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Analysis of Regulatory Mechanisms for T Cell Activation

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LIST OF ABBREVIATIONS

AIRE	Autoimmune regulator
AP-1	Activator protein-1
C1	Concerved domain 1
CBA	Cytokine bead array
CBM	Carma1, Bcl10 and MALT1
CRAC	Ca^{2+} release activated Ca^{2+} channels
cTEC	Thymic cortical epithelial cells
CTLs	Cytotoxic T lymphocytes
DAG	Diacylglycerol
DAMPs	Danger associated molecular patterns
DN1	Double negative stage 1
DN2	Double negative stage 2
DN3	Double negative stage 3
DN4	Double negative stage 4
DP	Double positive
EAE	Encephalomyelitis
ECM	Extracellular matrix

ER	Endoplasmic reticulum
Erk1	Extracellular signal-regulated kinase 1
FKBP12	FK506-binding protein 1A
FN3	Fibronectin type III domain
FVIII	Coagulation factor VIII
GARP	Glycoprotein-A repetitions predominant protein
GBS	Group B *Streptococci*
GEF	Guanidine exchange factor
GS	Glycine-serine
Hsp70	Heat-shock protein 70 kDa
IAM	Iodoacetamide
ICOS	Inducible co-stimulatory molecule
ICOSL	ICOS ligand
IKK	IkB kinase
IP3	Inositol 1,4,5-triphosphaate
ITAM	Immune receptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based motif
ITSM	Immunoreceptor tyrosine-based switch motif
JM	Juxtamembrane
JNK	c-Jun amino-terminal kinase
LAP	Latency associated peptide
LAT	Linker for activation of T cells
LBR	Non-selected phage display library

LLC	Large latent complex
LRRC	Leucin-rich-repeat-containing proteins
LTBP	Latent TGF-β binding protein
MAP	Mitogen activated protein
MH1	Mad homology 1
MHC	Major histocompatibility complex
MT1	MMP membrane type 1
Neu5Ac	N-Acetylneuraminic acid
NF-AT	Nuclear factor for activated T cells
NLS	Nuclear localization signals
PAMPs	Pattern associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PD-1	Programmed death-1
PI-3	Phosphatidyl inositol-3
PIP2	Phosphatidylinositol 4,5-biphosphate
PKC	Protein kinase C
pMHC	MHC peptide
PSLG-1	P-selectin glycoprotein-1
PTK7	Protein tyrosine kinase 7
RasGRP1	Ras guanyl nucleotide releasing protein
RBL	Rat basophil leukemia
ROS	Reactive oxygen species
RTEs	Recent thymic emigrants

S1P1	Sphingosine-1-phosphate receptor
SARA	Smad anchor for receptor activation
SFK	Src family kinase
SH	Src homology
Siglecs	Sialic acid-binding immunoglobulin lectins
SLC	Small latency complex
SLOs	Secondary lymphoid organs
SLP-76	SH2 domain-containing leukocyte phosphoprotein of 76 kDa
SP	Single positive
TCR	T cell receptor
TGF-β	Transforming growth factor β
TME	Tumor microenvironment
TNFRSF	Tumor necrosis factor receptor super family
Tr1	Type 1 regulatory T cells
Tregs	Regulatory T cells
TSLP	Thymic stromal lymphopoietin
TSP	T cell progenitors
VWF	Willebrand factor

ABSTRACT

The primary function of the immune system is to seek and eliminate altered or unhealthy cells. T cells are a significant component of the immune response and mediate their functions by recognizing specific antigens that eliminate infected or neoplastic cells. T cells have evolved strategies to discriminate self from non-self or healthy from altered and infected to avoid inappropriate activation and subsequent immune injuries. These strategies rely on the activation of receptors that restrict the T cell response.

CD33rSiglecs are a family of primarily inhibitory receptors that bind to sialic acids. Siglecs respond to specific sialic acid patterns characteristic for healthy and self and trigger tolerogenic signaling pathways that prevent activation of the immune response. To date, the expression of Siglecs in human T cells is not well appreciated. We found that one Siglec member, Siglec-5, is transiently expressed only on activated T cells. Using overexpression studies, we showed that Siglec-5 is a potent inhibitor of T cell activation. The expression pattern, along with the functional studies, suggested that Siglec-5 is a checkpoint-like receptor that negatively regulates T cell activation. Using a previously described protein-ligand, we tested if Siglec-5 engagement suppresses the functionality of human T cells. We found that Siglec-5 reduces the T cells effector functions, as measured by the production of cytokines and cytolytic molecules.

Cancers change their sialyation to evade immune recognition, and we hypothesized that Siglec-5 is a mechanism to enable this. If malignant cells can engage and activate the Siglec-5 receptor on T cells, they could suppress and evade the anti-tumor T cell response. Our data demonstrate that soluble Siglec-5 binds to cancer cell lines from different tissues, suggesting the expression of putative Siglec-5 ligands. We measured the T cell specific response when the Siglec-5 signaling axis is interrupted using engineered melanoma-specific T cells. We found that blocking the availability of Siglec-5 putative ligands on the cancer cells reinvigorates the T cells immune response against melanoma cancer cells.

