME) were subjected to liver injury and fibrosis with administration of carbon-tetrachloride (CCl4; Sigma-Aldrich). CCl4 was resuspended at a 1:2 ratio with corn oil (Sigma-Aldrich) and injected intraperitoneally (i.p.) into mice 0.5µl CCl4/g body weight twice weekly for 6 weeks. FIST-15 (2µg/dose) was administered i.p. on days alternating with CCl4 administration, twice weekly beginning on week 2 for a total of 4 weeks (8 doses total). Equivolume media conditioned with HEK293T cells were administered to a separate group of mice (control). An additional group of control mice were given PBS injections contemporaneously with the mice receiving CCl4 and conditioned media/IL-15. Mice were sacrificed at 6 weeks with liver and spleen extracted at time of sacrifice.

Intrahepatic lymphocyte isolation

A portion of the liver was incubated in a solution of 0.4% protease (Sigma-Aldrich), and 0.01% collagenase (Sigma-Aldrich) in DMEM media for one hour at 37°C. After gentle mechanical disruption, the digested suspension was filtered

through 70μm mesh and centrifuged at 50g for 3 min. The pellet was then washed in 2% FBS in PBS before cell surface marker staining.

Cell surface marker staining and flow cytometry

For cell surface marker staining, cells were resuspended in PBS with 2% FBS, incubated with anti-mouse FcR III/II for 15 minutes and labeled with conjugated antibodies specific for CD3, CD8, CD19, CD25, CD95L, CD314, KLRG1, NK1.1, and/or TRAIL. The expression of these cell surface markers was determined by FACS Canto cytometers (BD Biosciences) and analyzed by FlowJo software (Treestar, Inc).

3.2.3 Results

FIST-15 treatment in fibrotic livers rescues NK cell proportions to levels similar to healthy controls

Carbon tetrachloride (CCl_4) was administered i.p. twice weekly for 6 weeks to induce hepatic fibrosis or given PBS as control. Mice receiving CCl_4 were randomized into two groups: (1) conditioned media treatment (CM), and (2) FIST-15 treatment, receiving 4 i.p, injections of the treatment for once a week for the last four weeks of fibrosis induction (Fig. 3.5A). Mice that received PBS were administered additional PBS at the same time as FIST-15 or conditioned media as controls (healthy controls; HC). The proportion of NK cells in mice that had liver fibrosis but were treated with conditioned media was significantly lower than the proportion of intrahepatic NK cells seen in the livers of mice that were treated

with FIST-15 (Fig. 3.5B). Meanwhile, no significant differences in the size of NK cell compartment could be seen between healthy animals and animals treated with FIST-15.

FIST-15 enhances expression of NKG2D on intrahepatic NK cells

FIST-15 was able to significantly enhance the surface expression of activating receptor, NKG2D, on intrahepatic NK cells compared to both conditioned media treated animals and healthy controls (Fig. 3.5C). No differences were detected in between conditioned media treated controls and FIST-15 treated animals in terms of the proportion of cells expressing KLRG1. FIST-15 treatment, however, did result in a significantly decreased proportion of intrahepatic NK cells expressing Fas ligand compared to healthy controls, as well as conditioned media treated animals. Further, it appears that TRAIL expression is decreased in fibrotic animals treated with both conditioned media and FIST-15 compared to non-fibrotic animals. However, no significant differences in TRAIL expression could be seen between conditioned media and FIST-15 treated animals (Fig. 3.5C).

3.2.4 Discussion

This set of pilot experiments was undertaken to determine whether FIST-15 could potentially ameliorate liver fibrosis. Portions of the liver have been fixed for sectioning and histopathology and RNA has been extracted for qRT-PCR analysis of genes associated with fibrosis (e.g. type 1 collagen, αSMA). However,

the data that would provide a measure of the efficacy of FIST-15 is not yet available. Our interpretation of this data is only limited to the gross pathology seen, as well as from the flow cytometric analysis of intrahepatic NK cells. Grossly, livers from conditioned media treated mice were more tough and fibrotic. Nodules consisting of backed-up bile and dense fibrotic tissue could be seen throughout the organ. Bridging fibrosis and discoloration (whitening) of the periphery of the liver could also be appreciated. Healthy control mice had no such features in their livers upon extraction (Fig. 3.6). FIST-15 treated mice were of an intermediate phenotype. While nodules of fibrosis could be appreciated, bridging fibrosis at the liver periphery was absent or less pronounced in the majority of animals. While whole lobes of liver from all animals have been fixed. Sectioning and histopathological scoring of lesions within the liver are required for determining the true extent of fibrosis.

We were able to observe that FIST-15 treated animals had intrahepatic NK cells at proportions seen in healthy controls. While NK cells have been shown to be decreased in patients with fatty liver disease, it is uncertain if the same phenomenon occurs in the particular model of liver injury we have used [343]. The maintenance of the intrahepatic NK cell pool to levels seen in healthy controls is an encouraging observation. At the very least, the presence of NK cells in the liver hints at their ability to potentially induce cytolysis of activated HSCs in the setting of fibrosis. Further, intrahepatic NK cells from FIST-15 treated animals exhibit higher surface levels of NKG2D, which has been reported to be critical in their ability to detect stress ligands on activated HSCs. In a report

by Radaeva et al, blockade of NKG2D on NK cells with an antibody severely diminished their ability to lyse activated HSCs in a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) induced model of liver fibrosis [337]. These authors also reported that blockade of TRAIL also inhibited HSC lysis by NK cells stimulated with polyI:C, though its role seems to be less important that NKG2D. Interestingly, they also found that lack of Fas ligand expression on NK cells did not have any impact on their ability to lyse target HSCs. Our observations that FIST-15 treatment seems to downregulate expression of TRAIL and Fas ligand is intriguing insofar as they don't seem to play as important a role as NKG2D in their cytotoxic capacity against HSCs. The reason for which FIST-15 treatment decreases the expression of TRAIL and Fas ligand will require further investigation.

Although we have not been able to collect definitive outcome data from this set of experiments, preliminary results point to a possibility of therapeutic benefit in using FIST-15 as an immunotherapeutic agent in the setting of liver injury and fibrosis. Chronic liver injury and fibrosis remains a significant area of unmet medical need. Liver transplant remains the only curative option for patients with end stage liver disease. An agent that can target NK cells within the liver microenvironment and prevent the ongoing remodeling of the liver parenchyma during cycles of inflammation and wound repair would help to slow the progression of chronic liver disease to an irreversibly fibrotic state.

3.2.5 Figure Legends

Figure 3.5. FIST-15 treatment rescues intrahepatic NK cell numbers in CCl_4-indced hepatic fibrosis. Representative flow cytometric plots of $CD3^-NK1.1^+$ NK cells from non-fibrotic, healthy controls (HC), fibrotic animals treated conditioned media (CM), or FIST-15 (top panel) and the proportion of intrahepatic lymphocytes that are NK cells from the three groups (bottom panel) are shown in (A). Mean fluorescence intensity (MFI) of NKG2D activating receptor, Fas ligand, and TRAIL on the surface of intrahepatic $CD3^-NK1.1^+$ NK cells are in (B). Graphs depict the value of individual animals ± SD. Statistical significance was determined by one-way ANOVA and Dunnett's post-test for multiple comparisons. * *P < 0.05*, ** *P < 0.01*, *** *P < 0.001,* ****, *P < 0.0001.*

Figure 3.6 Gross pathology of CCl_4-induced hepatic fibrosis. Representative images from the livers of non-fibrotic, healthy control animals (HC), fibrotic animals treated with conditioned media (CM) or FIST-15 are shown. Green arrows depict regions of bridging fibrosis and extensive collagen deposition at the liver periphery.

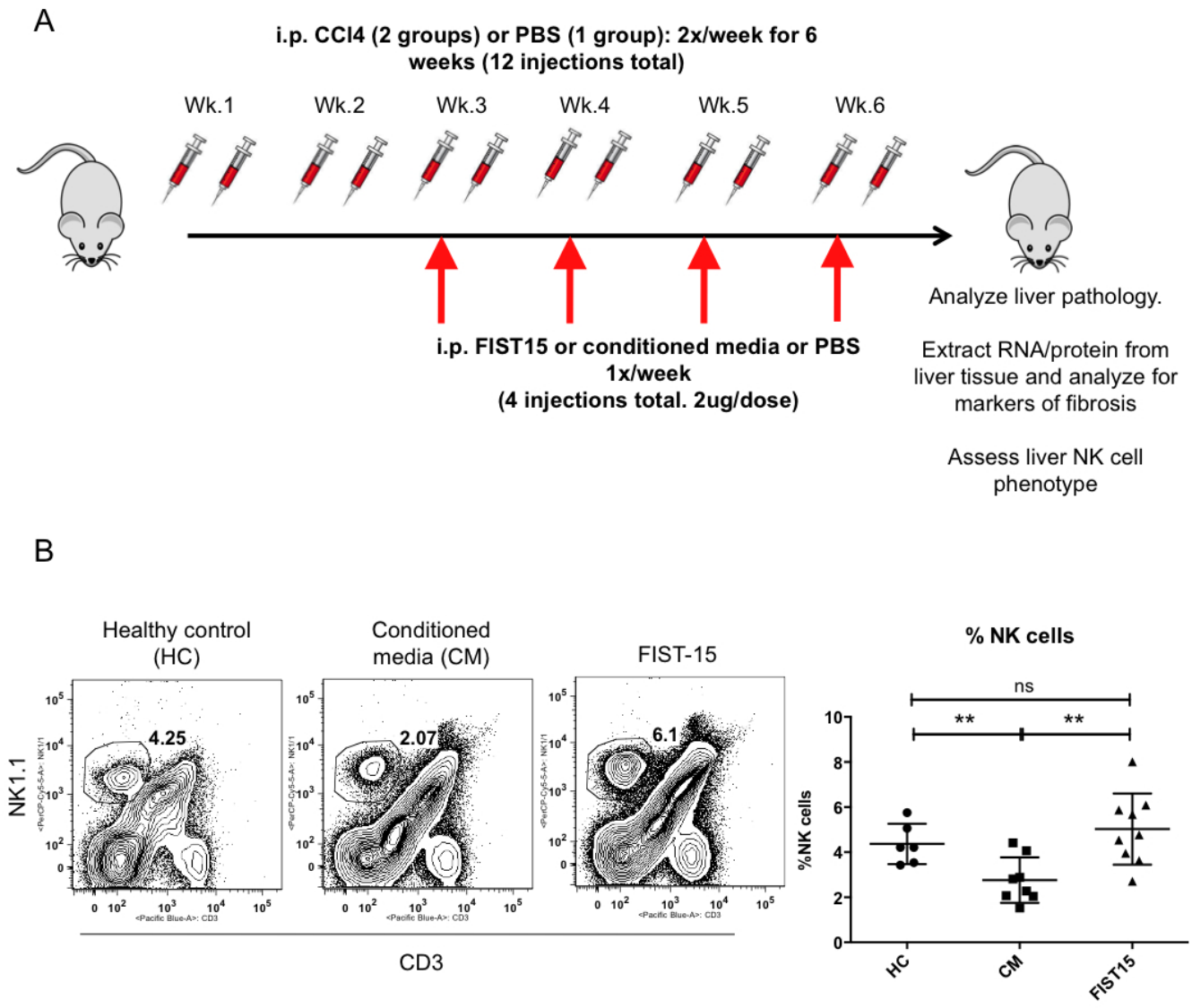
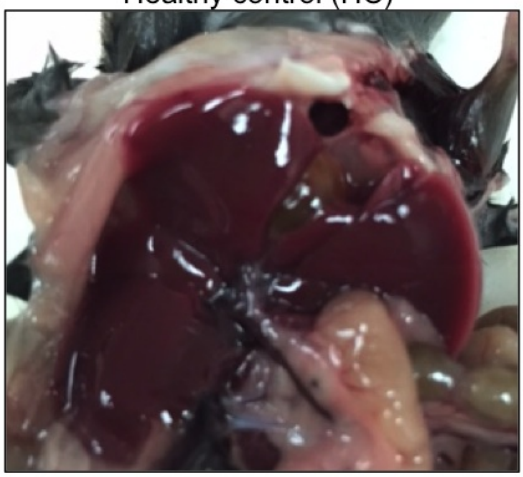
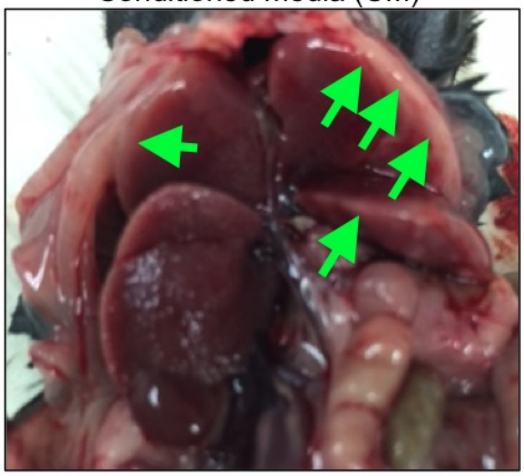

A
i.p. CCl4 (2 groups) or PBS (1 group): 2x/week for 6 weeks (12 injections total)
Wk.1
Wk.2
Wk.3
Wk.4
Wk.5
Wk.6
i.p. FIST15 or conditioned media or PBS 1x/week (4 injections total. 2ug/dose)
Analyze liver pathology.
Extract RNA/protein from liver tissue and analyze for markers of fibrosis
Assess liver NK cell phenotype
B
Healthy control (HC)
Conditioned media (CM)
FIST-15
NK1.1
CD3
4.25
2.07
6.1
% NK cells
%NK cells
ns
**
**
HC
CM
FIST15
C
NKG2D MFI: NK cells
NKG2D MFI
****
**
**
FasL MFI: NK cells
FasL MFI
****
ns
***
TRAIL MFI: NK cells
TRAIL MFI
**
*
ns

Figure 3.5

Healthy control (HC)



Conditioned media (CM)



FIST-15

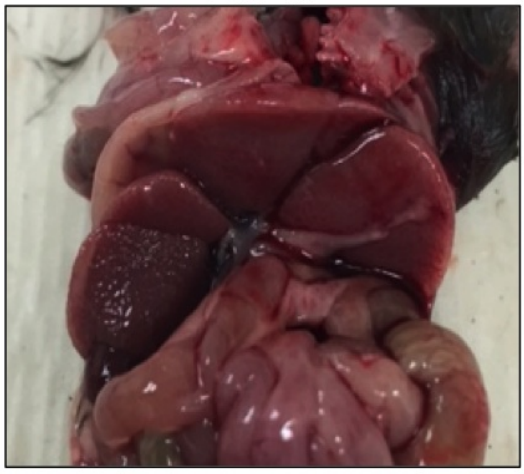


Figure 3.6

Chapter 4

GIFT-15 induced regulatory B cells (GIFT-15 B_{regs}) promote the development of $CD4^+CD25^+FoxP3^+$ regulatory T cells (T_{regs}) and $CD4^+FoxP3^-$ type-1 regulatory T cells (Tr-1) in murine experimental autoimmune encephalomyelitis (EAE)

Portions of this work was accepted for publication in the Journal of Neuroscience.

4.1.0 Abstract

The fusion of GMCSF with the common γ chain interleukin 15 (IL-15) produces a chimeric protein termed GIFT-15 (GMCSF and Interleukin-15 Fusion Transgene). Distinct from IL-15, GIFT-15 induces sustained activation of STAT3 in the absence of STAT5 activation, converting resting splenic B cells into IL-10 secreting regulatory B cells. Utilizing Vert-X GFP/IL-10 reporter mice, we show that GIFT-15 induced regulatory B cells (GIFT-15 B_{regs}) were superior at inducing IL-10 expression in activated $CD4^+$ T cells. In addition to increased FoxP3 expression in *ex vivo* MOG_{35-55} re-stimulated $CD4^+$ T cells from mice with experimental autoimmune encephalomyelitis (EAE), GIFT-15 B_{regs} also enhances the proportion of $CD49b^+CD223^+$ T regulatory type 1 (Tr1) cells. *In vivo*, adoptively transferred GIFT-15 B_{regs} home to mesenteric lymph nodes (MLN) and spleen leading to an induction of endogenous $FoxP3^+$ and Tr1 $CD4^+$ T cells, that were increased in the CNS of EAE mice and may play a direct role in attenuating EAE symptoms.

4.2.0 Introduction

Regulatory B cells (B_{regs}) have been ascribed important anti-inflammatory functions in a multitude of autoimmune conditions in both humans and mice, including colitis, arthritis, and neuroinflammatory disorders [252, 344-347]. B_{regs} are functionally defined by their ability to inhibit overt inflammatory responses via production of immunosuppressive cytokines, predominately interleukin-10 (IL-10), transforming growth factor-beta (TGF-β), and more recently interleukin-35 (IL-35) [348-351]. A functional definition remains the current standard of classifying B_{regs} due to a lack of consensus regarding subset-defining cell surface marker expression or a lineage-specific transcription factor [348, 352]. The role that IL-10 secreting B_{regs} play in disease has been most well documented in experimental autoimmune encephalomyelitis (EAE), a murine model of the human demyelinating disease, multiple sclerosis (MS). Various groups, including our own, have shown that the adoptive transfer of B_{regs} into mice with EAE can lead to durable remission of symptoms [249, 353, 354]. However, the basic mechanisms that underscore how B_{regs} may induce EAE remission *in vivo* remain unknown. The fact that IL-10 and MHC-II have been reported to be critical for B_{reg}-mediated immunosuppression in EAE have steered the investigation towards interactions between B_{regs} and $CD4^+$ T cells, which initiate and drive EAE through cognate antigen recognition by MHC-II and the pathologic secretion of interleukin-17 (IL-17) in a particular subset of these cells (T_H17) [249, 355].

While B cells have traditionally been thought of as augmenting pro-inflammatory responses of $CD4^+$ T cells, B_{regs} have been reported to suppress interferon-γ secreting T-helper 1 (T_H1) effector functions in favor of T-helper 2 (T_H2)-like responses [356]. B_{regs} have also been reported to dampen overt inflammation by inducing $FoxP3^+$ regulatory $CD4^+$ T cells (T_{regs}) in transplant models of islet allografts and collagen-induced arthritis [357, 358]. Further, B_{regs} have been shown to induce the formation of IL-10 secreting, $FoxP3^-$ regulatory $CD4^+$ T cells, known as T regulatory 1 (Tr1) cells, in mouse models of lupus and collagen-induced arthritis [346, 359]. However, B_{reg} induction of regulatory T cell function in EAE has not been demonstrated.

In an effort to elucidate the effect of B_{regs} on induction of regulatory T cell responses *in vivo*, we sought to determine how B_{reg} treatment of mice with MOG_{35-55}-induced EAE alters the $CD4^+$ T cell compartment in a GFP/IL-10 reporter mouse model. Utilizing a recombinant, bacterially derived fusion protein consisting of GMCSF and IL-15 (GIFT-15) [249, 360], we show that GIFT-15 can convert splenic B cells into IL-10 secreting B_{regs} (GIFT-15 B_{regs}). In addition to IL-10 secretion, we report for the first time that GIFT-15 B_{regs} also secrete IL-27, a cytokine critical for the induction of Tr1 cells [361-363]. In accordance with this observation, GIFT-15 B_{regs} are capable of not only inducing IL-10 producing $CD25^+FoxP3^+$ T_{regs}, but also $CD49b^+CD223^+$ Tr1 cells, in an antigen-specific manner. GIFT-15 B_{regs} home to the spleen and mesenteric lymph nodes (MLN) when adoptively transferred into mice, and induce remission of disease in mice

with EAE. Treatment of these mice with GIFT-15 B_{regs} correlated with increased T_{reg} and Tr1 subsets in the spleen, MLN, and the central nervous system (CNS). This is the first report to demonstrate that IL-10 secreting B_{regs} are capable of inducing regulatory $CD4^+$ T cell responses *in vivo* in an autoimmune, neuroinflammatory disorder.

4.3.0 Materials and Methods

Ethics statement

All animal experiments were approved by the Institutional Animal Care and Use Committee at Emory University and performed by accepted veterinary standards. C57BL/6, B6(Cg)-*Il10*$^{tm1.1Karp/}$J (Vert-X) mice were purchased from The Jackson Laboratory. C57BL/6-Tg(Act-EGFP)C14-Y01-FM131 Osb a gift from Dr. Masaru Okabe (Osaka University) and are maintained by Dr. David Archer at Emory University. The luciferase expressing (*luc*$^{+}$) transgenic C57BL/6 mice (B6-L2G85) were a kind gift from Dr. Edmund K. Waller. Mice were used between 6 and 20 week of age, and sacrificed using CO_2.

Generation of GIFT-15 B_{reg} and phenotyping

B cells were isolated from the spleen of wild type C57BL/6 mice by negative selection (Stem Cell Technologies) and stimulated with 10 μg/ml of recombinant bacterial derived GIFT-15 for 72-96 hours. GIFT-15 B_{regs} were phenotyped by flow cytometry of surface marker expression (CD1d, CD5, CD19, CD21, CD22, CD24, CD38, CD40, CD86, IgM,, H-2kb, I-Ab, PD-L1, PD-1 and their respective isotype controls, BD Biosciences, and CD80, IgD, PD-L2, and Rat IgG2aκ, eBioscience). Supernatant from untreated, GMCSF and IL-15 treated, and GIFT-15 treated B cells were analyzed for cytokine secretion (IL-10, IL-27, TGF-β, eBioscience; IL-35, Biolegend; TNFSF18/GITR ligand, RayBiotech).

In vitro co-culture

B cells and $CD4^+$ T cells were isolated by negative selection (Stem Cell Technologies) per manufacturer's protocol. $CD4^+$ T cells were placed into co-culture with freshly isolated splenic B cells (derived from C57BL/6 mice by negative selection) or GIFT-15 B_{regs} at a ratio of 2:1. $CD4^+$ T cells were stimulated with anti-CD3/28 DynaBeads (ThermoFisher) and analyzed by flow cytometry for GFP/IL-10 expression 72 hours post-culture. In MOG_{35-55} peptide restimulation co-cultures, $CD4^+$ T cells isolated from the spleen of mesenteric lymph nodes of Vert-X mice exhibiting EAE symptoms were placed into culture with B cells or GIFT-15 B_{regs} in the presence of MOG_{35-55} peptide (Sigma-Aldrich; see below for MOG_{35-55}-induced EAE). $CD4^+$ T cells were analyzed by flow cytometry for GFP/IL-10 expression 48 hours post-culture.

Western blot analysis

Cells were extracted in lysis buffer (Cell Signaling Technologies) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1x protease inhibitor (Thermo Scientific). Samples were separated by SDS-PAGE and immunoblotted for phospho-STAT3 (1:1000), phospho-STAT5 (1:1000), phospho-Akt (1:500), phospho-IKB (1:500), phospho-p38 (1:500), phospho-JNK (1:500), phospho-Erk1/2 (1:500), STAT3 (1:2000), STAT5 (1:2000), Erk1/2 (1:1000). All antibodies obtained from Cell Signaling Technologies.

Bio-distribution of GIFT-15 B_{regs}

GIFT-15 B_{regs} were generated by co-culturing $CD19^+$ B cells purified from B6/L2G85 mice splenocytes in complete R10 media with 10 ng/mL recombinant

mouse GIFT-15 at a cell density of 0.5×10^6 cells/mL for 4/5 days. 5×10^6 of B6-L2G85-GIFT-15-B_{regs} or B6-L2G85-B cells were intravenously injected into syngeneic EAE C57BL/6 mice with a clinical score of 1/2. The mice were injected subcutaneously with luciferin (150 mg/kg body weight) before imaging on the In Vivo Imaging System (IVIS) (Xenogen, Alameda, CA) at the core facility in Winship Cancer Institute.

Alternatively, GIFT-15-Bregs were generated from GFP-B cells and infused in EAE mice. After one week the animals were euthanized and MLN, spleen and CNS harvested for analysis. White cells were isolated and analyzed by flow cytometry for the presence of GFP.

Cells isolation and flow cytometry procedures for MLN, spleen and brain leukocytes

Spleens and mesenteric lymph nodes were dissected post-mortem and collected in RPMI (Lonza). For splenocytes and lymph node cells preparation, organs were mashed through a 70-μm cell strainer (BD Biosciences), as previously described[364] and erythrocytes from spleens were lysed using Red Cell Lysis Buffer (Sigma-Aldrich).

At different time points, mice (n=5) were perfused intracardially with ice-cold DPBS without Ca^{2+} and Mg^{2+}. Brains were extracted and immediately homogenized with a plunger in RPMI. The cell suspension was centrifuged at 300 x g for 5 min at room temperature. The supernatant was aspirated and cells were gently resuspended in 37% Percoll (GE Healthcare, Uppsala, Sweden).

The cell suspension was underlaid beneath 80% Percoll and centrifuged at 600 x g for 25 min with slow acceleration and deceleration rates. The cell ring at the interphase was collected and mixed thoroughly with DPBS containing 2% FBS (FACS buffer). Cells were then centrifuged at 300 x g for 5 min and washed twice with FACS buffer.

For detection of cell surface markers, cells were stained in FACS buffer with the different fluorochrome labeled monoclonal antibodies. Cells were incubated for 30 min on at 4 °C antibodies. Flow cytometry analysis and data acquisition were performed using a BD Canto II and the data analysis with BD FACSDiva software.

Experimental autoimmune encephalitis induction and analysis

We induced and scored experimental autoimmune encephalitis (EAE) as previously described [365, 366]. Briefly, EAE immunization was performed by subcutaneous injection of 50 µg $MOG_{35–55}$ (Sigma-Aldrich, St. Louis, MO, USA) emulsified in complete Freund's adjuvant (CFA; Difco, Detroit, USA) containing 5 mg/mL of H37Ra *Mycobacterium tuberculosis*. On day 0 and 2, 100 ng of pertussis toxin (PTX; Sigma-Aldrich, St. Louis, MO, USA) was administered by intraperitoneal injection. Three independent experiments were conducted (n = 5). EAE clinical scores were graded as follows: 0, normal; 1, flaccid tail; 2, hind-limb weakness; 3, flaccid tail with paralysis of one front or one hind leg; 4, complete hind-limb paralysis and partial front leg paralysis; 5, tetraplegia, moribund or death.

4.4.0 Results

Recombinant GIFT-15 induced B_{reg} phenotype

We have previously reported that a fusion protein consisting of GMCSF and IL-15 possesses the ability convert splenic B cells into IL-10 secreting regulatory B cells (GIFT-15 B_{regs})[249]. Beyond IL-10 secretion, we sought to determine whether published cell surface markers, CD1d and CD5 were expressed in GIFT-15 B_{regs}. To do so, we utilized the GFP/IL-10 (Vert-X) reporter mouse model[367] to specifically identify IL-10 secreting GIFT-15 B_{regs}. In contrast to wildtype C57BL/6 derived B cells, a subset of splenic B cells derived from Vert-X mice became GFP/IL-10^{+} after 72 hours of GIFT-15 treatment *in vitro* (Fig. 4.1A). Consistent with previous reports, this subset of GFP/IL-10^{+} GIFT-15 B_{regs} expressed higher surface levels of CD1d and CD5 compared to GFP/IL-10^{-} GIFT-15 B_{regs}. In addition to these two surface markers, and consistent with other reports of B_{reg} cell surface phenotypes, we found that GIFT-15 B_{regs} expressed higher surface levels of CD22, CD24, CD38, CD40, CD80, CD86, MHC-I, and MHC-II (Supplementary Fig. 4.1) compared to GMCSF and IL-15 treated B cells. In addition to markers related to the B cell receptor complex, co-stimulation, and antigen presentation, we interrogated expression levels of co-inhibitory molecules and their ligands, such as PD-1 (CD279), PD-L1 (CD273), and PD-L2 (CD274). We found that compared to control cytokine treatment, GIFT-15 B_{regs} uniformly upregulated PD-1 expression and expressed higher levels of PD-L1, but not PD-L2 (Fig. 4.1B). GIFT-15 B_{regs} secrete high levels of IL-10, which we verified by ELISA (Fig. 4.1C). We also detected the secretion of IL-27 by GIFT-15

B_{regs}, but did not detect any IL-35, TGF-β, or TNFSF18, a soluble ligand for glucocorticoid-induced TNFR-related protein (GITR) (Fig. 4.1D and data not shown). In order to determine how GIFT-15 may induce conversion of splenic B cells to GIFT-15 B_{regs}, GIFT-15 signaling was investigated. Treatment of mouse splenocytes with GIFT-15 showed that, distinct from GMCSF and IL-15 treatment, GIFT-15 induces STAT3 phosphorylation and activation in the absence of STAT5 activation with delayed kinetics (Fig. 4.1E). Biochemically, GIFT-15 drives STAT3 phosphorylation later than GMCSF and IL-15 treatment and STAT3 remains phosphorylated for at least 48 hours post-treatment in the absence of STAT5 phosphorylation (Fig. 4.1F). In addition to this unopposed STAT3 activation event, we interrogated other non-canonical signaling pathways that have been associated with GMCSF and γ-chain signaling (Fig. 4.1G). 15 minutes post-treatment, both GMCSF and IL-15 and GIFT-15 treatment induced phosphorylation of Erk1/2 (p44/42 MAP kinase) but this effect was transient. Both GMCSF and IL-15 and GIFT-15 also induced sustained Akt phosphorylation.

GIFT-15 B_{regs} induce IL10 expression in activated $CD4^+$ T cells *in vitro*

In order to determine the suppressive effects of GIFT-15 B_{regs}, we utilized an *in vitro* co-culture system. $CD4^+$ T cells isolated from naïve Vert-X mice were placed into co-culture with B cells or GIFT-15 B_{regs} to determine if GIFT-15 B_{regs} could induce IL-10 expression by $CD4^+$ T cells. In steady state conditions, without cognate antigen or polyclonal activation, neither B cells nor GIFT-15 B_{regs}

induced IL-10 expression in $CD4^+$ T cells (data not shown). $CD4^+$ T cells gained the ability to produce IL-10 when stimulated with anti-CD3/28 beads in the presence of GIFT-15 B_{regs}, but not in the presence of splenic B cells (Fig. 4.2A). $CD4^+$ T cells isolated from the spleen and MLN of Vert-X mice with EAE also produced more IL-10 in the presence of GIFT-15 B_{regs} and MOG_{35-55} peptide compared to splenic B cells (Fig. 4.2B). A significant amount of IL-10 expressing $CD4^+$ T cells derived from the spleen of mice with EAE were conventional $CD25^+FoxP3^+$ T_{regs}, as there were no appreciable differences in the $CD49b^+CD223^+$ Tr1 subsets. A large proportion of $CD4^+$ T cells from the MLN of mice with EAE expressed IL-10 in the presence of GIFT-15 B_{regs} and MOG_{35-55} peptide, exhibiting contemporaneous increases in both conventional $CD25^+FoxP3^+$ T_{regs} (2-fold increase), as well as Tr1 cells (4-fold increase) (Fig. 4.2B).

Dynamic distribution of GIFT-15 B_{regs}

Homing of GIFT-15 B_{regs} was first studied after a single intravenous infusion in EAE mice followed by *in vivo* imaging with the use of luciferase-expressing transgenic B cell. Purified B cells from B6-L2G85 mouse splenocytes were stimulated with recombinant mouse GIFT-15 to generate GIFT-15 B_{regs}, which were tail vein injected into EAE C57BL/6 mice (5×10^6 cells per mouse). Alternatively, non-stimulated B cells (5×10^6) were injected as a control. Mice were then imaged using a Xenogen IVIS bioluminescent imager for 3 min at

small binning. Pseudo-colored scale shows by day 2 varying light emittance throughout the gut (Fig. 4.3A). By day 5, the highest light intensity of the GIFT-15 B_{regs} was localized in the spleen. No signal was detectable after 10 days. Minor diffuse light was transiently noticeable in the gut of mice treated with B6-L2G85 B cells during the first 7 days (Fig. 4.3A).

To have a better understanding of the migratory patterns of GIFT-15 B_{regs}, transgenic animals expressing enhanced GFP under the chicken β-actin-promoter[368] were used as B cells donors. GIFT-15 B_{regs} generated from these mice (GFP-GIFT-15 B_{regs}) were then adoptively transferred into a wildtype recipient with EAE. After 5 days, the recipient mice were sacrificed and cell content within the spleen and MLN were analyzed by flow cytometry. GFP-labeled cells were observed in MLN and spleen of EAE mice that received GFP-GIFT-15 B_{regs}. No signal was detectable in the CNS of any animal neither in spleen and MLN collected from the animal treated with GFP-B cells (Fig. 4.3B).

T cell dynamics in MLN and spleen after adoptive transfer of GIFT-15 B_{regs}

We have shown that GIFT-15 B_{regs} generated with the mammalian derived GIFT-15 were able to reverse EAE in mice [249]. To verify whether this therapeutic effect was reproducible by GIFT-15 B_{regs} generated with bacterial derived GIFT-15 [360], we injected EAE mice with $2x10^6$ GIFT-15 B_{regs} every 10 days and followed disease score over time. Clinical attenuation of disease score was observed in mice treated with GIFT-15 B_{regs} (Fig. 4.4A). Complete and stable

remission was achieved one month post-adoptive transfer. In mice treated with B cells (control group) there was no suppression or clinical amelioration of EAE disease (Fig. 4.4A).

To better understand the effect of GIFT-15 B_{regs} on $CD4^+$ T cells in MLN and spleen, EAE mice were intravenously infused with GIFT-15 B_{regs} or B cells and organs were collected after 1, 2 and 4 weeks post-adoptive transfer, and lymphoid cells isolated and analyzed by flow cytometry (Fig. 4.4B-D).

Within the first week, a higher frequency of $CD4^+$ T cells were observed in the spleen of the animals that received GIFT-15 B_{regs} relative to the control group. The two most well-known types of regulatory $CD4^+$ T cells are $CD25^+$ and $FoxP3^+$, T_{regs}; and $CD49b^+$, $CD223/Lag\text{-}3^+$, and $IL\text{-}10^+$, Tr1 cells[369] (Supplementary Fig. S4.2C). To determine whether GIFT-15 B_{regs} treatment altered the regulatory of $CD4^+$ T cell content, and if so, whether it was due to differences in T_{regs} or Tr1 populations, we carried out further subset analyses. An increase of T_{regs} was observed in the GIFT-15 B_{regs} treated group as well as Tr1 cells (P=0.006, P=0.001 respectively and Fig. 4.4E). A moderate increase of $CD4^+$ T cells was also observed in the MLN with an increased frequency of Tr1 cells (P=0.03) and T_{regs} (P=0.002 and Fig. 4.4B and E).

After 2 weeks no major differences were noticeable in the spleen between GIFT-15 B_{regs} treated and control groups, whereas in the MLN of GIFT-15 B_{regs} treated mice a modest surge of $CD4^+$ T cells was still present. Further analysis showed

expansion of both T_{regs} and Tr1 cells in the MLN of GIFT-15 B_{regs} treated group compared to controls (P=0.03 and P=0.05 respectively, Fig. 4.4C and E).

At one month post-adoptive transfer, no major differences were noticeable in the MLN whereas in the spleen of the mice that received GIFT-15 B_{regs}, there was a sustained increase in Tr1 cells (P=0.02 and Fig. 4.4D and E).

Leukocyte dynamics in the CNS of EAE mice after adoptive transfer of GIFT-15 B_{regs}

In order to determine both the extent and the composition of CNS-infiltrating cells and subsequent changes in clinically improved animals, spinal cords and brains were examined for leukocyte subset content at 2 weeks, 4 weeks, and 12 weeks after GIFT-15 B_{regs} adoptive transfer.

Relative CD45.2 surface expression levels can be used to distinguish between microglia ($CD45.2^{dim}CD11b^{+}$) and infiltrating macrophages ($CD45.2^{high}CD11b^{+}$). We evaluated the relative dynamics of the CNS microglial and macrophage response following adoptive transfer of GIFT-15 B_{regs} by assessing the percentage of $CD11b^{+}$ cells that expressed low or high levels of CD45.2. We did not find major differences in the CNS leukocyte profile in GIFT-15 B_{regs}-treated mice relative to controls during the first month (Supplemental Fig. S4.2). In contrast, at three months we observed that blood-derived infiltrate macrophages ($CD45^{high}$) were increased in the CNS of mice treated with B cells (i.e. mice with ongoing EAE) whereas resident microglia ($CD45^{low}$) content was similar to that

observed in naïve mice (Fig. 4.5A). Mice in the GIFT-15 B_{regs} treated group showed a comparable percentage of infiltrated macrophages to that observed in normal mice.

There was no obvious correlation between disease severity and the proportion of GR-1/Ly-6G$^+$, neutrophils (always less than 1%), in all groups of mice. Lymphocytes were increased in the CNS of B cell treated mice who showed signs of disease (CD45.2$^+$CD11b$^-$ or CD45.2$^+$GR1$^-$).

A detailed analysis of the lymphocyte compartment revealed increases of both CD3$^+$ T cells (4-fold increase) and CD19$^+$ B cells (5-fold increase) in EAE mice. In mice treated with GIFT-15 B_{regs}, the percentage of B and T cells was similar to normal mice.

Accumulation of T_{regs} was detectable in both EAE groups (i.e. mice treated with B cells or GIFT-15 B_{regs}) when compared to normal mice. However, only in the group treated with GIFT-15 B_{regs}, was a significant increase in Tr1 frequency observed compared to non-EAE mice (P=0.006, Fig. 4.5B).

4.5.0 Discussion

Regulatory B cells (B_{regs}) are a functional subpopulation of B cells, which exert their immune suppressive function via the production of regulatory cytokines, such as IL-10 [349], TGF-β [370], IL-27 [371], IL-35 [372] and the expression of inhibitory surface molecules that suppress pathogenic T cells and auto-reactive B cells in a cell-to-cell contact-dependent manner [373]. Akin to our previously published report [360], we show that recombinant, bacterial derived GIFT-15 is able to convert a subset of splenic B cells into IL-10 secreting B_{regs}. Consistent with other reports [374, 375], we show with the GFP/IL-10 (Vert-X) reporter mouse model that the B cells capable of secreting IL-10 are enriched for expression of CD1d and CD5. Interestingly, GIFT-15 B_{regs} express high levels of PD-1 and PD-L1, but not PD-L2. Moreover, we demonstrate that in addition to IL-10 secretion, GIFT-15 B_{regs} also secrete IL-27, but not TGF-β or IL-35. Mechanistically, GIFT-15 leads to asymmetrical signaling through the IL-15R complex, which manifests as STAT3 hyperphosphorylation in the absence of STAT5 signaling for up to 48 hours post-stimulation. In an effort to identify other potential signal transduction pathways activated by GIFT-15 stimulation, we interrogated non-canonical pathways of GMCSF and IL-15 signaling. We found that both GIFT-15 and control cytokine treatment results in transient activation of Erk and sustained Akt activation.