Altogether, this work identifies Siglec-5 as a novel checkpoint receptor that suppresses the activation of T cells. Several checkpoint receptors with similar functions to Siglec-5 already serve as successful targets for cancer immunotherapies. However, such therapies work in only a small fraction of cancer patients. Our work shows that blocking Siglec-5 strongly reinvigorates the T cell response. Alone, or in combination with other checkpoint targets, blockade of Siglec-5 can serve as a strategy to prevent cancer immune evasion.

CHAPTER I: REVIEW OF THE LITERATURE

T Cell Development

The Discovery of the Thymus

T cell development beings in the thymus, an organ that supports the development and selection of T cells. The thymus was Initially considered a useless organ; however, in 1960, J. Miller made a bold postulation that “the thymus at birth may be essential to life”[1]. Miller provided vital evidence that the thymus is the main source of immunocompetent lymphocytes during the neonatal period. Using thymectomized neonatal mice, he showed that skin grafts from up to four different strains with different coat colors are not rejected[1]. Based on these studies, he proposed that key T cell development happens in the thymus during embryogenesis, from where fully developed lymphocytes migrate to other sites at the time of birth[2]. Remarkably, Miller’s studies established that unlike previously thought, thymus lymphocytes are not immune-incompetent, and instead can respond to antigen stimulation that can further stimulate bone marrow-derived cells to produce antibodies [3]. Thymic seeding progenitors (TSPs) originate from adult bone marrow and arrive in the thymus via the thymic cortico-medullary junction. Once in the thymus, progenitor cells undergo progressive differentiation as they migrate across the morphologically similar but functionally different regions of the thymus, and before their export into the periphery [4]. It is now apparent that for commitment into the T cell lineage a complex network of different transcriptional regulators, proliferative and survival signals from

thymic epithelial cells, and signals from the developing thymocytes, must interplay into a cooperative activity to support proper development.

Entry of T Cell Progenitors into the Thymus and Early Thymic Development

Upon entry into the thymus, TSPs are defined as $CD4^{-}CD8^{-}CD25^{-}CD44^{hi}$, or lineage negative, double negative stage 1 (DN1). At this point TSPs have lost the ability to enter the erythroid or myeloid lineage, and instead are biased towards the lymphoid lineage. However, DN1 cells are not yet entirely restricted to the T cell lineage, and can undertake the B cell, NK cell, or dendritic cell lineage as well[5,6]. For TSPs to commit to the T cell lineage, a network of transcriptional proteins is induced and tightly regulates the process. E-proteins are a subfamily of helix-loop-helix transcription factors that play an important role during several thymocyte developmental processes. Mice deficient in E2A gene products have tenfold decrease in thymocyte numbers, as compared to wild-type littermates. As E2A expression is induced when TSPs begin to restrict towards a potential T cell lineage, the underlying reason for the E2A null mice phenotype is a developmental defect preceding T cell lineage commitment and initiation of TCR rearrangemen[7,8]. Further evidence that supports the role of E2A proteins in T cell development comes from studies showing that Id proteins, which are negative regulators of E-protein activity, block T cell and instead favor NK cell lineage commitment. Altogether, the balance between E- and Id-protein activity is essential in determining T versus NK cell lineage commitment[9,10]. Notch is another transcription factor critical for T cell development and essential for blocking B cell lineage commitment of TSPs enteringthe thymus. Mice lacking functional Notch1 postnatally show a defect in thymocyte development presented with a loss of both $\alpha\beta$ or $\gamma\delta$ T

lineages, and enrichment in cells carrying all the typical B cell markers[11]. When TSPs enter the double negative stage 2 (DN2), defined as $CD4^{-}CD8^{-}CD25^{+}CD44^{hi}$, they have already lost the ability to become NK or B cells but can still become T cells or dendritic cells[12]. During this stage, developing cells begin T cell receptor (TCR)-β, TCR-γ, and TCR-δ gene rearrangement, The rearrangement of the TCR chains is a process mediated by RAG1 and RAG2, and drives TSPs towards total commitment to the T cell lineage[13–15]. However, at this stage, cell proliferation is arrested and requires the formation of the pre-TCR complex for cell growth to continue. Thus, when entering the double negative stage 3 (DN3), defined as $CD4^{-}CD8^{-}CD25^{+}CD44^{lo}$, cells that have successfully rearranged their TCR-β chain now pair up with an invariant, germline-encoded pre-TCR-α and CD3 to form the pre-TCR. Pre-TCR signaling leads to forcing the β-chain selection, rescuing cells from apoptosis, supporting further cell proliferation, and silencing the TCR-γ rearrangement[16–19]. The selection of the β-chain mediates loss of CD25, which leads to the final stage of the lymphopoiesis, double negative stage 4 (DN4), a transient state that is also considered as the pre-double positive (DP) stage. Pre-TCR signaling is also essential for initiating the TCR-α rearrangement, which is completed in the DP stage. If TCR-α successfully rearranges at the double positive $CD4^{+}CD8^{+}$ state, thymocytes will express αβ-TCR, allowing for positive and negative selection and differentiation into single positive $CD4^{+}$ helper, or $CD8^{+}$ killer lineage[20,21]. Alternatively, if the developing thymocytes successfully rearrange both their TCR-γ and TCR-δ loci, they will express a γδ-TCR. Expression of γδ-TCR also initiates a burst in proliferation, however, these cells avoid entering the DP stage of development and instead enter the periphery as $CD4^{-}CD8^{-}$ cells. Interestingly, it is suggested that the