While IL-10 expression is considered essential to the immunosuppressive

properties of GIFT-15 B_{regs}, our result suggests that IL-27 may play a role as well. IL-27 is a member of IL-6/IL-12 cytokines family and has been shown to have anti-inflammatory properties. *In vitro*, IL-27 elicited the differentiation of Tr1-like cells, which express IL-10 and have been more recently described to co-express CD49b and lymphocyte activation gene 3 (CD223/Lag-3), a negative regulator of T cell function [376]. The secretion of IL-27 by GIFT-15 B_{regs} may promote the differentiation of Tr1 cells *in vitro* and *in vivo*. Contrary to a recent report describing the role B_{reg}-derived IL-35; we did not detect this cytokine in media cultured by GIFT-15 B_{regs}. It is possible that the mechanism by which GIFT-15 promotes differentiation of B_{regs} is distinct from the one dependent on IL-35 [372]. Independent of IL-10, B cell production of TNFSF18, a soluble ligand for GITR, was reported to be required for the induction of protective T_{regs} in mice with EAE [377]. However, we were also unable to detect this molecule in media cultured by GIFT-15 B_{regs}.

Utilizing Vert-X reporter mice, we show that GIFT-15 B_{regs} were able to induce IL-10 expression in activated $CD4^+$ T cells. $CD4^+$ T cells isolated from the spleen of Vert-X mice and cultured with GIFT-15 B_{regs} were not induced to secrete IL-10. However, Vert-X $CD4^+$ T cells stimulated with anti-CD3/28 beads and co-cultured with GIFT-15 B_{regs} displayed a dramatic increase in the proportion of cells secreting IL-10. More physiologically, we wished to determine if $CD4^+$ T cells could respond in a similar fashion to their cognate antigen. To test this, $CD4^+$ T cells were isolated from the spleen and MLN of MOG_{35-55}-immunized Vert-X mice

and stimulated *ex vivo* with MOG_{35-55} peptide in the presence of GIFT-15 B_{regs} or B cells. We found that in the presence of MOG_{35-55} peptide and GIFT-15 B_{regs}, $CD4^+$ T cells from both spleen and MLN exhibited increased IL-10 expression. While $CD4^+$ T cells from both compartments had increased proportions of $CD25^+FoxP3^+$ T_{regs} cells, only $CD4^+$ T cells derived from MLN displayed increased proportions of $CD49b^+CD223^+$ Tr1 cells. While there have been reports of B_{reg} induction of T_{regs} , we believe this is the first report of *in vitro* induction of Tr1 cells by B_{regs} [378, 379].

In vivo, adoptively transferred GIFT-15 B_{regs} home to MLN and spleen leading to an increase frequency of endogenous $FoxP3^+$ and Tr1 $CD4^+$ T cells, which may play a direct role in attenuating EAE. One week post-adoptive transfer of GIFT-15 B_{regs}, we observed an intense increase in the proportion of both T_{regs} and Tr1 cells in the spleen. To a lesser extent, but in a more sustained fashion, we observed an expansion of T_{regs} and Tr1 cells in MLN. In the CNS, an increase of T_{regs} and Tr1 was evident only one month post-adoptive transfer (Fig. 4.6). While this is the first demonstration of GIFT-15 B_{reg}-dependent increase of T_{regs} in mice with EAE, the role that T_{regs} play in the autoimmune pathogenic scenario of EAE is well-established [380]. Less well-characterized is the role of Tr1 cells in EAE. Several reports indicate that Tr1 cells are protective through their provision of IL-10 and inhibition of T_H17 differentiation in an IL-27-dependent manner[381]. Interestingly, in humans, it has been reported that MS patients exhibit impaired Tr1 differentiation and IL-10 secretion by Tr1 cells [382, 383]. We hypothesize that adoptive transfer of GIFT-15 B_{regs} induces an expansion or differentiation of

regulatory T cells, both T_{regs} and Tr1, with IL-10 secreting capabilities, which ultimately relocate to areas of active inflammation within the CNS of EAE mice.

Mice treated with GIFT-15 B_{regs} showed a much fewer infiltrating macrophages compared to B cell treated mice with EAE; with levels similar to the one observed in normal, non-EAE C57BL/6 mice. In the CNS of mice with EAE, microglia/macrophage activation has been shown to lead to the secretion of pro-inflammatory cytokines and antigen presentation. Macrophages are also known dominate the inflammatory infiltrates and their degree of infiltration correlates with clinical score severity. We show here that GIFT-15 B_{reg} treated mice with EAE exhibit less CNS-infiltrating macrophages compared to EAE mice treated with B cells, a finding that correlates with their disease remission. Additionally, mice with EAE showed a higher recruitment of lymphocytes in the CNS, both T cells and B cells, a sign of compromised blood-brain barrier function. In contrast, mice treated with GIFT-15 B_{regs} showed levels of lymphocyte infiltration similar to normal, non-EAE mice. Further analysis of the $CD4^+$ T cells reveled a moderate increase of T_{regs} compared to normal mice. Increase in T_{regs} population in mice with EAE has been previously observed [384]. Indeed, during EAE, T_{regs} enter the CNS, where they may locally regulate pathogenic inflammation [385], a phenomena clearly augmented by adoptive transfer of GIFT-15 $B_{regs.}$

We have previously shown that adoptive transfer of syngeneic, but not allogeneic, GIFT-15 B_{regs} can induce EAE remission in a MHC-II and IL-10 dependent manner[249]. These aggregate findings suggest that GIFT-15 B_{regs} likely interact with $CD4^+$ T cells *in vivo* as part of their physiology. We here have shown that GIFT-15 B_{regs} do not directly migrate to inflamed EAE CNS, but rather home in to MLN and spleen and dissipate within two weeks. We show that GIFT-15 B_{regs} home to the MLN and spleen where they may directly interact with $CD4^+$ T cells in a manner which leads to an augmentation of endogenous IL-10^+ T_{regs} and Tr1 which latterly home and accumulate in inflamed CNS, altering the lymphomyeloid brain compartment to a pattern seen in non-inflamed normal brain (Fig. 4.6). These observations support the notion that pharmacological augmentation of autologous B cells to a GIFT-15 B_{reg} functionality may allow for adoptive cell therapy of multiple sclerosis (MS). Others and we have shown that endogenous content of circulating B_{regs} in human subjects with and without MS is vanishingly low and that less than 1% of blood B cells fulfill a B_{reg} definition [386].

In our EAE murine model, we administered the equivalent of three doses of 100 million cells/kg intravenously. Extrapolating to human translation, any attempt to collect and enrich a sufficient number of endogenous B_{regs} from blood would be logistically unfeasible. Therefore, the demonstrated property of GIFT-15 to *ex vivo* convert resting blood B cells to B_{regs} would foreshadow the possibility of exploiting an autologous augmented B cell therapy for MS. This strategy differs from the clinical use of alternate autologous suppressor cell types for

autoimmune disorders, such as T_{regs} (NCT02428309), as much as we show that B_{regs} appear to launch a pan-IL-10^{+} CD4 T cell response *in vivo* that is durable and CNS tropic, far outlasting the B_{regs} initiators. Lastly, though GIFT-15 B_{regs} are pharmacologically activated, we speculate that their functionality likely reflects that of endogenous B_{regs} and may provide the insight that B_{reg} biology may play a key role in the physiopathology of maladapted immune response seen in EAE/MS and possibly other autoimmune disorders.

Figure Legends

Figure 4.1 GIFT-15 induced regulatory B cell (GIFT-15 B_{regs}) phenotype and cell signaling profile. GFP/IL-10 expression in GIFT-15 treated C57BL/6 and Vert-X B cells after 3 days. Comparison of CD1d and CD5 expression on GFP/IL-10^{-} and GFP/IL-10^{+} B_{regs} by mean fluorescence intensity (MFI) (A). Surface expression of PD-1, PD-L1, and PD-L2 on GMCSF and IL-15 treated B cells compared to GIFT-15 B_{regs}. Shaded histograms represent isotype staining and solid black lines represent indicated surface marker staining (B). IL-10 (n=2) and IL-27 (n=4) levels in GIFT-15 B_{reg} cultured media compared to controls (C and D). STAT3 and STAT5 phosphorylation following treatment with GIFT-15 or GMCSF and IL-15 at 15 minutes and 5 hours (E). STAT3 and STAT5 phosphorylation time course (F). GIFT-15 activation of major MAPK (p38/Erk/Jnk) and PI3K/Akt signaling pathways (G). Flow cytometry plots are representative of a biological replicate from two to four independent experiments. Bar graphs in A-D display the mean ± S.D. *P* values were calculated using two-tailed Student's *t* test. * $P <$

0.05; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. NS: no stimulation, GFP: green fluorescent protein.

Figure 4.2. GIFT-15 B_{regs} induce IL-10 expression in activated $CD4^+$ T cells and enhance *ex vivo* formation of $CD25^+FoxP3^+$ T_{regs} from the spleen and $CD49b^+CD223^+$ Tr1 cells from the MLN of mice with EAE. αCD3/28 Dynabead stimulated Vert-X $CD4^+$ T cells cultured with splenic B cells or GIFT-15 B_{regs} (A). $CD4^+$ T cell analysis from the spleen Vert-X mice with EAE, clinical score of 1 or 2, cultured with splenic B cells or GIFT-15 B_{regs} following MOG_{35-55} peptide stimulation (B). $CD4^+$ T cell analysis from the MLN of Vert-X mice with EAE, clinical score of 1 or 2, cultured with splenic B cells or GIFT-15 B_{reg} following MOG_{35-55} peptide stimulation (C). Flow cytometry plots are representative of a biological replicate from three independent experiments. Bar graphs in A-C display the mean ± S.D across triplicate samples. *P* values were calculated using two-tailed Student's *t* test. * $P < 0.05$; ** $P < 0.01$. Tr1: Type 1 regulatory $CD4^+$ T cells, T_{regs}: $CD25^+FoxP3^+$ regulatory $CD4^+$ T cells.

Figure 4.3. Dynamics of GIFT-15 B_{regs} biodistribution. Real-time IVIS imaging with GIFT-15-Luciferase-B_{regs} or Luciferase-B cells. MOG immunized mice were injected with 5×10^6 B6-L2G85-GIFT-15 B_{regs} or B6-L2G85 B cells and they were imaged every day for two weeks (A). Flow cytometry plot of GFP-GIFT-15 B_{regs} or GFP-B cells after adoptive transfer in EAE mice. Immunized mice were injected

with 5 × 10^6 cells. After 7 days the animals were euthanized (n=5) and spleen, MLN and CNS collected. Leukocytes were isolated and run by flow cytometry Data are representative of 2 independent experiments with n=10 mice in each group (B).

Figure 4.4. GIFT-15 B_{regs} ameliorate EAE and induce *in vivo* formation of $CD25^+FoxP3^+$ T_{regs} and $CD49b^+CD223^+$ Tr1 cells in mice with EAE. Clinical score evolution in MOG_{35-55} immunized Vert-X mice treated with GIFT-15 B_{regs} or B cells (2 × 10^6 cells/mouse, n=10 per group). Data are the mean ± s.e.m. The data is representative of 2 different experiments (A). Two-parameters contour plots show CD3 *versus* CD4, T_{regs} (CD25 *versus* FoxP3), Tr1 (CD49b *versus* CD223) expression in spleen and MLN after 1 weeks (B), 2 weeks (C) or 4 weeks (D) of GIFT-15 B_{regs} or B cells adoptive transfer in EAE mice. The gating strategy: the lymphocytes were gated according to their side- and forward-scatter properties, and $CD4^+$ cells were gated from lymphocytes. $CD4^+$ cells were stained for Tregs or Tr1 phenotype. GFP/IL-10 *versus* SSC is the frequency of either T_{regs} or Tr1 cells respectively (Supplementary Fig S4.2A). Bar graphs are the mean ± S.D. of two independent experiments (biological replicates) with n=10 per group (E). *P* values were calculated using two-tailed Student's *t* test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 4.5. Profile of infiltrating leukocytes in the CNS of mice with EAE three months post-adoptive transfer of GIFT-15 $B_{regs.}$ CNS monuclear cells from naïve C57BL/6, MOG_{35-55} immunized mice with EAE treated with B cells or GIFT-15 B_{regs} were analyzed after 3 months from immunization. The gated populations represent microglial cells ($CD45.2^{int}$ $CD11b^{+}$), macrophages ($CD45.2^{hi}$ $CD11b^{+}$), and leukocytes ($CD45.2^{hi}$ $CD11b^{+}$). Infiltrating lymphocytes were stained for the presence of $CD4^{+}$, $CD8^{+}$, $CD25^{+}$, $FoxP3^{+}$, $CD49b^{+}$ and $CD223^{+}$. The data are representative of 2 independent experiments with n=10 per group (A). Bar graph is the mean ± S.D. of two independent experiments (biological replicates) with n=10 per group (B). *P* values were calculated using two-tailed Student's *t* test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 4.6. Kinetics of GIFT-15 B_{regs} versus B cell treatment in mice with EAE. Kinetics of occurance of GIFT-15 B_{regs}, T_{regs}, and Tr1 cells over time in the spleen, MLN and CNS in MOG_{35-55} immunized mice adoptively transfer with GIFT-15 B_{regs}.

Supplemental Figure Legends

Supplementary Fig. 4.1 GIFT-15 B_{regs} surface marker expression. Gating strategy for GIFT-15 B_{regs} derived from Vert-X GFP/IL-10 mice is shown in (A). GFP gating was determined by generating GIFT-15 B_{regs} from wildtype C57BlL/6 mice as a control for background fluorescence. IgD, IgM, H-2Kb, I-Ab, CD80, CD86,

CD40, PD-L1, PD-L2, PD-1, CD21, CD23, CD24, and CD38 expression levels were compared between GFP/IL-10^- and GFP/IL-10^+ populations of GIFT-15 B_{regs} in (b). Expression of these markers were also assessed between GMCSF and IL-15 treated splenic B cells compared to GIFT-15 B_{regs} generated from wildtype C57BL/6 mice (c and Fig. 1b).

Supplementary Fig. 4.2 Profile of infiltrating leukocytes in the CNS of mice with EAE two weeks and one month post-adoptive transfer of GIFT-15 B_{regs}. (A) Gating strategy for identifing T_{regs} and Tr1 populations. After selecting singlets (R1) an selection for $CD4^+$ cells cells were characterized for the expression of CD25 and FoxP3 (T_{regs}) or CD49b and CD223 (Tr1). The two populations were then independently analzyed for the expression of IL-10 (GFP). (B-C) Monuclear cells from the CNS of naïve C57BL/6, MOG_{35-55} immunized mice with EAE (5 mice per group) analyzed after 2 weeks (B) or 1 month (C) post adoptive transfer with B cells or GIFT-15 B_{regs}. The gated populations represent microglial cells ($CD45.2^{int}$ $CD11b^+$), macrophages ($CD45.2^{hi}$ $CD11b^+$), and leukocytes ($CD45.2^{hi}$ $CD11b^+$). Infiltrating lymphocytes were stained for the presence of $CD4^+$, $CD8^+$, $CD25^+$, $FoxP3^+$, $CD49b^+$ and $CD223^+$. The data are representative of 2 independent experiments.

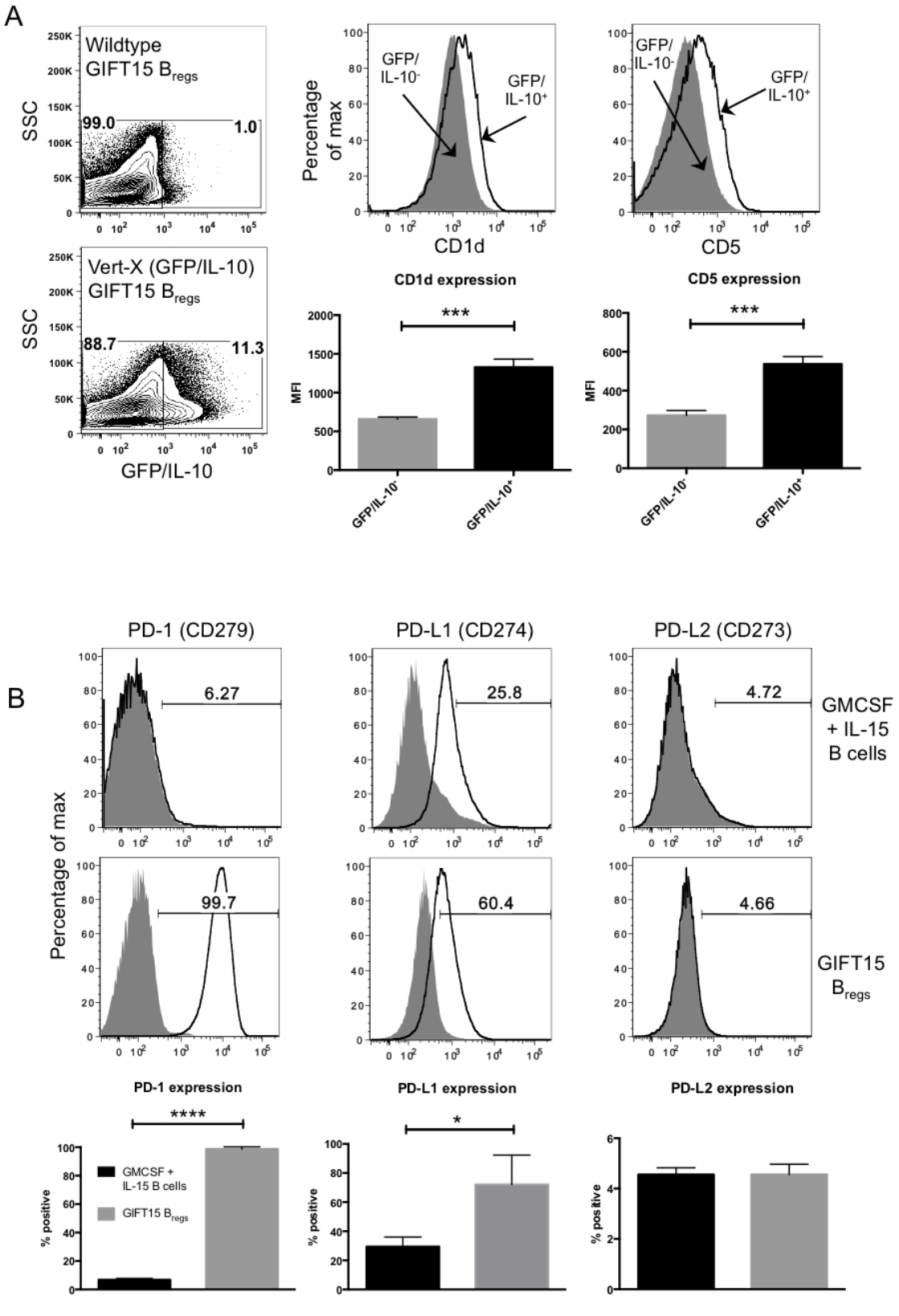
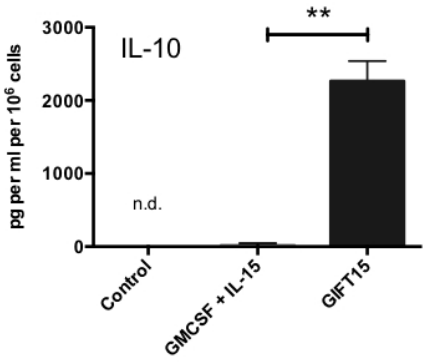

A
Wildtype
GIFT15 Bregs
99.0
1.0
SSC
Vert-X (GFP/IL-10)
GIFT15 Bregs
88.7
11.3
GFP/IL-10
Percentage of max
GFP/ IL-10-
GFP/ IL-10+
CD1d
CD5
CD1d expression
CD5 expression
***
MFI
GFP/IL-10-
GFP/IL-10+
B
PD-1 (CD279)
PD-L1 (CD274)
PD-L2 (CD273)
6.27
25.8
4.72
99.7
60.4
4.66
GMCSF + IL-15 B cells
GIFT15 Bregs
PD-1 expression
PD-L1 expression
PD-L2 expression
****
*
% positive
GMCSF + IL-15 B cells
GIFT15 Bregs

Figure 4.1

C


IL-10
**
pg per ml per 10^6 cells
3000
2000
1000
0
n.d.
Control
GMCSF + IL-15
GIFT15

D

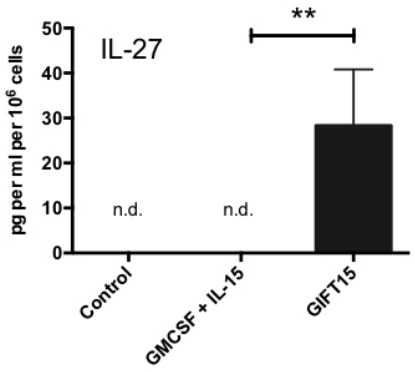

IL-27
**
pg per ml per 10^6 cells
50
40
30
20
10
0
n.d.
n.d.
Control
GMCSF + IL-15
GIFT15

Figure 4.1

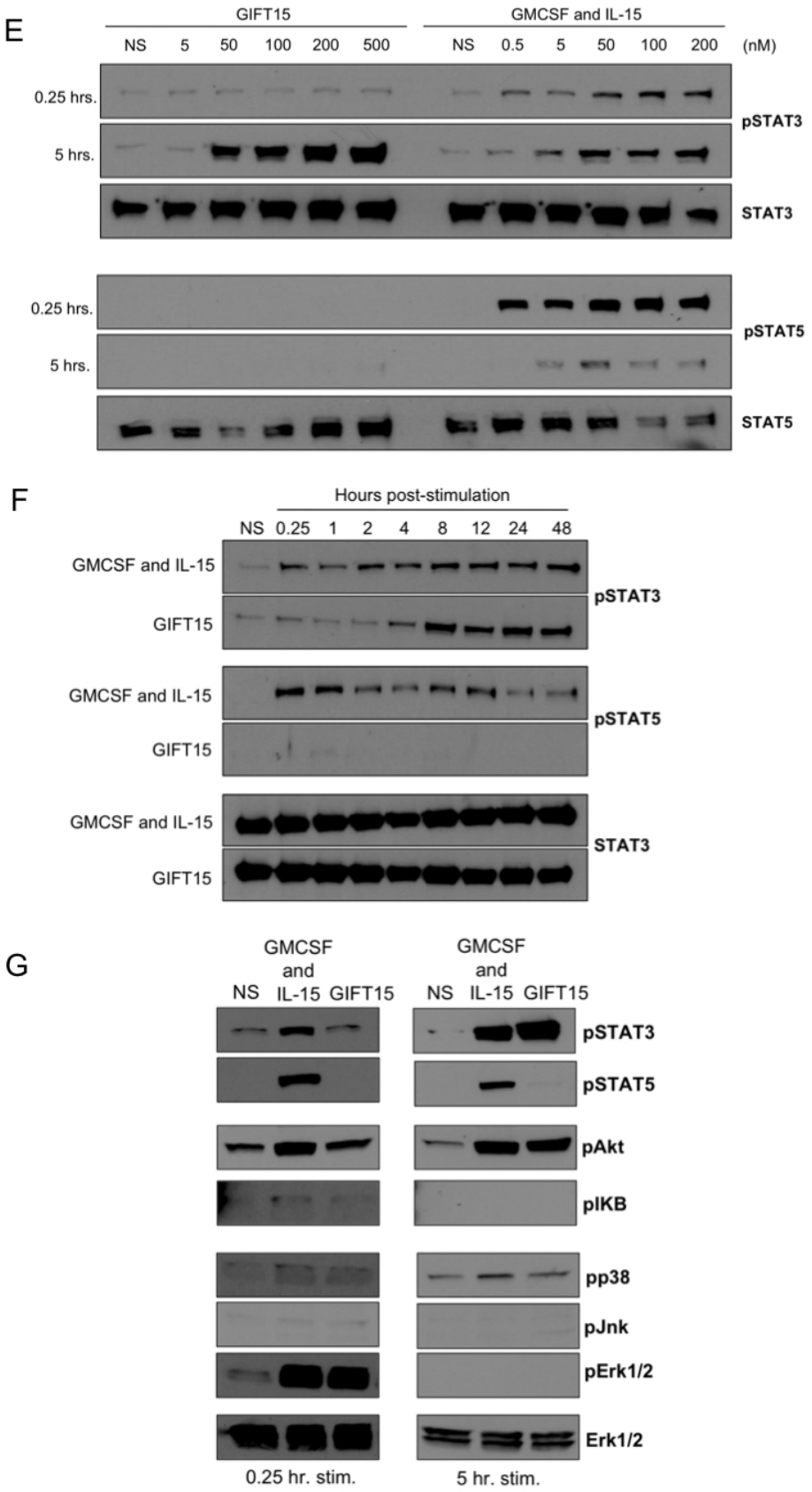

E
GIFT15
GMCSF and IL-15
NS 5 50 100 200 500
NS 0.5 5 50 100 200 (nM)
0.25 hrs.
5 hrs.
pSTAT3
STAT3
0.25 hrs.
5 hrs.
pSTAT5
STAT5
F
Hours post-stimulation
NS 0.25 1 2 4 8 12 24 48
GMCSF and IL-15
GIFT15
pSTAT3
pSTAT5
STAT3
G
GMCSF and
NS IL-15 GIFT15
pSTAT3
pSTAT5
pAkt
pIKB
pp38
pJnk
pErk1/2
Erk1/2
0.25 hr. stim.
5 hr. stim.

Figure 4.1

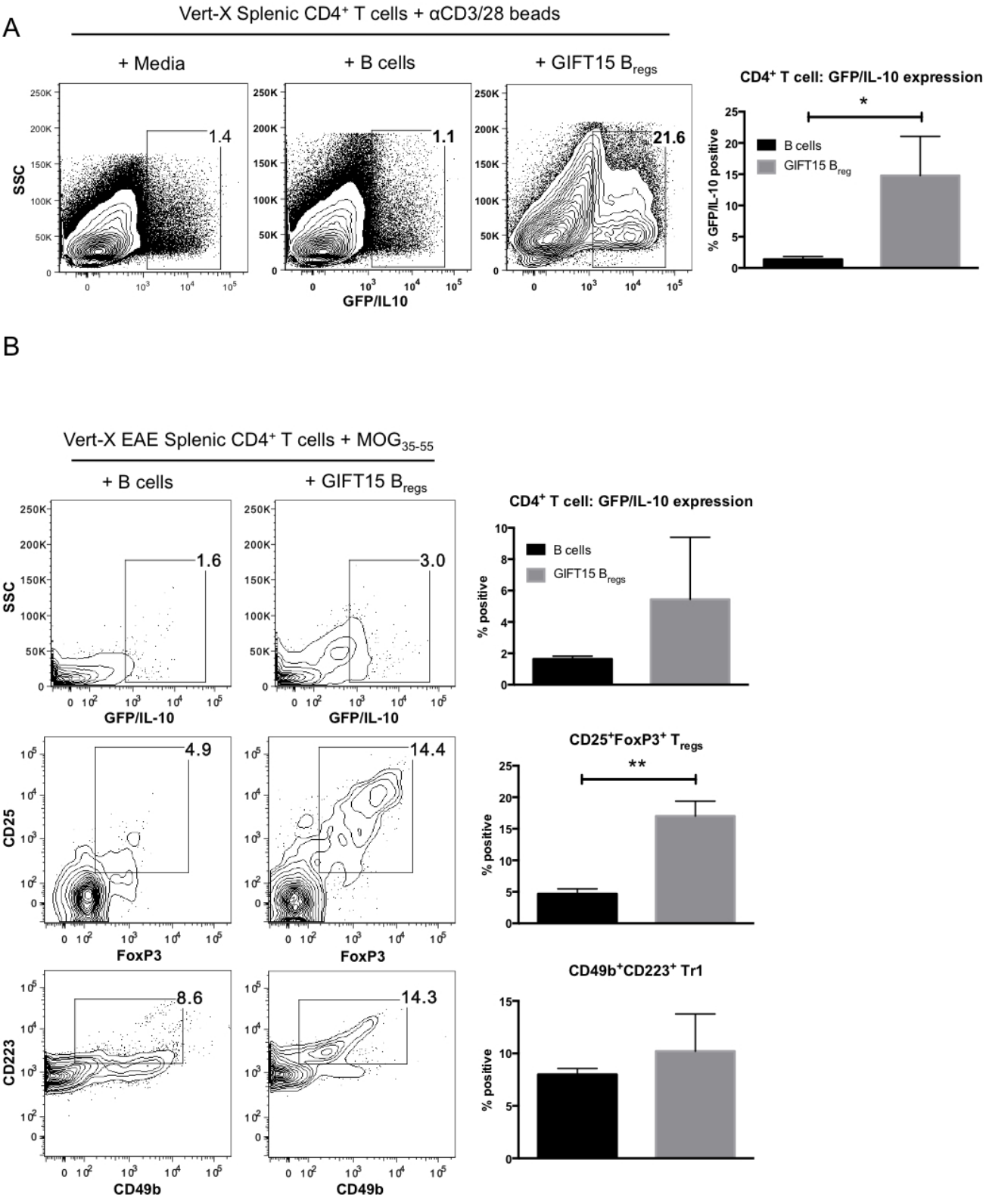

A
Vert-X Splenic CD4+ T cells + αCD3/28 beads
+ Media
+ B cells
+ GIFT15 Bregs
SSC
GFP/IL10
1.4
1.1
21.6
CD4+ T cell: GFP/IL-10 expression
% GFP/IL-10 positive
B cells
GIFT15 Breg
*
B
Vert-X EAE Splenic CD4+ T cells + MOG35-55
+ B cells
+ GIFT15 Bregs
GFP/IL-10
1.6
3.0
CD4+ T cell: GFP/IL-10 expression
% positive
B cells
GIFT15 Bregs
CD25
FoxP3
4.9
14.4
CD25+FoxP3+ Tregs
**
CD223
CD49b
8.6
14.3
CD49b+CD223+ Tr1

Figure 4.2

C

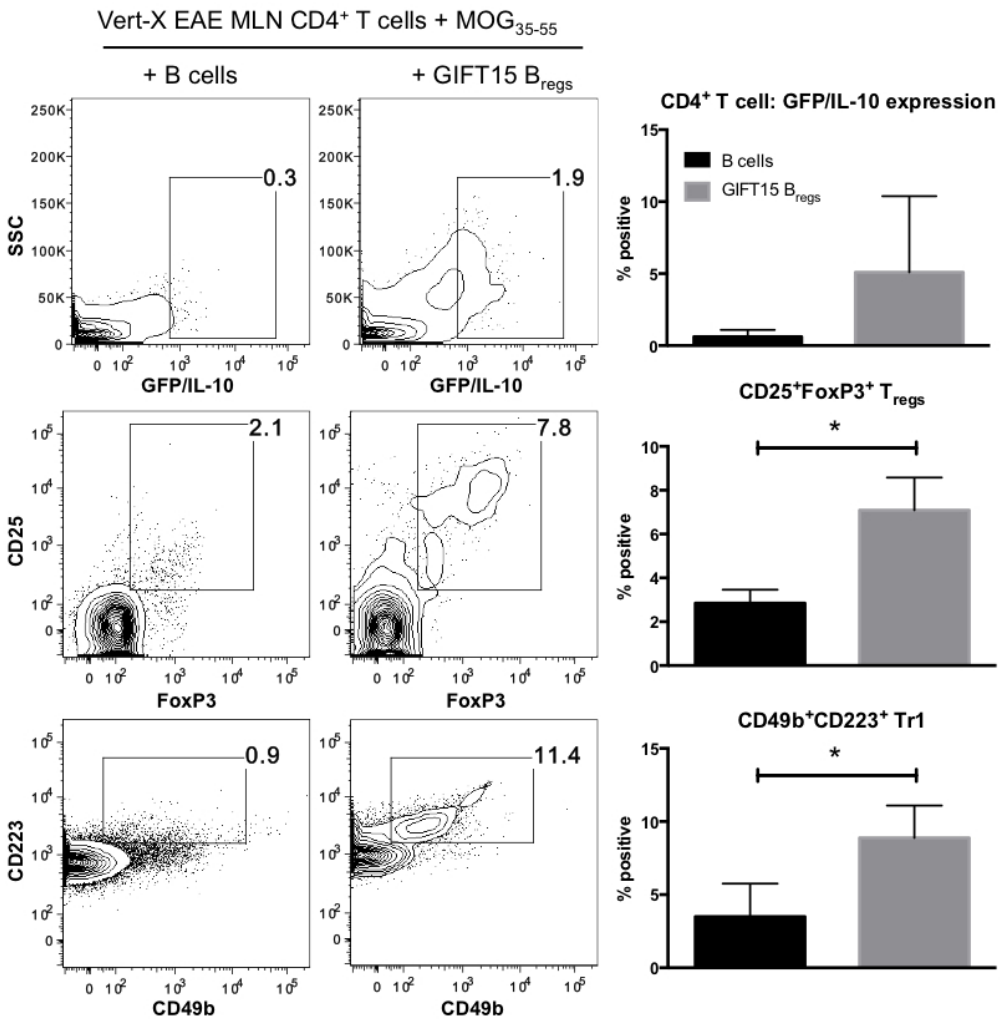

Vert-X EAE MLN CD4+ T cells + MOG35-55
+ B cells
+ GIFT15 Bregs
SSC
GFP/IL-10
0.3
1.9
CD25
FoxP3
2.1
7.8
CD223
CD49b
0.9
11.4
CD4+ T cell: GFP/IL-10 expression
B cells
GIFT15 Bregs
% positive
CD25+FoxP3+ Tregs
*
CD49b+CD223+ Tr1
*

Figure 4.2

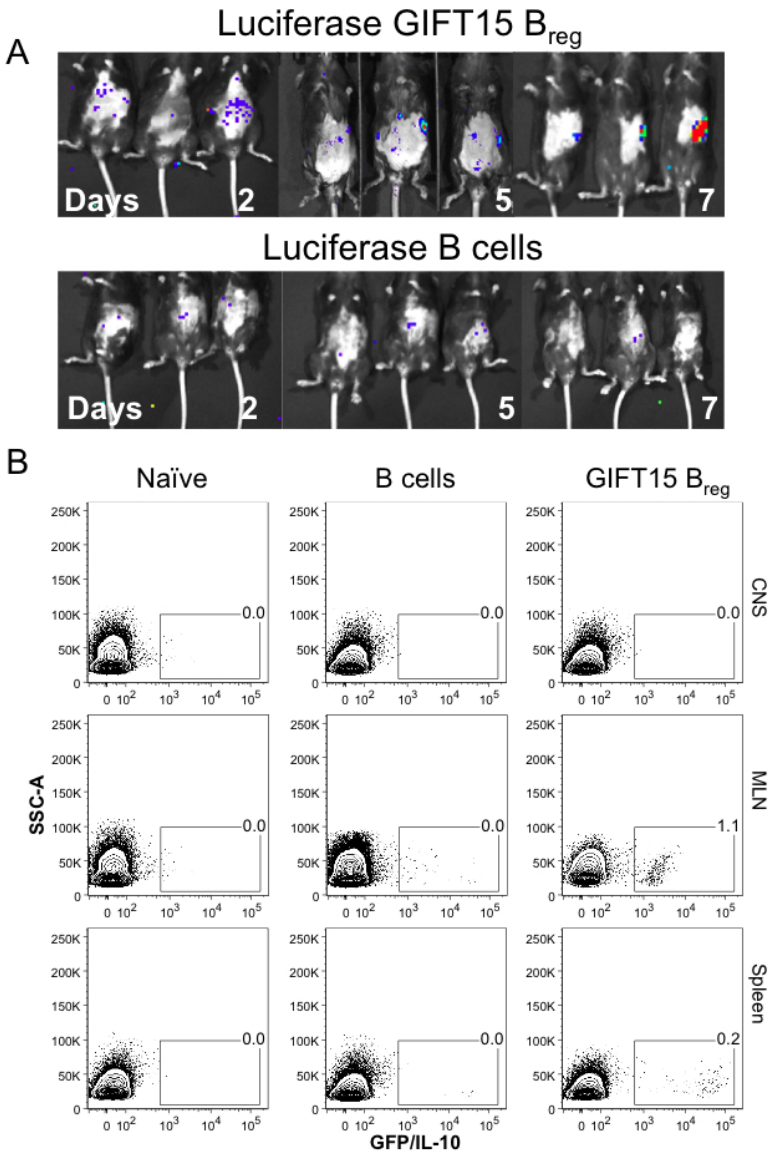

A
Luciferase GIFT15 Breg
Days
2
5
7
Luciferase B cells
Days
2
5
7
B
Naïve
B cells
GIFT15 Breg
CNS
MLN
Spleen
SSC-A
GFP/IL-10
0.0
0.0
0.0
0.0
0.0
1.1
0.0
0.0
0.2

Figure 4.3

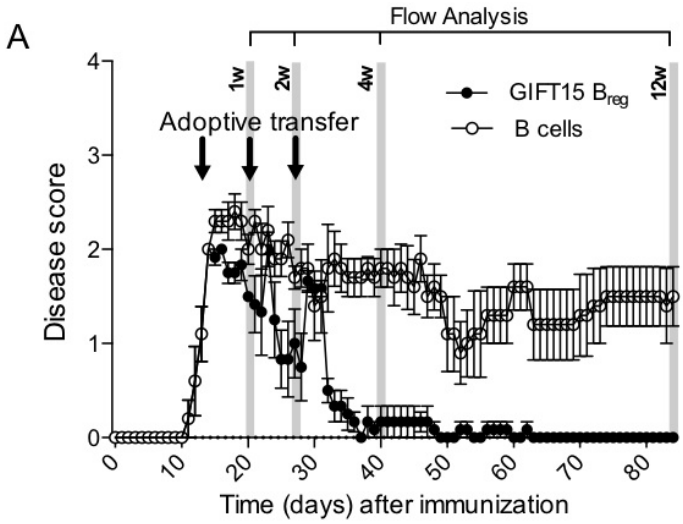

A
Flow Analysis
1w
2w
4w
12w
Adoptive transfer
GIFT15 Breg
B cells
Disease score
0
1
2
3
4
0
10
20
30
40
50
60
70
80
Time (days) after immunization