TCR type, αβ or γδ is not what determines the lineage fate of the T cells; instead, it is the strength of the TCR signal that makes a choice. Compared to the pre-TCR, composed of a rearranged β and invariant α chain, the γδ-TCR induces stronger TCR signaling events (high levels of phosphorylated Erk and strong induction of Egr family of transcription factors) which favor the γδ-T cell lineage. However, manipulations designed to reduce the strength of γδ-TCR signaling by altering the surface levels of γδ-TCR, the amount of signaling molecules, or the removal of the γδ-TCR ligands, favor the αβ-TCR lineage cells[22–24]. Yet other studies suggest a pre-commitment model, where the lineage choice is made prior to TCR expression[25,26]. The reconciliation between the two models has been that the TCR signal strength is essential to confirm the pre-commitment choice for lineage differentiation. In the case that the two are incompatible, the cell gets eliminated.

Positive and Negative Selection

Upon β-chain rearrangement, and as soon as pre-TCR signaling begins in DN precursor cells, gene rearrangement of the α-chain is initiated. Even though low levels of α-chain rearrangement can be detected during the final DN4 stage of development, active and efficient rearrangement occurs once the cells become quiescent DP. Nevertheless, rearrangement of the α-chain does not stop until the TCRs successfully engage with a major histocompatibility complex (MHC) peptide during positive selection. In other words, surface expression of an αβ-TCR does not reduce the activity of RAG1 and RAG2, and thus α-chain rearrangement continues. It is postulated that the developing T cell uses these events to try different combinations of αβ-TCRs to choose only those that successfully bind to MHC peptides (pMHC)[27,28]. DP thymocytes that

interact poorly with MHC-peptide complexes and thus don't receive the intracellular threshold signaling required for survival, die by a process known as death by neglect. Conversely, a small fraction of the DP thymocytes has a strong affinity for the MHC-peptide. If permitted to egress in the periphery, these cells could cause autoimmune related pathologies. Intracellular signaling triggered by strong affinity interactions results in rapid apoptotic death, also termed negative selection. Thus, only cells with intermediate affinity for MHC-peptide are permitted to survive and differentiate into single positive (SP), $CD4^+CD8^-$ or $CD4^-CD8^+$, T cells[29,30]. But how does the TCR-pMHC interaction determine whether a DP thymocyte will become $CD4^+$ or $CD8^+$? A critical step in positively selecting a DP thymocyte is the quality of the interaction between the TCR and pMHC. TCR and pMHC interactions dictate the proximal signaling events that will be triggered in that T cell, and the pairing of either CD4 or CD8 co-receptors plays a crucial role in the quality of that signal. The lineage commitment only occurs when TCR-pMHC class I engage with CD8, and TCR-pMHC II engage with CD4 co-receptor. In fact, the strength of the signal generated when CD4 or CD8 engage with the appropriate TCR-pMHC complex makes the decision, with prolonged signals leading to $CD4^+$ and short signals leading to $CD8^+$ T cells. Molecularly, the CD4 and CD8 co-receptors bind to the TCR-pMHC complex and stabilize the receptor-ligand interaction, thus decrease the dissociation rate between the TCR and pMHC. Furthermore, the co-receptors also increase the strength of the signal as they can directly bind and recruit Lck to the immunological synapse[31–33]. Lck is a cytoplasmic kinase which activity dictates the duration and strength of the downstream signaling cascade, and thus the fate of the developing thymocyte[34–37]. CD4 associates with Lck more efficiently and thus brings

more Lck to the TCR complex for signaling. Subsequently, when CD4 engages with the right TCR-pMHC complex the developing thymocyte receives a stronger signal that drives the cell into a CD4 lineage[38,39]. The efficiency of positive selection is highly dependent on the thymic microenvironment and the thymic cortical epithelial cells (cTEC). Unlike other epithelial cells, cTECs possess a unique protein degradation machinery, the thymoproteasome, which generates the pool of ligands necessary to select for vast TCR pool that reacts to foreign, but not self-antigens. Crosstalk between the developing thymocytes and the cTEC is an essential component in the process of positive selection, as DN thymocytes generate the signals for the development of cTECs[40].