Figure 4.4

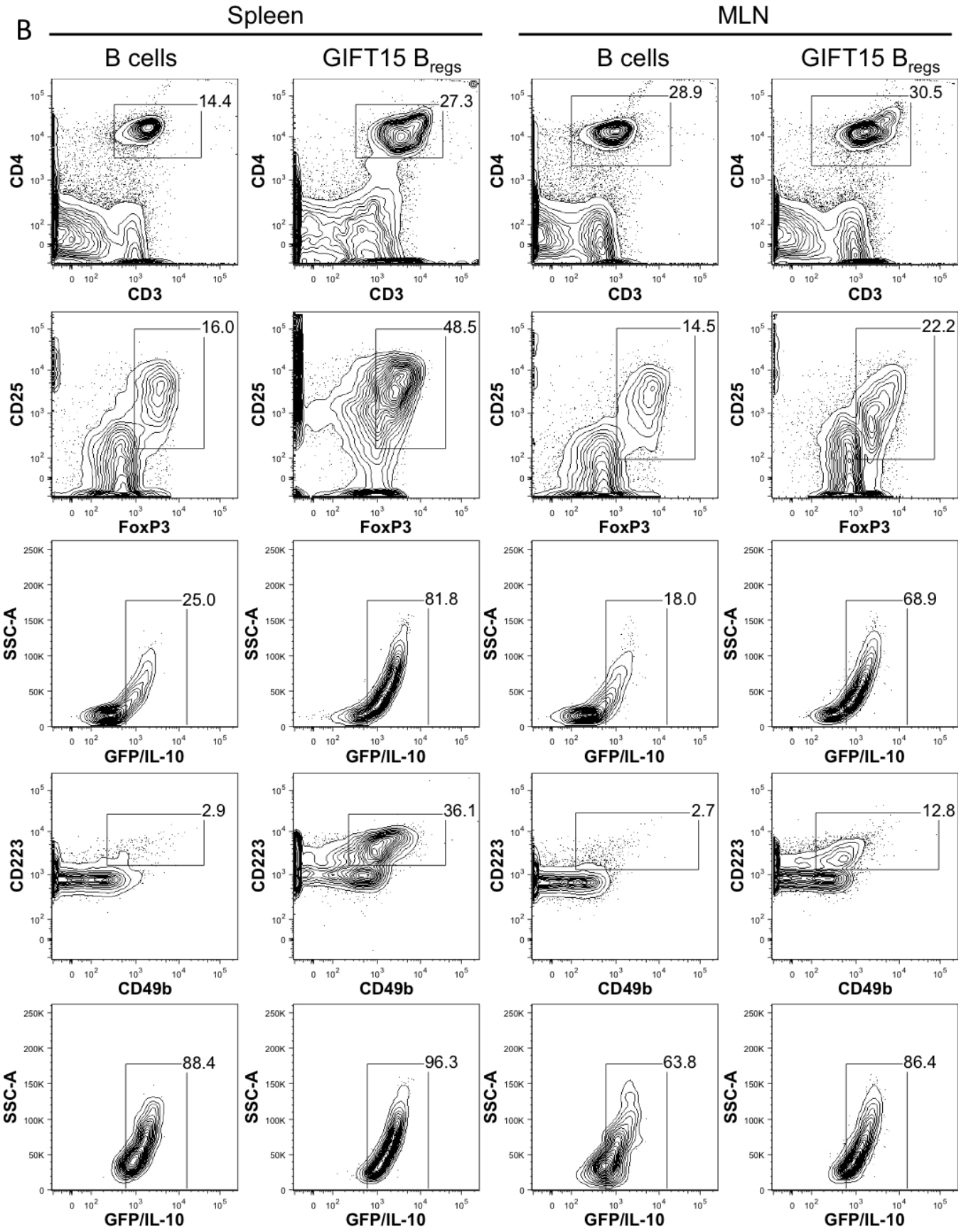

B
Spleen
MLN
B cells
GIFT15 Bregs
B cells
GIFT15 Bregs
CD4
CD3
14.4
27.3
28.9
30.5
CD25
FoxP3
16.0
48.5
14.5
22.2
SSC-A
GFP/IL-10
25.0
81.8
18.0
68.9
CD223
CD49b
2.9
36.1
2.7
12.8
SSC-A
GFP/IL-10
88.4
96.3
63.8
86.4

Figure 4.4

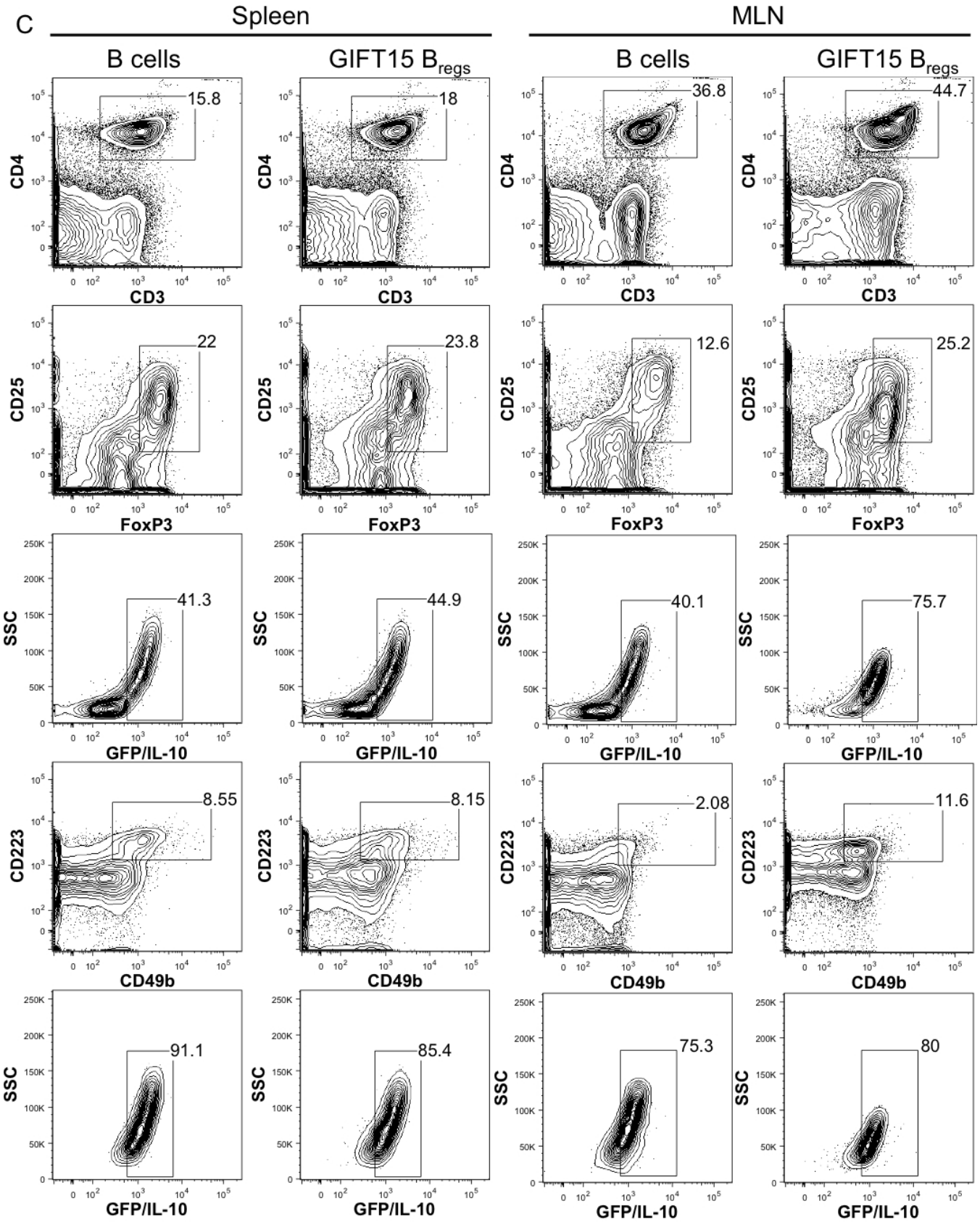

C
Spleen
MLN
B cells
GIFT15 Bregs
B cells
GIFT15 Bregs
15.8
18
36.8
44.7
CD4
CD3
22
23.8
12.6
25.2
CD25
FoxP3
41.3
44.9
40.1
75.7
SSC
GFP/IL-10
8.55
8.15
2.08
11.6
CD223
CD49b
91.1
85.4
75.3
80

Figure 4.4

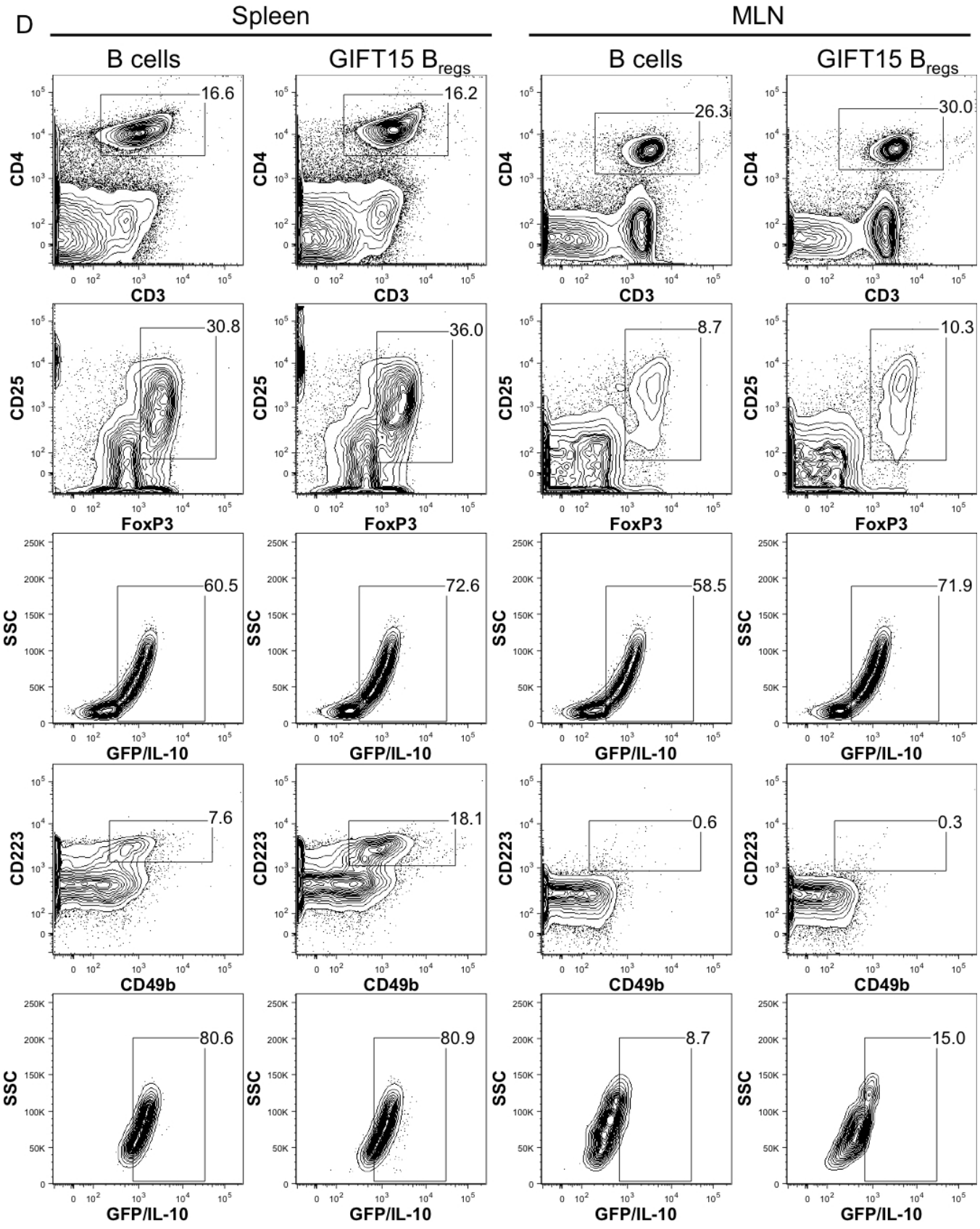

D
Spleen
MLN
B cells
GIFT15 Bregs
B cells
GIFT15 Bregs
CD4
CD3
16.6
16.2
26.3
30.0
CD25
FoxP3
30.8
36.0
8.7
10.3
SSC
GFP/IL-10
60.5
72.6
58.5
71.9
CD223
CD49b
7.6
18.1
0.6
0.3
SSC
GFP/IL-10
80.6
80.9
8.7
15.0

Figure 4.4

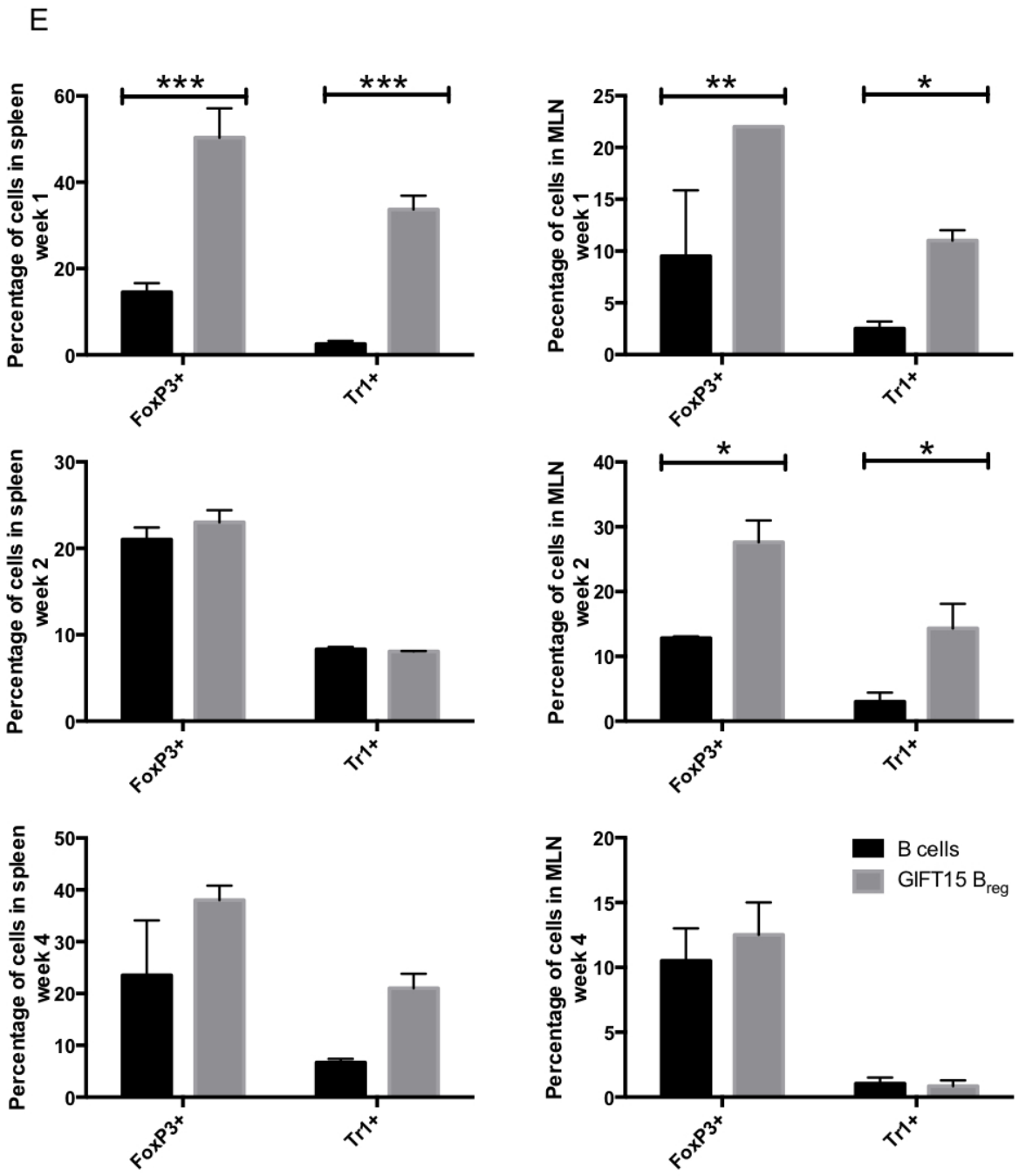

E
Percentage of cells in spleen week 1
***
***
FoxP3+
Tr1+
Pecentage of cells in MLN week 1
**
*
Percentage of cells in spleen week 2
Percentage of cells in MLN week 2
Percentage of cells in spleen week 4
Percentage of cells in MLN week 4
B cells
GIFT15 Breg

Figure 4.4

A

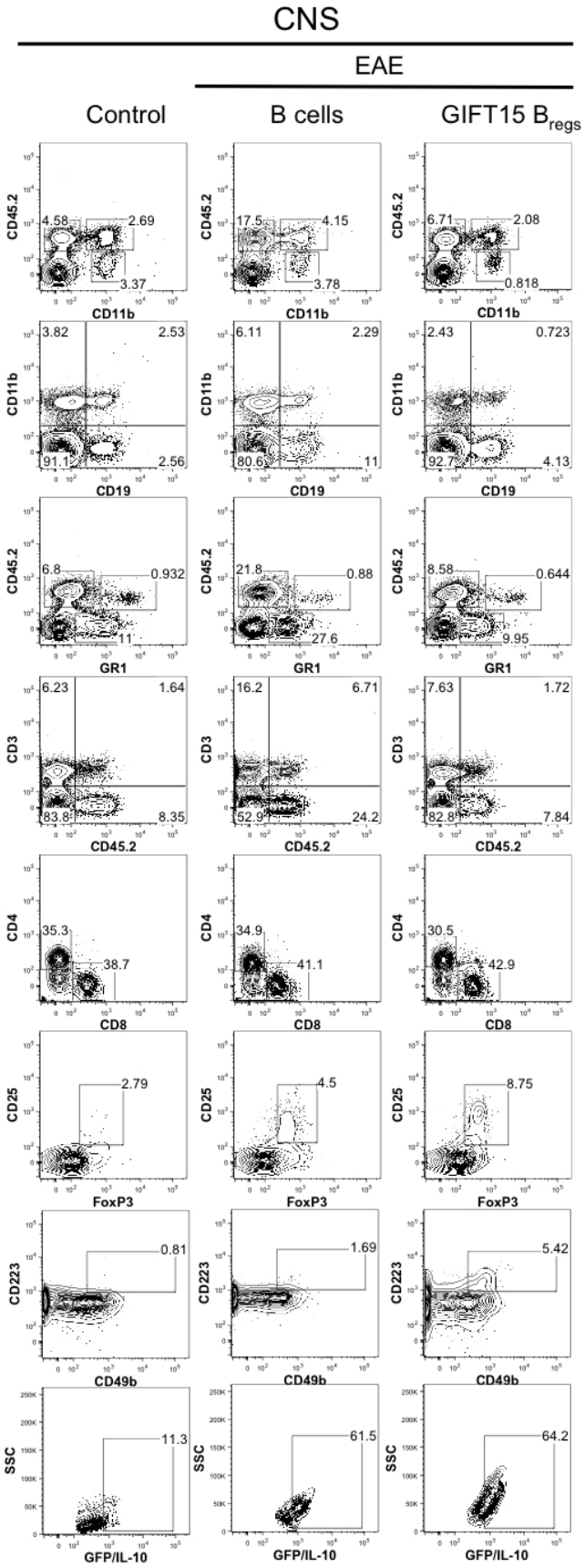

CNS
EAE
Control
B cells
GIFT15 Bregs
CD45.2
CD11b
CD19
GR1
CD3
CD4
CD8
CD25
FoxP3
CD223
CD49b
SSC
GFP/IL-10
4.58
2.69
3.37
17.5
4.15
3.78
6.71
2.08
0.818
3.82
2.53
91.1
2.56
6.11
2.29
80.6
11
2.43
0.723
92.7
4.13
6.8
0.932
11
21.8
0.88
27.6
8.58
0.644
9.95
6.23
1.64
83.8
8.35
16.2
6.71
52.9
24.2
7.63
1.72
82.8
7.84
35.3
38.7
34.9
41.1
30.5
42.9
2.79
4.5
8.75
0.81
1.69
5.42
11.3
61.5
64.2