Completion of Thymocyte Development

Regardless of the strength of the TCR signal, when developing thymocytes receive TCR stimulation, they upregulate CCR7. This chemokine receptor allows their migration from the cortical to the medullary region of the thymus, where CCR7 ligands (CCL19 and CCL21) are expressed. However, since negatively selected T cells are destined to die, only positively selected T cells to migrate. Crosstalk between the developing thymocytes and the medulla is essential for the cells to migrate, as successful positive selection is required for formation of the architecture of the medulla[41,42]. When SP T cells arrive in the medulla, they are functionally incompetent, and susceptible to apoptotic signals, and need to undergo maturation processes before being sent into the periphery[43]. Furthermore, the maturation processes are also accompanied by a second round of selection and deletion of tissue-reactive thymocytes that have escaped cortical negative selection. Negative selection in the medulla is

mediated in part by the activity of the transcription factor autoimmune regulator element (AIRE), and the presentation of peripheral tissue-specific peptides by medullary TECs[44–47]. Additionally, the medulla is where natural regulatory T cells (Tregs) are generated as well. A specialized structure within the medulla, known as the Hassal's Corpuscle, plays a vital role in the generation of Tregs via production of the cytokine thymic stromal lymphopoietin (TSLP). TSLP activates medullary dendritic cells that drive the induction of Foxp3+ regulatory T cells. Altogether, the medulla is essential for central tolerance, as it ensures deletion of auto-reactive T cells while also producing regulatory T cells specific for peripheral self-antigens[48–50].

Export of Mature T cells into Circulation

When thymocytes reach the mature state, they begin expressing high levels of the sphingosine-1-phosphate receptor 1 (S1P1), a G-protein coupled receptor. The ligand for S1P1, S1P, is highly expressed in serum, and not so much in tissues, and thus acts as a chemoattractant that drives mature thymocytes to egress from the thymus and into the perivascular space, which channels into the post-capillary venules, arterioles, and lymphatics[51,52].

Naïve T cells

Upon positive and negative selection in the thymus, mature thymocytes egress into the periphery as naïve or antigen inexperienced T cells. However, with age, the thymus regresses, loses its tissue organization, and thus results in less efficient T cell development and reduced naïve T cell output[53,54]. As the ability of the adaptive immune system to mediate adequate immune responses against foreign antigens depends mainly on the TCR diversity generated during thymopoiesis, a functional pool of naïve T

cells must be maintained throughout an individual lifetime. Interestingly, unlike mouse naïve T cells, which derive from continuous thymic output throughout life, human naïve T cells derive from continuous peripheral T cell division and can persist for 5-10 years[55,56]. Thus, besides loss in thymic output and a decline in the overall frequency, human naïve T cells preserve their diversity and functionality even at old age through peripheral post-thymic expansion[57,58].

Recent Thymic Emigrants

Naïve T cells that have just egressed from the thymus are also called recent thymic emigrants (RTEs). Phenotypically, RTEs are characterized by the expression of IL7Rα^{lo}TCR^{hi}CD3^{hi}CD28^{lo}CD24^{hi}Qa2^{lo}CD45RB^{lo}, as well as the protein tyrosine kinase 7 (PTK7) and CD31 for CD4 and CD103 for CD8. RTEs are functionally different than mature naïve T cells as they manifest reduced proliferation and cytokine production in response to TCR/CD3 and CD28 stimuli. In fact, the functional differences are reflected by the different epigenetic states, with RTEs naïve T cells having hypermethylated promoters for the *Il2* and *Il4* genes, compared to both the precursors of RTEs and mature naïve T cells[59]. Furthermore, RTEs also have a much more diverse TCR repertoire compared to mature naïve T cells[60,61]. Altogether, RTEs are a transitional but important T cell compartment in which functional responses are tightly regulated.

Naïve T cell Maturation

In order to enter the fully functional, long-lived mature naïve T cells state, RTEs must undergo a T cell maturation process that occurs specifically in secondary lymphoid organs (SLOs)[62]. Compared to RTEs, mature naïve T cells have a less diverse TCR

repertoire, suggesting that TCR-pMHC interactions in the SLOs might lead to the deletion of certain RTE clones. Even though the details of RTE maturation are precise, it is known that the process is independent of TCR signaling, even though TCR engagement is required for shaping the TCR repertoire of mature naïve T cells[63,64].

A transcriptional repressor, NKAP, is involved in the maturation process, as T cells lacking NKAP remain stuck at the RTE stage. However it is unclear how NKAP mediates its effects[65]. An explanation for the requirement of extra-thymic naïve T cell maturation could be that it provides another checkpoint to ensure self-tolerance. Even though central tolerance mechanisms (i.e., negative selection) in the thymus are highly efficient processes, self-reactive T cells, especially ones with lower affinity for self-antigens, manage to egress to the periphery.

Within SLOs, extrathymic *Aire*-expressing cells present self-antigens and are capable of deleting of self-reacting cells[66]; since RTEs require entry in the SLOs in order to transition to mature naïve T cells, it could be that self-reacting RTEs are also deleted before becoming mature naïve T cells. The fact that the TCR repertoire of mature naïve T cells is much less diverse compared to RTEs could also serve as circumstantial evidence that RTEs clones that react to self-antigens within the SLOs are eliminated.

T cell Subsets

When naïve $CD4^+$ and $CD8^+$ T cells encounter their cognate antigen, in the context of MHCII and MHCI, respectively, they differentiate into various cellular subsets, helper or regulatory, for CD4+, or cytotoxic or regulatory, for CD8+. Besides the TCR stimuli[67], efficient and appropriate activation and differentiation also require two additional signals, one coming from co-stimulatory molecules, most often CD28, which

amplify or modify the signaling cascade initiated by the TCR[68,69], and the other from cytokines in the environment, which skew the signaling cascades toward polarization of the cells into different subsets[70].

CD4+ T cells

CD4+ T cells, or helper T cells, play a central role in mediating adequate immune responses via direct or indirect involvement. Not only do helper T cells respond themselves to antigens, but they also provide critical help in the production of antibodies by B cells, the cytolytic activity of CD8 T cell, and the activity of various innate immune cells. The diverse functions that helper cells can perform are enabled by the differentiation of naïve CD4+ T cells into specific subsets upon stimulation with their cognate antigen. While antigen concentration or different co-stimulation molecules can influence the helper subset that naïve CD4 can become, the critical regulators in the process are the different cytokines present in the milieu.

In the late 80s, Mossman and Coffman first reported two T helper subsets, Th1 and Th2, each producing distinct set of cytokines, and thus shaping the immune response in a different direction[71,72].

Th1. The critical cytokines that drive Th1 differentiation are interleukin-12 (IL-12)[73] and interferon-γ (IFN-γ)[74], which synergize into a signaling cascade that leads to the expression of the Th1 master transcription factor, T-box expressed in T cells (T-bet)[75]. The signature cytokine for Th1 cells is IFN-γ, but they also produce IL-2 and tumor necrosis factor-α (TNF-α). Using cytokine-mediated mechanisms, Th1 cells direct cell-mediated immune responses to ensure eradication of intracellular pathogens, but also mediate autoimmune tissue inflammation.

Th2. On the other hand, IL-4 plays a central role in the differentiation of the Th2 helper subset. IL4 triggers a STAT6 dependent signaling cascade that upregulates expression of the transcription factor GATA3[76,77]. GATA 3 is the master regulator of the Th2 cell lineage and potentiates the cells into producing more IL-4 and other cytokines, such as IL-5, IL-9, and IL-13[78,79]. Via the cytokines they produce, Th2 cells orchestrate immune responses that target helminth infections and facilitate repair of damaged tissues and contribute to chronic inflammatory diseases such as asthma and allergies.

Th17. In 2005, a third, distinct lineage of CD4 T-cells, known as Th17, was reported[80,81]. The key cytokines for Th17 differentiation are a combination of transforming growth factor-β (TGF-β) and IL-6, with TNF-α and IL-1β further enhancing the differentiation process[82–85]. The signature cytokine of Th17 cells is IL-17A (commonly known as IL-17), but they also produce IL-17F, TNF-α, IL-21, IL-22, and IL-26. The master regulator of Th17 cell differentiation is the orphan nuclear receptor ROR-γt, which regulates the transcription of IL-17 and the related cytokines[86]. Th17 responses are critical in mediating mucosal immune responses against both intracellular and extracellular bacteria and some fungi. However, the Th17 subset is also the primary mediator of several autoimmune diseases such as rheumatoid arthritis, psoriasis, Chron's disease, etc.

Tregs. The existence of multiple CD4+ helper subsets can be explained by the unique programs triggered by each subset in orchestrating the immune response. However, an inappropriate or overly active immune response can often enable the development of immune pathologies and autoimmune disorders. Such improper

responses are often initiated against self-antigens, innocuous antigens found in food, and commensal bacteria or fetal antigens.

A critical CD4+ T cell subset, known as regulatory T cells, is dedicated to constraining the expansion and activity of such pathogenic T cells. The idea for the existence of suppressive T cells dates back to the late 60s[87]; however, it was not until 1995 when strong evidence was provided that T cells can induce immune tolerance. In a seminal paper, Sakaguchi *et al*. described a unique CD4+ T cell subset, defined by the constitutive expression of CD25, the IL-2 receptor α-chain, that is responsible for down-regulating immune responses against self- and non-self antigens[88]. It was later elaborated that Tregs can be generated in two ways: 1) in the thymus, during negative selection (naturally arising regulatory T cells) and 2) in the periphery by co-stimulation of naïve T cells with antigen, TGF-β and IL-2 (peripheral Tregs)[89]. The master regulator for the development and function of both thymic and peripheral regulatory T cells is the forkhead transcription factor Foxp3, which maintenance is dependent on TGF-β[90,91].

Another cell subset involved in mediating immune tolerance against self-antigens is the type 1 regulatory T cells (Tr1). Tr1 cells are generated by chronic antigen stimulation in the presence of IL-10. Tr1 lacks expression of Foxp3 but constitutively produces high levels of IL-10 and low levels TGF-β and thus suppresses pathological immune responses[92].