Figure 4.5

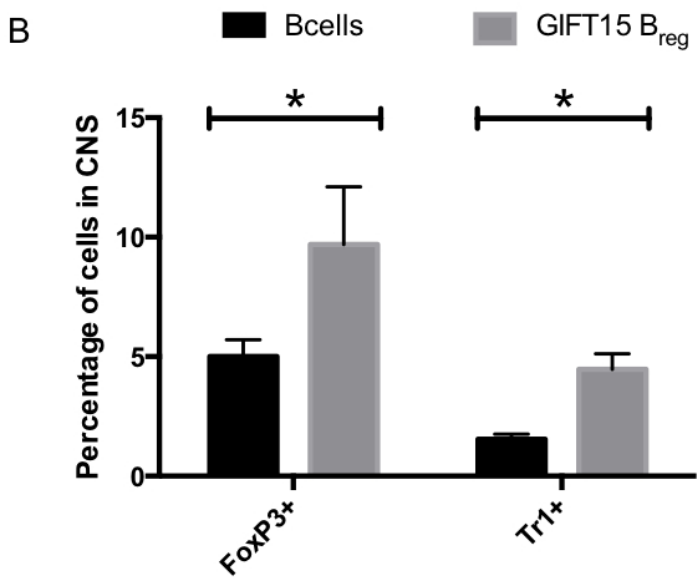

B
Bcells
GIFT15 Breg
*
*
Percentage of cells in CNS
15
10
5
0
FoxP3+
Tr1+

Figure 4.5

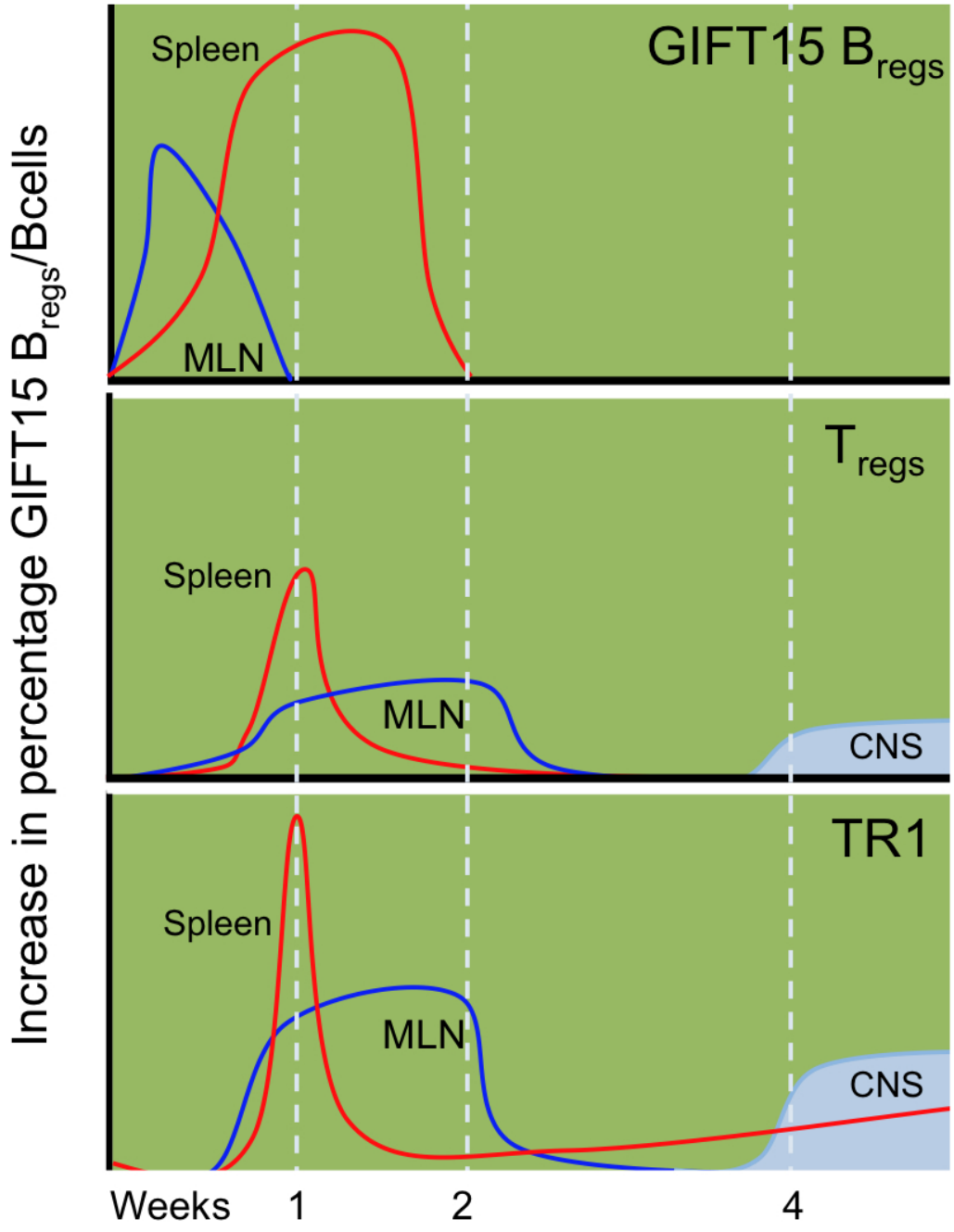

Increase in percentage GIFT15 Bregs/Bcells
Spleen
GIFT15 Bregs
MLN
Tregs
Spleen
MLN
CNS
TR1
Spleen
MLN
CNS
Weeks
1
2
4

Figure 4.6

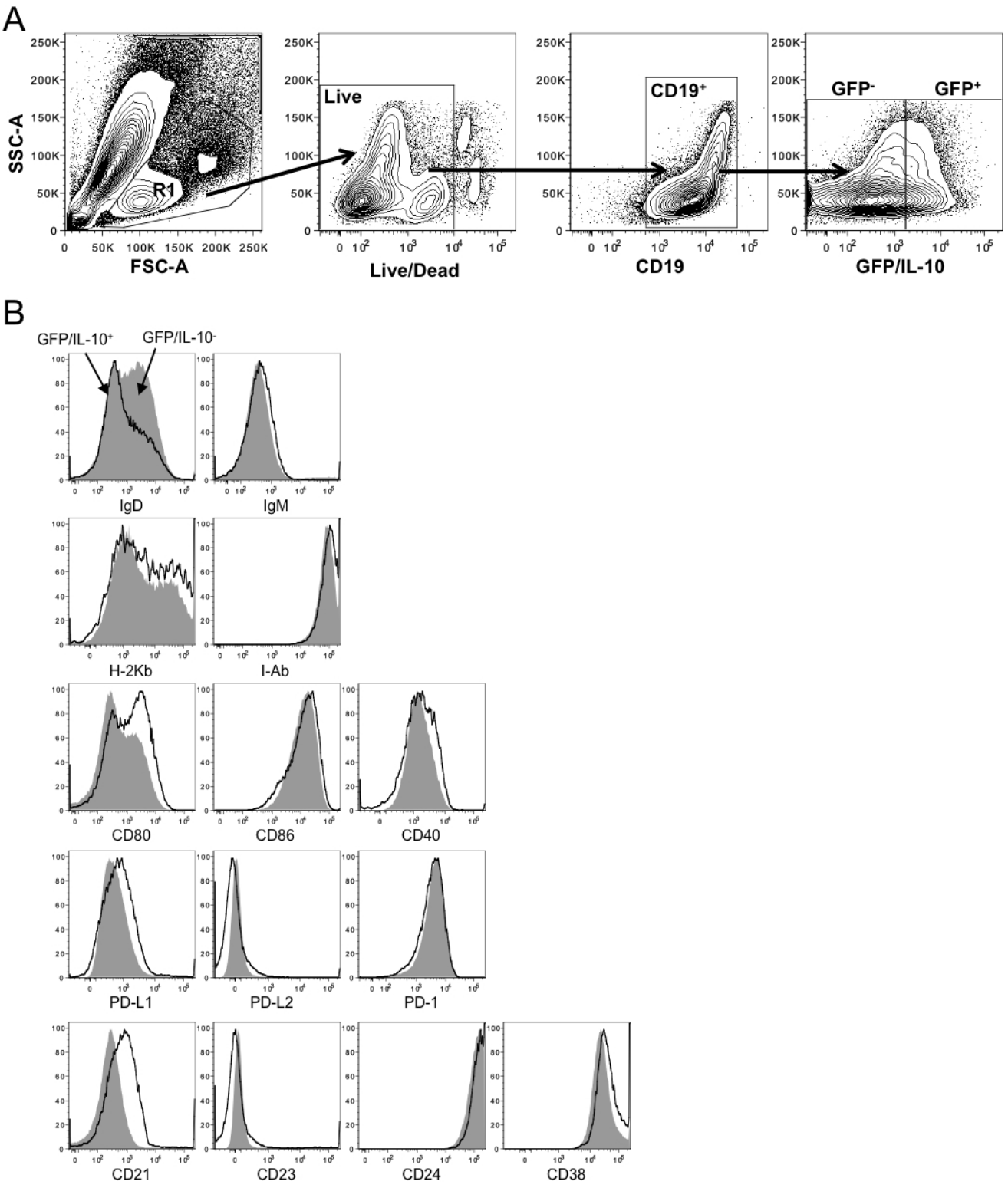
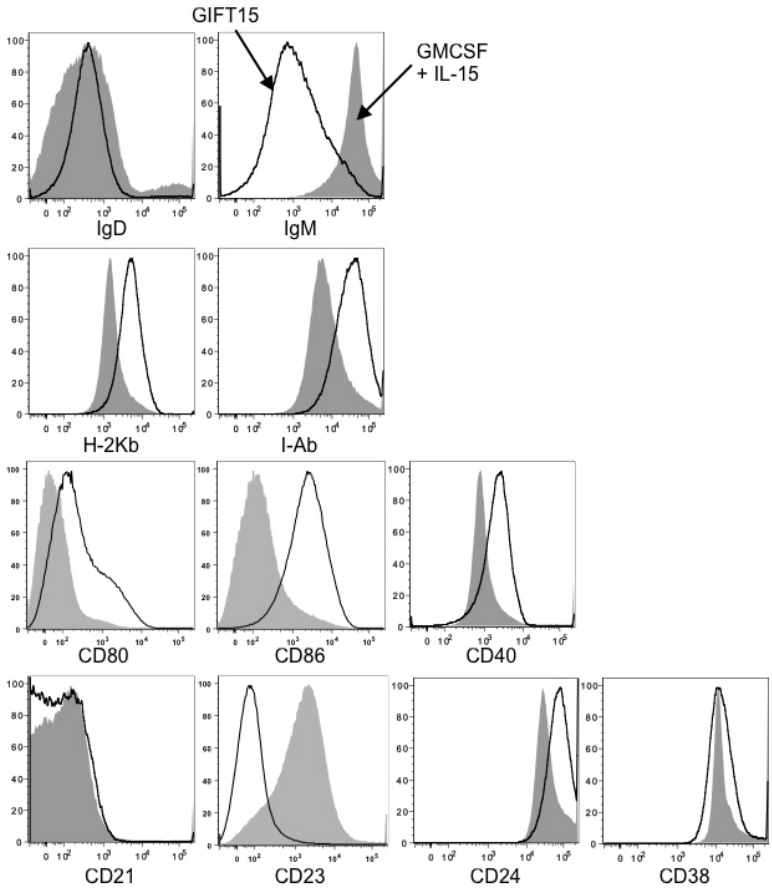

A
SSC-A
FSC-A
R1
Live
Live/Dead
CD19+
CD19
GFP-
GFP+
GFP/IL-10
B
GFP/IL-10+
GFP/IL-10-
IgD
IgM
H-2Kb
I-Ab
CD80
CD86
CD40
PD-L1
PD-L2
PD-1
CD21
CD23
CD24
CD38

Figure S4.1

C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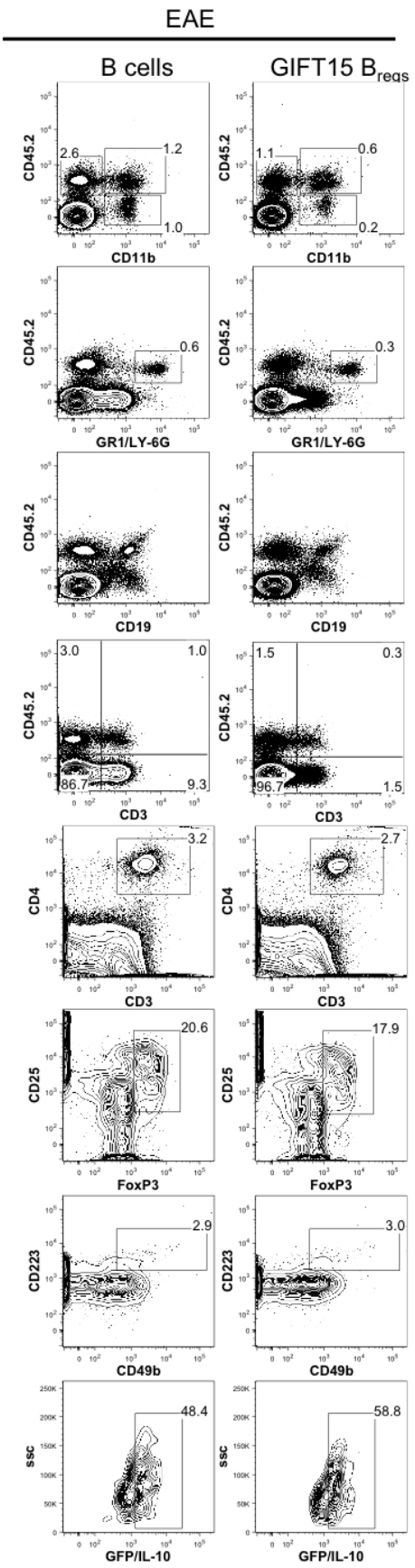
GMCSF
+ IL-15
IgD
IgM
H-2Kb
I-Ab
CD80
CD86
CD40
CD21
CD23
CD24
CD38

Figure S4.1

A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B cells
GIFT15 Breg
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CD11b
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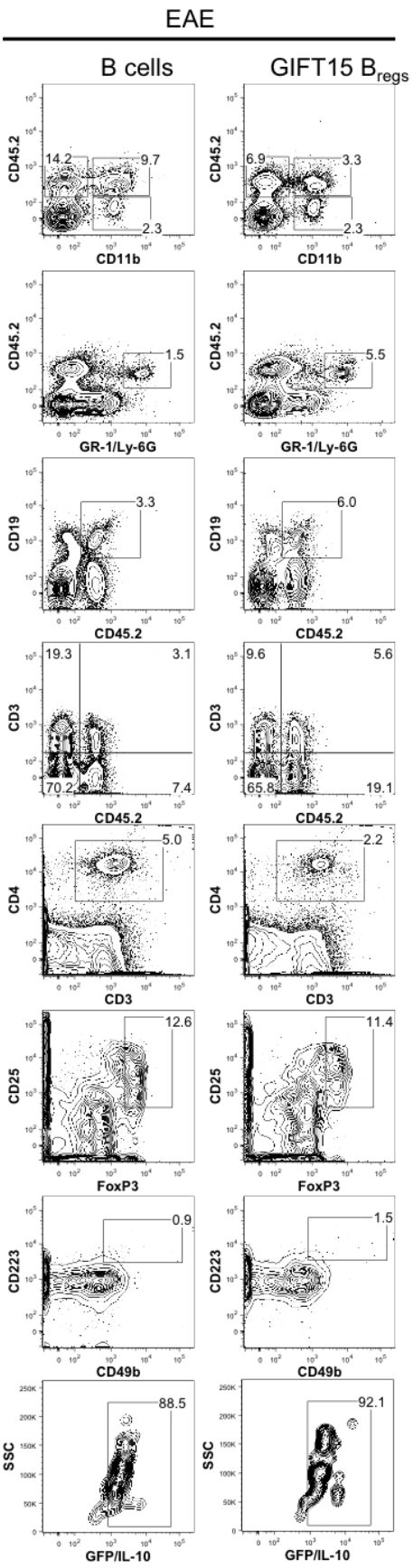
1.2
1.0
1.1
0.6
0.2
GR1/LY-6G
0.6
0.3
CD19
3.0
1.0
86.7
9.3
1.5
0.3
96.7
1.5
CD3
CD4
3.2
2.7
CD25
FoxP3
20.6
17.9
CD223
CD49b
2.9
3.0
ssc
GFP/IL-10
48.4
58.8

Figure S4.2

B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B cells
GIFT15 Bregs
CD45.2
CD11b
14.2
9.7
2.3
6.9
3.3
2.3
GR-1/Ly-6G
1.5
5.5
CD19
3.3
6.0
19.3
3.1
70.2
7.4
9.6
5.6
65.8
19.1
CD3
CD4
5.0
2.2
CD25
FoxP3
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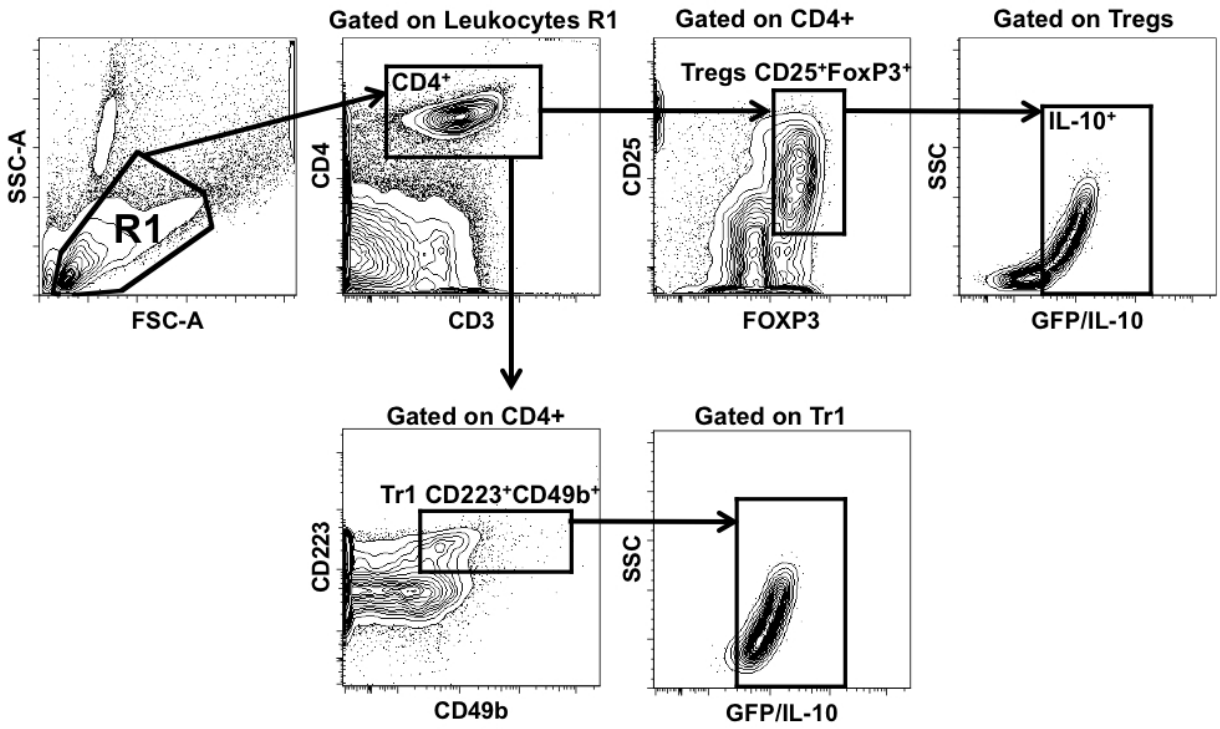
1.5
SSC
GFP/IL-10
88.5
92.1

Figure S4.2

C


Gated on Leukocytes R1
Gated on CD4+
Gated on Tregs
SSC-A
R1
FSC-A
CD4
CD4+
CD3
CD25
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FOXP3
SSC
IL-10+
GFP/IL-10
Gated on CD4+
Gated on Tr1
CD223
Tr1 CD223+CD49b+
CD49b
SSC
GFP/IL-10

Figure S4.2

Chapter 5

Discussion

5.1.0 Summary

The main objective of the work within this dissertation was to modify γc cytokines in a rational, hypothesis-driven way, learn how such modifications alter their biology, and to find a suitable experimental model of pathology to test the fusion protein for therapeutic potential. We have covered how modification of IL-15-derived fusion proteins, FIST-15 (fusion of IL-15 to a TGF-β receptor antagonist) and GIFT-15 (fusion of IL-15 to GMCSF), changes their biology and their function, particularly on immune cell subsets. FIST-15 mainly acts upon NK cells and $CD8^+$ T cells, enhancing their proliferation, activation, and enhancing their effector functions beyond the effects. We have demonstrated that in the setting of pre-established melanoma in mice, that administration of FIST-15 can inhibit tumor outgrowth and prolong survival (Chapter 2). Further, we found that the FIST-15's effect *in vivo* was mainly dependent on an intact and functional NK cell response. In our experimental tumor models, we found that FIST-15 was superior to IL-15 and a commercially available TGF-β receptor antagonist. We were also able to generate preliminary data to suggest that FIST-15 may improve innate immune responses against acute viral infections and may also have beneficial effects in the setting of hepatic fibrosis (Chapter 3). While the biological outcome of FIST-15 seemed to match our initial predictions that the combination of IL-15 with TGF-β blockade would result in enhanced IL-15 bioactivity, especially in the TGF-β rich conditions, fusion of IL-15 to GMCSF in GIFT-15 did not behave according to our initial predictions. The generation of GIFT-15 yielded a protein that was potently immunosuppressive. While it was initially hoped that the

combination of these two cytokines would yield a protein that would enhance anti-tumor immunity by driving DC maturation and $CD8^+$ T cell expansion through the GMCSF and IL-15 moieties, respectively. The ultimate result was that GIFT-15 acted on B cells, converting them to a regulatory phenotype (Chapter 4). We found that culturing naïve B cells *ex vivo* in the presence of GIFT-15 greatly enhanced their ability to secrete IL-10 and also induce T cells to also take on a regulatory phenotype both *in vitro* and *in vivo*. Adoptive transfer of these GIFT-15- B_{regs} can ameliorate autoimmune conditions, such as experimental autoimmune encephalomyelitis, in mice.