Th9 and Th22. In vitro studies suggest that little to no plasticity occurs between Th1 and Th2 subsets, with cells fixating on a specific lineage shortly after stimulation. However, it has been suggested that plasticity might occur between effector programs that are more closely related. Such examples are the Th9 and Th22 cell lineage. Th9

cells are characterized by IL-9 expression, and so far, lineage-specific transcription factors have not been described. These cells develop when naïve helper T cells are activated in the presence of both IL-4, a Th2 inducing cytokine, and TGF-β, a driver for Tregs and Th17 lineages. It is postulated that in this setting, TGF-β acts as a regulatory "switch" whereby in combination with IL-4 changes the fate of the developing cells from the Th2 into the Th9 lineage[93,94]. Th9 cells are implicated to contribute to anticancer immunity, with IL-9 mediating activation of dendritic cells, mast cells, natural killer cells, and CD8 T cells[95,96].

Similarly, it is not clear whether Th22 cells are a separate lineage, or a derivative of the Th17 cells as they both share the production of the cytokine IL-22. The immune contribution of Th22 cells is not well defined, and it is primarily thought that the function of their main cytokine, IL-22, is context-dependent. For example, in the presence of IL-17, IL-22 plays a proinflammatory role, whereas in the absence of IL-17, it is tissue protective[97–99].

CD8+ T cells

Upon encountering an antigen in the context of MHC I, naïve CD8 T cells differentiation into effector cytotoxic T lymphocytes (CTLs). Naïve CD8 T cells are developmentally pre-programed for clonal expansion and differentiation, and thus upon a brief period of antigen stimulation (2-24hrs) they undergo rapid and robust expansion[100–102]. CTLs are equipped with specialized cytotoxic mechanisms, such as cytokine production and cytolysis to eliminate infected or unhealthy cells. However, effector CTLs are not long-lived; instead, after clonal expansion peaks, effector CTLs enter a contraction phase. During the contraction phase, 90-95% of the cells die via

apoptosis; the remaining 5-10% of effector cells survive as long-lived memory cells. This memory population is of great importance as they can rapidly mobilize their effector mechanisms in response to re-encounter of the cognate antigen[103,104].

T Cell Receptor Signaling

Antigen Recognition

T cells possess clonal identity due to the unique TCRs generated because of genomic DNA rearrangement during development in the thymus. Therefore, naïve T cells use their TCRs as antigen detectors to survey antigenic peptides, complexed with MHC I or II (pMHC), that are novel or have never been experienced before. Initially, it was thought that efficient immune response against foreign antigens was due to the massive number of TCRs, where one clonotype is specific for a single antigenic peptide[105]. This view, based on the clonal selection theory[106], was later abandoned, as it was shown that T cell clones can recognize and respond to alternative peptide/MHC ligands that were significantly different from the cognate antigen that the clones were selected for[107,108].

The human naïve T cell pool is estimated to have $<10^8$ distinct TCRs[109], while the estimated pool of antigenic peptides is $>10^{15}$, based on the 20 amino acids available[110]. Since the number of potential antigens exceeds the number of available TCRs, an effective immune response is only possible if each TCR recognizes more than one peptide. The reactivity of T cells is regulated by their antigen sensitivity, a measure for the activation threshold in response to an antigen. Antigen sensitivity is greatly affected by the TCR affinity for the peptide, the expression levels of the TCR on the cells, and the expression of co-stimulation molecules. Additionally, the co-receptors CD4 and CD8

play a direct role in modulating the T cell response against antigens. In the case of helper T cells, CD4 augments T cell activation mainly by bringing Lck to the TCR signaling complex, but some effects also come from CD4 binding to the TCR-pMHC complex[38,39]. On the other hand, CD8 augments the CTL response by reducing the “off” and enhancing the “on” rate of TCR-pMHC engagement[111–113]. Altogether, even though one TCR can cross-react with multiple antigens, the threshold for activation is affected by several parameters that ensure a proper immune response.

Initiation of TCR Signaling

When the TCR engages with a pMHC, the mechanical information is translated into a chemical reaction by recruiting the Src family kinase Lck to the TCR complex. Lck phosphorylates tyrosine residues within the ITAM of the CD3 zeta chain, which serve as docking sites for the SH2 domains of the Zap70 kinase. Once at the plasma membrane, the autoinhibition of Zap70 is relieved by Lck mediated phosphorylation, and Zap70 can trigger the downstream signaling cascade[114]. How engagement of TCR with pMHC triggers the TCR signaling cascade, or how the TCR distinguishes engagements of different affinity are not well understood, but several different models are proposed.

According to the “kinetic proofreading” model, engagement of a TCR to pMHC is translated into a signal only if the TCR complex is phosphorylated before dissociation occurs. In other words, discrimination between antigens is based on the on- and off-rates or the duration of the TCR-pMHC interaction, with low affinity or non-specific interactions failing to elicit a response because dissociation occurs faster than signal generation[115–117]. The ‘co-receptor scanning’ model extends the “kinetic proofreading” model and suggests that the kinetics of co-receptor mediated delivery of Lck to the TCR

complex is the rate-limiting step that allows for kinetic proofreading to occur. As co-receptors CD4 and CD8 bind to Lck via their cytoplasmic tail, they recruit Lck to the TCR complex, triggering the TCR signaling cascade[118]. However, only a minority of CD4 and CD8 are bound to catalytically active Lck (2% and 0.2% respectively). Thus the “co-receptor scanning” model proposes that in the duration when a TCR is bound to a pMHC, the complex scans several hundreds of co-receptors until it finds an Lck-loaded one. The “co-receptor scanning” model proposes a mechanism for initiation of TCR signaling where the weak agonists, which have shorter half-times of pMHC-TCR interaction, have less time to recruit Lck loaded co-receptors and thus fail to elicit a response[119]. This model is supported by the fact that increased availability of active Lck enhances TCR responsiveness to low affinity pMHC, further suggesting that Lck might be the first necessary step in triggering of response[120].

However, *in vivo* resting T cells have a fraction of Zap70 already bound to phosphorylated ITAM ζ chains, although these Zap70 molecules require Lck mediated activation[121]. So a modification for the “co-receptor scanning” model is proposed, whereby basal levels of active Lck allow continuous ITAM phosphorylation and Zap70 recruitment. Instead, the rate limiting step for activation of the TCR signaling cascade is Lck mediated activation of Zap70, which is only triggered upon longer-lived TCR-pMHC interactions[122]. Another model, “kinetic segregation” suggests that Lck randomly encounters and phosphorylates the TCR complex. Still, phosphatases, such as CD45 which continuously counteract the Lck activity, keep the net phosphorylation of the TCR ζ chain at low levels. However, when a T cell encounters an APC, close-contact zones are formed, where the TCR and other proteins bind to their ligands, while large

molecules are excluded due to their steric hindrance effects[123]. According to the “kinetic segregation” model, as TCR-pMHC interaction leads to the formation of an immunological synapse where bulky molecules, such as CD45, are excluded, the balance between Lck and CD45 is disrupted and now T cell activation can proceed as net phosphorylated TCRs increase[124–126]. Other models suggest that the mechanical forces exerted upon TCR-pMHC interaction trigger and potentiate the TCR signaling cascade. Many proteins undergo conformational changes, which trigger their active state; interestingly, this is not the case for the TCR. Upon TCR-pMHC interactions, noticeable conformational changes only occur within the TCR CDR loops, which interact with the pMHC. However, besides no conformational changes in the distal parts of the TCR, mechanical forces do trigger TCR signaling[127,128], and evidence for a mechanosensitive mechanism of TCR activation has been reported. When the TCR-CD3 complex assembles, the juxtamembrane (JM) region of the ζζ are forced apart from what is proposed to be an “off” conformation; TCR engagement reorients the ζζ JM regions so that they move together into an “on” conformation. These pivot points at the linkage between the TCR and ζζ JM regions are thought to translate the mechanical force of TCR-pMHC engagement into a sequence of biochemical events that lead to T cell activation[129,130]. Because all models discussed are based on experimental observations, it is likely that all or some together can be applied to explain how TCR signaling is initiated.

Proximal TCR Signaling

Because the TCR lacks enzymatic activity, it requires the activities of the tyrosine kinases Lck and Zap70 which turn the extracellular recognition of an antigen into a

biochemical signaling cascade that culminates into T cell activation, proliferation, and differentiation. Lck is a Src family kinase (SFK), and its activity is mainly controlled by a balance between phosphorylation and de-phosphorylation of the inhibitory, Y505, and activating, Y394, residues. Structurally, Lck comprises of Src homology (SH) 2, SH3, kinase domain and an N-terminal cysteine rich motif that allows its association with the co-receptors CD4 and CD8[131]. The C-terminal Src kinase, Csk, phosphorylates Lck on the C-terminal end at Y505 and creates a docking site for the SH2 domain. In this form, Lck is in a closed, or autoinhibited conformation, which is further stabilized by the SH3 domain binding to a polyproline helix region in the linker region[132–134]. Csk activity is counteracted by the plasma-membrane localized tyrosine phosphatase CD45, which dephosphorylates Y505 and generates a pool of Lck in the open or primed conformation[135–137]. Autophosphorylation at Y394 allows for Lck activation and downstream substrate phosphorylation[138]. A pre-existing pool of already active Lck is thought to exist at all times[119,139], but *de novo* activation, mediated by T cell activation, contributes as well[140]. On the other hand, TCR-pMHC interaction is required for Lck-mediated phosphorylation of ITAMs within TCR-associated CD3 and ζ chains. Phosphorylation of both tyrosine residues in the ITAM creates docking sites for the SH2 domain of Zap70. Zap70 is thought to be in an autoinhibited, close conformation in the cytoplasm, and its recruitment to the TCR complex partially relieves that autoinhibition. Lck phosphorylates Zap70 at Y315, Y319, and Y493 which entirely relieves its autoinhibition by promoting an open conformation and full catalytic activation[141]. To sustain its localization to the TCR complex, and enhance the catalytic activity of both kinases, the SH2 domain of Lck binds p-Y319 of Zap70, an interaction critical for T cell