There is one outstanding point of discussion remaining to the fusion protein platform that remains to be addressed in this Discussion. This pertains to the synergistic or novel functions of fusing two proteins together. Posed another way, would be the question: why does fusing of two proteins together not yield the same result net biological activity of the two proteins separately? With FIST-15, a synergistic effect was seen as the fusion protein outperformed IL-15 and TGF-β receptor antagonists added separately. GIFT-15, on the other hand, displayed novel functions that could neither be ascribed to GMCSF or IL-15.

5.2.0 Discussion

FIST fusion protein gain of function

The fusion protein platform offers an interesting way to combine the effects of two different proteins and deploying them in the same time and space. The FIST family of proteins was conceived to provide γc cytokines with the ability to act in

the immunosuppressive tumor microenvironment. Penafuerte et al. initially showed that B16 melanoma cells secreted high levels of TGF-β, which hindered the effects of IL-2-based cytokine immunotherapy. Due to this observation, our group created a protein combining the IL-2 to a single TGF-β receptor ectodomain (FIST2), the first of the FIST family of proteins to be generated. Penafuerte et al. was able to show that FIST2 was superior to the combination of IL-2 and a dimeric TGF-β receptor-Fc fusion protein (sTβRII-Fc) in models of B16 melanoma and PANC02 pancreatic adenocarcinoma [387]. It was found that FIST2 could attenuate TGF-β-mediated angiogenesis and promote pro-inflammatory immune cell infiltration into tumors that secreted the fusion protein locoregionally. Most importantly, Penafuerte et al. showed that FIST2's gain-of-function over IL-2 and sTβRII-Fc was likely driven by STAT1 hyperphosphorylation and induction of Smad7 expression in immune cells, a regulatory Smad that inhibits antagonizes TGF-β induced Smad2/3 signaling [388]. Distinct from IL-2 and sTβRII treatment, mouse lymphoma cells (CTLL-2) phosphorylated STAT1 and 3 at greater levels compared when treated with FIST-2. Similarly, Smad7 was also phosphorylated to a greater degree when primary mouse splenocytes were used as responder cells. FIST-2 mediated Smad7 induction was shown to be blocked in splenocytes derived from STAT1 knockout mice. The authors' working model for FIST-2's gain-of-function was that FIST-2 acted as a hyperagonist on the IL-2 receptor complex, initiating phosphorylation of STAT5 (at similar levels to IL-2), but greater STAT1 and 3 phosphorylation. STAT1 phosphorylation induces a number of downstream of

genes, including Smad7, which renders the responding cells resistant to TGF-β-mediated inhibition, in addition to being able to bind soluble TGF-β in the tumor microenvironment [389]. This allows FIST-2 stimulated cells to produce more cytokines (particularly IFNγ, which is STAT1-driven) and enhance the effector functions of tumor-infiltrating cells, particularly NK and T cells, which were significantly increased in matrigel plugs of B16 melanoma tumors transduced to express FIST-2 (B16-FIST2). *In vivo*, FIST-2 was found to act primarily through NK cells to mediate its anti-tumor effect, since B16-FIST2 cells were only able to grow in *Beige* and NOD-SCID mice, which have defective and diminished numbers of NK cells, respectively.

Although we did not assess the level of STAT1 phosphorylation with FIST-15 treatment, we observed a similar gain-of-function with FIST-15 compared to IL-15 and sTβRII-Fc. Like FIST-2, FIST-15 stimulated NK cells and $CD8^+$ T cells also exhibited greater levels of IFN-γ production. IFN-γ is a potent inducer of STAT1 activation and its release by both FIST-2 and FIST-15 stimulated NK and $CD8^+$ T cells likely drives STAT1 signaling, which antagonizes inhibitory TGF-β signaling. In FIST-2, this was clear with induction of Smad7 phosphorylation. In FIST-15, however, we were unable to ascertain the status of Smad7 phosphorylation, since the antibody utilized in the FIST-2 studies are no longer in production. We hypothesize that FIST-15's gain-of-action is mechanistically similar to that of FIST-2. However, we would have to conduct further studies on the signaling profile of FIST-15, in particular STAT1 and Smad7 phosphorylation status, to

determine if this is the case. Interestingly, it was noted that Smad7 phosphorylation only occur in the presence of both FIST-2 and exogenous TGF-β. The authors surmise that TGF-β bound to the TGF-β receptor ectdomain (type II) of FIST-2 could conceivably be *trans* presented to a type 1 TGF-β receptor (or sequester TβRI away from other surface TβRII) on the surface of the cell, while the IL-2 moiety of the fusion protein was engaged with the IL-2 receptor complex. Although, there's no evidence to support that this tandem signaling event could occur, differences in the structure of FIST-2 and FIST-15 could potentially affect the way that any FIST-bound TGF-β would interact with TGF-β receptors on the surface of the cell. Importantly, FIST-15 contains an extra TβRII-domain compared to FIST-2. This difference in stoichiometry would certainly affect FIST-15 binding of TGF-β and its ability to be *trans* presented. Most evidence in literature points to a ratio of 1:2:2 (ligand:type 1 receptor:type 2 receptor) stoichiometry of binding, where a ligand monomer brings two homodimers of TβRII and TβRI together [390]. Whether a 1:2:1 pattern of binding and signaling is possible (as would be the case for FIST-2) or a 1:2:2 pattern (predicted for FIST-15, where the 2 TβRII are in *trans* to the TβRI) is not known. Spatially in terms of geometry of the fusion proteins, FIST-2 and FIST-15 also differ. Whereas, the N'-terminus of FIST-2 contains the IL-2 domain, the N'-terminus of FIST-15 contains the double TβRII-domains. This difference in geometry could also alter the way any FIST-bound TGF-β could engage with surface TGF-β receptors. A further difference lies in the orientation of how the respective γc cytokine of each FIST protein binds to surface receptors. The IL-2 moiety of

FIST-2 presumably binds to all three components of the IL-2 receptor complex in *cis.* The orientation of how the IL-15Rα-*sushi* and IL-15 domain of FIST-15 engages with the γc and βc of NK and $CD8^+$ T cells remain unclear, as IL-15Rα typically *trans* presents IL-15 to these cells.

FIST-15, like FIST-2, also depends on the effects of NK cells *in vivo* to mediate its anti-tumor effect. Whether FIST-15 enhances recruitment of lymphocytes into the tumor microenvironment like FIST-2 also warrants further investigation, although given their similarities *in vitro*, we believe that FIST-15 would behave in the same fashion. FIST-2 preferentially directs the recruitment of γc and βc secreting cells into the tumor microenvironment, and since FIST-15 acts predominately on cells expressing these receptors, we hypothesize it would behave in a similar manner as FIST-2 in this regard. Interestingly, FIST-2 was not demonstrated to be a more potent mitogen for CTLL-2 lymphoma cells *in vitro* beyond the effects of IL-2 and sTβRII-Fc. We believe that since we observed FIST-15 to be a more potent mitogen for primary $CD8^+$ T cells and NK cells, that we would see a corresponding increase in FIST-15-mediated recruitment of these cell types *in vivo*. However, *ex vivo* analysis of matrigel implants with tumor cells transduced to express FIST-15 protein or FIST-15 treatment of mice with pre-established tumors in matrigel plugs would be required to determine the true extent of FIST-15's affect on migration of lymphocytes into the tumor microenvironment. In follow-on studies, Penafuerte et al found that FIST-2 could convert naïve B cells into type 1 effector B cells,

characterized by the secretion of IFN-γ, GMCSF, TNF-α, and IL-6 [85]. This effect was not dependent on STAT1, as it was on NK cells in prior experiments, because B cells derived from STAT1 null animals produced similar levels of IFN-γ. It was also shown that, unlike T and NK cells in the prior study, B cells stimulated by FIST-2 did not show enhanced phosphorylation of STAT1, 3, or 5. Only after 3 days of culture with FIST-2 did the B cells appear to have enhanced STAT3 and 5 phosphorylation, compared to IL-2 and sTβRII-Fc treatment. Not surprisingly, the authors found that the increase in FIST-2-mediated IFN-γ production was correlated with increases in T-bet expression in the B cells. T-bet is known to be a transcription factor for IFN-γ and critically regulates the T_H1 differentiation programming of CD4+ T cells, which are known to secrete high levels of IFN-γ. Moreover, FIST-2 was shown to enhance the antigen-presentation properties of B cells (e.g. higher levels of MHC-I, MHC-II, and co-stimulatory molecules, CD80 and CD86). Adoptive transfer of FIST-2 treated B cells co-cultured with OVA into mice with pre-established tumors expressing OVA showed significant delays in tumor growth and even tumor rejection in 20% of mice. Similar to FIST-2, we were unable to detect appreciable differences in STAT3 or 5 phosphorylation with FIST-15 within a 30 minute treatment window. Despite this, it was shown that FIST-2 treated B cells had enhanced STAT3 and 5 signaling after 3 days in culture. It would be instructive to determine whether FIST-15 may have a similar delayed effect in enhancing signaling, as this may be the key to its gain-of-function. In the FIST-15 studies investigating the status of STAT5 phosphorylation by intracellular flow cytometry, we only interrogated the

status of substrate phosphorylation up to 2 hours post-stimulation. How FIST-2 has no effect in signaling on these key molecules at early time points, but causes changes at later timepoints is unknown. Interrogating timepoints in between 2 hours and 3 days would provide more information on the dynamics of how this signaling change occurs. It was also interesting that despite a lack of STAT1 hyperphosphorylation, B cells treated with FIST-2 could express high levels of IFN-γ, and that this was correlated with increased T-bet expression. While we did not ascertain the STAT1 activation status with FIST-15 treated cells, FIST-15 did not significantly alter the levels of T-bet expression in $CD8^+$ T cells after 72 hours of treatment (data not shown) compared to IL-15 or media only treatment conditions in a set of pilot experiments. While T-bet is known to be required for optimal production of IFN-γ in CD4+ T cells, its role in controlling IFN-γ production in $CD8^+$ T cells and NK cells is less well understood. In $CD8^+$ T cells, nuclear factor of activated T cells (NFAT-1), cAMP response element binding protein (CREB), activating-transcription factor (ATF), and activator protein (AP-1), are also important transcription factors in IFN-γ production [391]. FIST-15, however, induced T-bet expression to a similar degree as IL-15, both of which were elevated over T-bet expression in NK cells that were untreated. It was also observed that the presence or absence of TGF-β did not significantly alter the induction of T-bet expression by either IL-15 or FIST-15. Akin to $CD4^+$ T cells, T-bet has a role in regulating IFN-γ expression in NK cells. However, like $CD8^+$ T cells, the presence of T-bet is not required for induction of IFN-γ production [392]. The maintenance of IFN-γ production seems to be impaired in the NK cells of

mice lacking T-bet. It is also worth noting that T-bet plays an important role in the expression of cytotoxic molecules, such as perforin and granzymes, the latter of which was found to be highly upregulated in FIST-15 treated NK cells [393]. Since IL-15 and FIST-15 induced similar levels of T-bet, we hypothesize that FIST-15 must additionally activate other signaling pathways or enhance the persistence of signaling for its gain-of-function.

One important potential reason for FIST-2 and FIST-15's gain-of-function that was alluded to in the Discussion of Chapter 2 is the size of these molecules. The larger size of the fusion proteins compared to their individual constituents may allow them to remain in circulation for longer periods of time, as smaller proteins tend to be passively cleared from circulation quite quickly. The association of small proteins prone to proteolysis or clearance can also be stabilized by binding to chaperone proteins. This is most certainly the case for IL-15 and the IL-15Rα, which enhances the half-life of IL-15 and its bioavailability [394]. IL-2 can similarly be stabilized by conjugating it to anti-IL-2 antibodies, increasing its half-life and bioactivity [395, 396]. While we have not formally performed pharmacokinetic studies, we believe the added TGF-β receptor ectodomains to the γc cytokines prevents them from being cleared from circulation as quickly as endogenous cytokines.

GIFT fusion protein gain of function

In order to understand how GIFT fusokines deploy their biological effects, we must look at the way in which they transduce their signal within immune cells. The ability of GIFTs to bring together receptors belonging to different superfamilies result in radically altered signaling events downstream that are distinct from the signals transduced by monomeric cytokines. GMCSF typically triggers signaling by inducing the heterodimerization of cell surface GMCSF receptor (GMCSFR) alpha (αc)- and beta (βc)-chains. The dimerization results in conformational changes that result in the phosphorylation and activation of GMCSFR βc-associated JAK2. In turn, JAK2 will phosphorylate the cytoplasmic tails of GMCSFR α- and β-chains, resulting in the recruitment and activation of STAT5 in a SH-2 dependent manner. γc interleukins trigger a similar signaling cascade as described in Chapter 1. Of note, none of the γc interleukins utilize JAK2 as part of their signal transduction machinery.

While natural cooperativity between unrelated cytokine receptors have been observed, we endeavored to engineer a family of proteins that bridge unrelated receptors together [397, 398]. Co-immunoprecipitation experiments using GIFT-4 and 9 have shown that GMCSFR βc pulls down significant amounts of IL-4 and 9 receptor α-chains, respectively. This suggests that in the presence of GIFT fusokines, GMCSF and interleukin receptors are able to interact with one another. Direct evidence of receptor clustering was provided by immunofluorescent staining coupled with confocal microscopy. These studies revealed that antibodies directed towards GMCSFR βc and the γc on MC/9 mast cells were co-localized when treated with GIFT-9, but remained separated across the cell

surface when treated with GMCSF and IL-9, alone or in combination. Deng et al. were readily able to replicate this data, showing extensive co-localization of IL-4Rα and GMCSFR [261]. Despite our observation of receptor co-clustering events with GIFT-4, we were unable to determine whether such events occur for GIFT-15 on the surface of naïve B cells for technical reasons. It appears that GMCSFR βc expression was quite low relative to the expression of γc on the surface of primary B cells, making it difficult to visualize and quantify clustering events between the GMCSFR βc and the γc.

Heterologous receptor clustering has significant physiological consequences for immune cells. In addition to bringing GMCSF and interleukin receptors together, the kinases and molecules of signal transduction that are uniquely associated with these receptors are also co-localized as a result of GIFT-binding. In a set of mechanistic experiments, Li et al. showed that GIFT-9 leads to hyperactivation of IL-9R-associated STAT1 through a JAK2 dependent mechanism. This finding is significant because JAK2 is typically associated with GMCSFR βc and does not physiologically interact with γc interleukin receptor complexes. The ability of GIFT-9 to hyperactivate STAT1 is a result of its capacity to recruit and activate GMCSFR βc-associated JAK2 into a complex that also includes IL-9R-associated STAT1. In other words, JAK2 is able phosphorylate STAT1 because GIFT-9 acted as a chaperone clustering the IL-9R and its associated signaling molecules to GMCSFR βc-associated JAK2. Specific blockade of JAK2 with a small molecule inhibitor abrogated GIFT-9's ability to hyperactivate STAT1, demonstrating that this effect was JAK2 specific. In essence, an environment is

created where JAK1, JAK2, and JAK3 are assembled in a manner not otherwise physiologically permissible, hence resulting in a gain-of-function (Figure 5.1).

GIFT-9 provided a proof-of-concept that receptor clustering is responsible for the altered signaling that is observed across the GIFT family of fusokines. By bringing together different combinations of activated JAKs and STATs that do not physiologically interact with one another, GIFT fusokines are able to launch novel downstream signaling events. The phenotypic outcome and eventual effector functions of GIFT-responsive cell types rely on the balance of STATs that become activated [399]. While we were unable to ascertain whether GIFT-15 truly clusters the GMCSF receptor and IL-15 receptor complexes together, GIFT-15 behaves in an atypical fashion, because it is the only GIFT fusokine that acts as a partial agonist. While GMCSF and IL-15 are both activators of STAT5 signaling, GIFT-15 stimulation activates STAT3 without concurrent activation of STAT5, driving a transcriptional program that coaxes naïve B cells to take on a regulatory phenotype. This would suggest that GIFT-15 engages its receptors in a way that alters the ability of adaptor molecules to transduce an intracellular signal [246]. Molecular modeling of mammalian GIFT-15 showed that while the GMCSF domain remain unchanged in its ability to bind the GMCSFR complex, the IL-15 moiety which lies C'-terminally to the GMCSF moiety may be inhibited in its ability to bind the γc, while the residues responsible for IL-15Rβc binding remained exposed. Surface plasmon resonance experiments utilizing IL-15Rα and GIFT-15 also showed that GIFT-15 was able to bind to IL-15Rα with high affinity (e.g. compared to IL-15 alone). Thus, GIFT-15 theoretically should be

able to bind to the GMCSF receptor complex, the IL-15Rα and βc, but not the γc. This fits with the general signaling profile that was seen with mammalian GIFT-15, whereby GIFT-15 was unable to induce STAT5 phosphorylation because of its inability to engage the γc (and therefore its inability to activate JAK3), but could induce STAT3 phosphorylation, given its interaction with the IL-15Rβc (and therefore its ability to activate JAK1). Although the GMCSF moiety should be able to bind to the GMCSFRβc, activating JAK2 and phosphorylating STAT5, we were unable to detect any STAT5 activation with GIFT-15 treatment, suggesting that somehow GIFT-15 is unable to fully induce JAK2 activation. While we were able to demonstrate that GIFT-15 is incapable of inducing JAK3 phosphorylation, experiments interrogating JAK2 phosphorylation have been uninterpretable. Interestingly, Rafei et al. showed similarly that mammalian GIFT-15 was unable to induce meaningful STAT5 phosphorylation in unfractionated splenocytes akin to our own observations with bacterially derived recombinant GIFT-15. Rafei et al. showed that mammalian GIFT-15 could only induce visible STAT5 phosphorylation by immunoblot on the GMCSF-dependent macrophage cell line, JAWS-II, which express the GMCSFR βc at very high levels. The lack of glycosylation in bacterially-derived GIFT-15 may additionally hinder its ability to bind the GMCSFR βc.

We also cannot exclude the possibility that potential receptor clustering by GIFT-15 of the GMCSFR and IL-15 receptor complexes can alter the way negative regulators of cytokine signaling function. Protein phosphatases such as the SH-2 domain containing protein tyrosine phosphatase-1 (SHP-1), suppressor of

cytokine signaling family members (e.g., SOCS1 and 3), CD45, and protein inhibitors of activated STATs (PIAS), which negatively regulate the JAK/STAT pathway could inadvertently be affected by receptor clustering, a hypothesis that bears further testing by immunoblot [400-403].

Ultimately, both mammalian and bacterially-derived GIFT-15 results in the transduction of a strong STAT3 signal without concomitant STAT5. Although IL-2, IL-7, IL-15, and IL-21 (all pro-inflammatory γc cytokines) induce some element of STAT3 signaling, this transcription factor is known to exert many immunoregulatory effects [404]. STAT3 has become increasingly implicated in immunosuppressive networks in conditions such as cancer and T cell exhaustion/dysfunction in the face of chronic viral infections [405, 406]. Although STAT3 signaling has been documented to be important in the survival and development of T cells, B cells, and DCs, its overactivation is associated with net immunosuppressive effects or inappropriate activation of the immune response towards autoimmunity [407-409]. Conditional knockouts generated for individual cell subsets, such as DCs, have shown that STAT3 is a negative regulator of their function [410]. STAT3 is known to drive the expression of tolerogenic markers, such as PD-L1 on the surface of APCs, such as DCs and B cells, a phenotype we observe with GIFT-15 induced B_{regs} [411]. STAT3 signaling also inhibits both $CD4^+$ and $CD8^+$ T cell proliferation and their production of IL-2 [412]. Importantly, STAT3 is known to drive the expression of immunosuppressive IL-10, the defining characteristic of GIFT-15 induced B_{regs} [413, 414]. While STAT5 is known to provide a balance to some of the regulatory actions of STAT3, the fact

that GIFT-15 induces an unopposed STAT3 signal (that is in the absence of STAT5), likely leads to the regulatory phenotype of the B cells seen [415]. Most of the literature on STAT3 and the immune response comes from T cells and myeloid cells, with few groups examining its effect on B cells. STAT3 signaling in B cells is mainly appreciated for its role in the development and differentiation of germinal center B cells, particularly via the provision of IL-21 by T_{FH} cells [416, 417]. Selective deletion of STAT3 in bone marrow progenitor cells resulted in a lack of pro-, pre-, and immature B cells in the bone marrows, although pre-pro-B cells were increased suggesting that STAT3 signaling plays an early role in the B cell differentiation [418]. STAT3 signaling seems to be important for human naïve B cells to differentiate in plasmablasts [419]. The role of STAT3 signaling in the emerging field of regulatory B cell is less well understood. Importantly, IL-10 secreted by regulatory B cells is a potent activator of STAT3 signaling, suggesting that the induction of B_{regs} may create a sort of positive feedback loop, wherein STAT3 signaling induces IL-10 production, which leads to additional STAT3 signaling [420, 421]. Interestingly, a new cytokine shown to be secreted by B_{regs}, IL-35, has also been shown to be activated via STAT3 (as well as STAT1) [372]. B_{regs} induced by IL-33 (a member of the IL-1β family) have also been shown to signal via STAT3 [422, 423]. It is thought that IL-1β itself also signals via STAT3 in conjunction with IL-6 and innate activation via TLRs to endogenously commit naïve B cells to a B_{reg} fate [424].

Due to wide array of other possible signaling pathways that both GMCSF and IL-15 may signal through, predominately Erk1/2, p38, or JNK pathways, we

attempted to determine whether GIFT-15 may exert its effect via these alternative pathways. Our immunoblot analysis, at least for the time points interrogated, did not show any significant differences between GIFT-15 and control cytokines, suggesting that GIFT-15 does not confer its physiological influence on B cells via these specific pathways.

5.3.0 Future directions

The eventual goal of the pre-clinical work surrounding the GIFT and FIST fusion family of proteins is to see their translation into the clinical setting. In addition to the continued mechanistic work required to understand the mechanisms behind how these proteins function, additional strategies warrant further testing, given clinical trial information surrounding the use of IL-15 for cancer immunotherapy (for FIST-15) or pre-clinical data gleaned from the use of regulatory B cells for autoimmune ailments or in settings where immunosuppression is desired (for GIFT-15).

Now that we have generated, characterized, and found a useful *in vivo* model of experimental pathology in which FIST-15 proves beneficial, our goal for FIST-15 is to advance its efficacy. Although it was hoped initially that the combination of TGF-β neutralization and a pro-inflammatory IL-15 signal may be enough to help overcome tumor-immune suppressive mechanisms, the use of FIST-15 as a therapeutic agent does not result in complete tumor regression, even in a fairly immunogenic model of cancer, such as B16 melanoma. In order to improve upon the efficacy of FIST-15, we are looking at other pre-clinical and current clinical

studies to guide the future direction of FIST-15 development. Many IL-15-based clinical trials have been conducted in conjunction with autologous transfer of NK cells [425]. In essence, adoptive cell transfer followed by IL-15 treatment seems to result in greater efficacy than the use of IL-15 as a therapeutic agent alone. In our opinion, this would be the next rational model in which we should test FIST-15. Rather than simply culturing NK cells *ex vivo* with IL-15 and adoptively transferring the NK cells into the tumor bearing host, expansion of NK cells with FIST-15 *ex vivo*, proceeded with additional FIST-15 treatment may yield the best results. This is due to the fact that while FIST-15 can expand NK cells to a high degree (greater than IL-15), the real rationale for its design was to neutralize tumor-derived TGF-β. Expansion with FIST-15 *ex vivo*, followed by adoptive transfer of these cells and subsequent FIST-15 delivery into the tumor-bearing host will give the transferred NK cells the best change at infiltrating and destroying the tumor. While we did not assess the toxicity profile of FIST-15, other groups have shown that IL-15/IL-15Rα superagonists may have untoward side effects due to excess IFN-γ production and secretion [426]. To mitigate this effect, we are also interested in pairing FIST-15 with effective tumor-targeting strategies, such as the use of nanoparticles, which can bind or contain biologics and enrich for their concentration within the tumor microenvironment. This would decrease likely systemic side effects, while enhancing local anti-tumor responses [427]. Finally, from an NK cell immunotherapy standpoint, any additional interventions that enhance the immunogenicity of the tumor would likely also improve the efficacy of subsequent FIST-15 therapy. NK cells require the

recognition of surface markers of stress and transformation before engaging optimally in their cytolytic functions [428]. Neoadjuvant therapy such as chemotherapy or radiation prior to treatment with adoptive transfer of NK cells and FIST-15 treatment would likely improve anti-tumor outcomes.

It appears that while FIST-15 acts on $CD8^+$ T cells, as well as NK cells, *in vitro*, the predominant immune subset that mediates FIST-15's anti-tumor effect are NK cells. Our observations are consistent with others who report that NK cells are most sensitive to the mitogenic effects of IL-15 signaling [429]. However, the importance of $CD8^+$ T cells in the anti-tumor response cannot be understated, especially as it pertains to long-term, durable anti-tumor immunity. The ability of $CD8^+$ T cells to form robust memory against tumor antigens and protect from subsequent tumor challenge is indisputable [174]. Since IL-15 agonism has profound effects on the development and maintenance of memory $CD8^+$ T cells, we believe that FIST-15 may be used to boost anti-tumor memory. Although the models we utilized favor enhancement of innate responses for tumor clearance (e.g. NK cell activation with FIST-15, use of B16 melanoma tumors lacking MHC-I expression, etc.), we cannot preclude the possibility that FIST-15 could induce strong memory $CD8^+$ T cell responses. The kinetics of FIST-15's effect on $CD8^+$ T cells, particularly in the context of memory formation, is required to determine when FIST-15 may best be administered to provide signals required for optimal memory formation (and not just quantity of memory formation). We would favor whole cell tumor vaccine strategies, utilizing FIST-15 as an adjuvant, or FIST-15 transduced tumor cells as a vaccine, to determine the extent to which FIST-15

can boost memory responses. Further, with the advent and widespread utilization of chimeric antigen receptor (CAR) T cells in cancer immunotherapy, we would be curious to see the effect of FIST-15 on adoptively transferred CAR-T cells. We would hypothesize that FIST-15 could provide crucial assistance to CAR-T cells in the tumor microenvironment by neutralizing TGF-β, which would likely hamper their antigen-specific cytolytic functions. Further, should CAR-T cells go on to form a stable population of anti-tumor memory T cells, FIST-15 would likely provide important homeostatic signals to boost their longevity and persistence in the host. Finally, although we did not conduct extensive phenotypic analysis on NKT cells with FIST-15 treatment, recent literature has begun to shed light on how important these cells may be in anti-tumor surveillance and function. Innate-like lymphocytes (ILCs), particularly those that are tissue-resident also depend heavily on IL-15 for homeostatic maintenance [430]. Further research investigating the effects of FIST-15 on ILCs and NKT cell subsets could yield interesting avenues to new therapies with FIST-15.

While cancer immunotherapy has been the main focus of FIST-15's development, with alternative applications of FIST-15 in immunotherapy (Chapter 3), we are now seeing other potential areas of unmet medical need that FIST-15 could prove to be therapeutically beneficial. Particularly in the context of viral infections, we would be curious to determine whether FIST-15 could boost immune responses in the setting of chronic viral infections, where both innate and adaptive immune responses are suppressed. While experimentally we tested the effects of FIST-15 and NK cells in liver fibrosis, the role of NK cells and

innate immune cells in fibrotic processes within the liver and other organs (for which we have few efficacious treatments) remain relatively unknown. The use of FIST-15 in other models of end-organ fibrosis would likely yield interesting information regarding the role of NK cell biology in these organs at the very least, or FIST-15 may prove to behave therapeutically in this area of unmet medical need as well.

With FIST-2 and FIST-15 completed and characterized, the remainder of the γc interleukins that remain to be coupled to TGF-β neutralization include IL-4, 7, 9, and 21. To that end, we have generated prototype fusion proteins for FIST-7 and FIST-21, although they remain to be fully characterized. The generation of the human orthologue of FIST-15 is also currently underway.

The future of GIFT-15 as an agent for immunomodulation remains bright. The fact that GIFT-15 acts predominately on B cells makes it a useful agent in that B cells are very abundant and easy to access. Although regulatory B cells are exceeding rare under physiological conditions, the ability of GIFT-15 to readily convert a significant population of splenic B cells into regulatory B cells makes it an attractive candidate for translational development [431]. Current technologies to convert B cells to regulatory B cells remain limited and are may be unfeasible in a large-scale context. The use of GIFT-15 to convert B cells into B_{regs} could potentially overcome some of the limitations that other groups have encountered. The mechanism by which GIFT-15 converts B cells to B_{regs} still remains relatively obscure despite our attempts to dissect it. In so far as we are able to recapitulate the effects of mammalian-derived GIFT-15 with bacterially-derived recombinant

GIFT-15, we are confident the effect we see is specific to this protein. The ability to generate this protein utilizing recombinant bacterial approaches further makes GIFT-15 an attractive candidate for translation into the clinical setting. The general consensus on regulatory B cell biology is that they be defined by their functional capacity to modulate and regulate overt inflammatory responses and GIFT-15 is readily able to do this [352]. While the exact phenotype of GIFT-15 B_{regs} look to be different than other regulatory B cell subsets described, the ultimate goal (from a translational standpoint) is that the these cells are indeed capable of inhibiting inappropriate immune activation [432].

To that end, GIFT-15 would likely prove to be efficacious in a variety of autoimmune settings. In this dissertation, we provided evidence that GIFT-15 B_{regs} can suppress neuroinflammatory responses via induction of peripheral regulatory T cell subsets. GIFT-15 B_{regs} may very well be utilized as a therapeutic agent for other T cell-mediated autoimmune conditions, such as Type 1 diabetes mellitus, polymyositis, dermatomyositis, etc. Further, in situations where immunosuppression is desired, GIFT-15 B_{regs} may prove to be useful. One major example in which a major unmet medical need exists is in the realm of solid organ transplantation. Regulatory B cells could very well dampen allograft immune responses against transplanted organs to prolong graft survival [433]. Further, in allogeneic hematopoietic transplant settings, GIFT-15 B_{regs} may provide benefits in prevention or treatment of life-threatening graft-versus-host-disease [434].

5.3.0 Conclusion

In summary, much of the biology γc cytokines and their effects on the immune response remain to be uncovered. As we continue to understand the potential and limitations of these cytokines in normal biology, we can improve on strategies to optimize their use in a translational setting. From what was known about their biology and physiological effects, we have attempted to rationally improve upon their function through their modification by fusions to other protein moieties. These fusion families, specifically the GIFT and FIST family of proteins, have been demonstrated to enhance the effect of these cytokines, allow them to reach their full potential in environments where their activity may be inhibited, or even change their biology altogether. Although the ultimate goal of the fusion protein platform is to see their translation and use in a clinical setting to mitigate disease, the insights into cytokine biology that we have gleaned from these modifications are equally important, and will continue to help us better design the cytokine-based therapies of the future.

5.4.0 Figure Legends

Figure 5.1 GIFT-induced receptor clustering

GIFTs are able to bring together activated GMCSF and interleukin receptors belonging to the common gamma-chain (γ-c) family. GMCSF ligand binding to the GMCSF receptor (GMCSFR) triggers the dimerization of alpha (αc)- and beta (βc)-chains, resulting in the activation of β-chain-associated JAK2/STAT5. γ-c

cytokines initiate a similar signaling cascade by bringing together the γ-c and a cytokine-specific IL-R alpha (αc)-chain. JAK3 associates exclusively with the γ-c in lymphomyeloid cells and activates STAT5 upon interleukin (IL) binding. JAK1 associated with the IL-Rα chain will activate different STATs (STAT-X), depending on the IL bound. GIFTs trigger the co-clustering of all four activated receptor components, resulting in transphosphorylation of IL-Rα chain-associated STAT-X substrates by JAK2 (dotted arrows). Changes in the balance of STAT phosphorylation events induce a unique GIFT-mediated response that is distinct from canonical GMCSF- and IL-mediated responses.

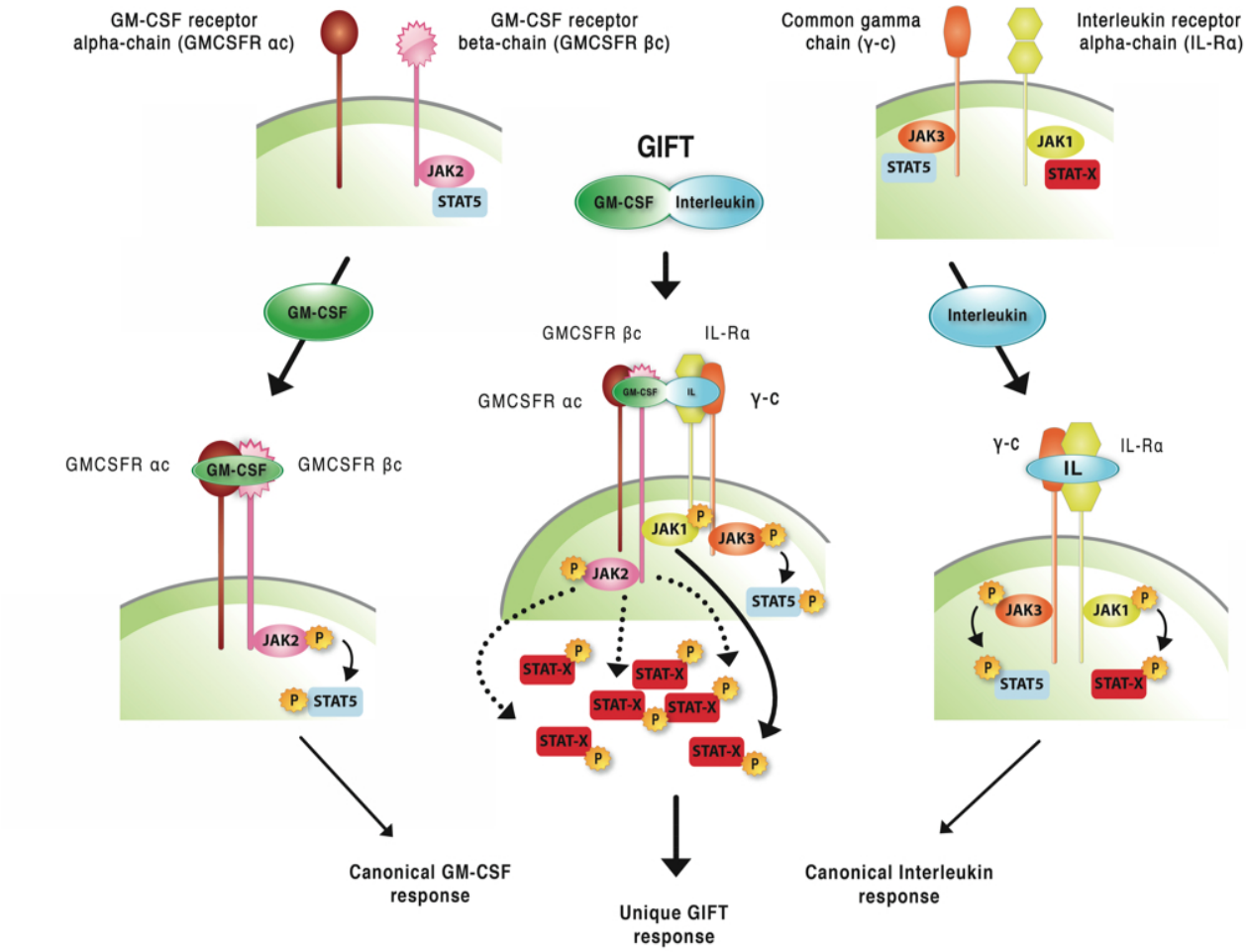
GM-CSF receptor alpha-chain (GMCSFR αc)
GM-CSF receptor beta-chain (GMCSFR βc)
JAK2
STAT5
GIFT
GM-CSF
Interleukin
Common gamma chain (γ-c)
Interleukin receptor alpha-chain (IL-Rα)
JAK3
JAK1
STAT5
STAT-X
GM-CSF
Interleukin
GMCSFR αc
GM-CSF
GMCSFR βc
JAK2
P
STAT5
GMCSFR βc
IL-Rα
GMCSFR αc
γ-c
JAK1
JAK3
JAK2
STAT5
STAT-X
γ-c
IL-Rα
IL
JAK3
JAK1
STAT5
STAT-X
Canonical GM-CSF response
Unique GIFT response
Canonical Interleukin response

Figure 5.1

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