activation[122,142]. Downstream of Lck and Zap70 are the adaptor molecules LAT (the linker for activation of T cells) and SLP-76. LAT and SLP-76 play a critical role in mediating TCR signaling as they serve as nucleating sites for multi-protein complex formation, essential for T cell activation[143,144]. Zap70 phosphorylates LAT and SLP-76 at multiple tyrosine residues, which serve as docking sites for recruitment of downstream signaling molecules essential to trigger signals such as calcium mobilization and MAPK (mitogen-activated protein kinase) activation[145]. Considering that to be fully activated, Zap70 localizes at the cytoplasmic portion of the TCR complex,and that Zap70 and LAT for example are spatially segregated, it is not understood how LAT phosphorylation is maintained during T cell activation. One possible mechanism is by “catch-and-release” which suggests a cycle of recruitment, activation, and dissociation of Zap70 upon TCR stimulation. The dissociated, but active Zap70 diffuses within the plane of the plasma membrane to sites where it can encounter and phosphorylate its substrates, i.e., LAT. The TCR signal is then amplified as the unoccupied sites of the TCR become available for activation of additional Zap70 molecules that go through the same cycle[146]. A caveat for this proposed mechanism is that it can uncouple the TCR signaling cascade and cause a premature end of Zap70 kinase activity via phosphatases or ubiquitin ligases[147,148]. Another model favors the idea of active recruitment of LAT to Zap70, where Lck serves as a molecular bridge. Based on this model, Lck binds to Zap70 using its SH2 domain and binds a conserved proline-rich motif in LAT via its SH3 domain, thus enhancing Zap70 mediated phosphorylation of LAT[149]. LAT phosphorylation is thought to serve as a critical kinetic bottleneck in the propagation of TCR signaling. In fact, among the several tyrosine residues, Y132 is suggested to play the unique role of

discriminating between different ligands and strengths of TCR activation[150]. LAP Y132 is of great importance because it recruits PLC- γ1 and mediates its activation by the Tec family kinase Itk[151]. Activation of PLC- γ1 leads to calcium mobilization and MAPK activation, and eventually T cell response. What makes LAT Y132 so critical in regulating T cell activation is a glycine residue at the G131 position. Zap70 strongly favors tyrosine residues preceded by an acidic residue, such as Aspartic or Glutamic acids, and thus G131 in LAT makes Y132 a poor substrate. G131 slows down phosphorylation of Y132 by Zap70 which perhaps serves as a mechanism for the T cell to discriminate between different signal strengths and appropriately activate PLC-γ1 and the events downstream of it[150].

Distal TCR Signaling

Proximal TCR signaling culminates in the activation of PLC- γ1, an event critical for T cell activation. The two events are bridged together via the adaptor molecule linker for activation of T cells (LAT). LAT is a transmembrane protein that localizes to membrane lipid rafts via posttranslational modifications, and as a substrate of Zap70 links TCR-pMHC engagement to T cell activation[152]. Upon phosphorylation at multiple tyrosine residues, LAT serves as a nucleating site for recruitment of downstream SH-2 containing signaling molecules, notably Grb2, Gads and PLC- γ1. Via the intermediate linker Gads, LAT associates with the src homology (SH)2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76), and together promote downstream TCR signaling by creating a platform for membrane localization and subsequent activation of PLC-γ1[153–155]. Following activation, PLC- γ1 hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) and generates the second messengers inositol 1,4,5-triphosphate

(IP3) and Diacylglycerol (DAG)[156,157]. IP3 and DAG simultaneously activate several signaling pathways, which lead to the activation of transcription factors critical for the induction of T cell activation-related genes. These transcription factors are: Nuclear factor for activated T cells (NF-AT), NF-kB and activator protein-1 (AP-1).

DAG-mediated Signaling Pathways

DAG is a lipid second messenger which in T cells binds to conserved domain 1 (C1) containing proteins[158], such as protein kinase C (PKC) θ and Ras guanyl nucleotide releasing protein (RasGRP1). In response to increased DAG levels, both PCKθ and RasGRP1 translocate to the internal membranes[159,160], triggering signaling pathways critical for T cell activation. RasGRP1 is a guanidine exchange factor (GEF) that links TCR activation to the MAPK signaling pathway by activating the small GTPase protein Ras[161,162]. Even though RasGRP1 plays a dominant role, Ras can also be activated by a second GEF, SOS, which gets recruited to the LAT signaling complex via the adaptor molecule Grb2. Ras triggers the MAPK signaling pathway by sequential activation of Raf-1, a MAPKKK, which then activates MEK, a MAPKK, which in turn activates the MAPK's extracellular signal-regulated kinase 1 (Erk1) and Erk2. Erk activates the transcription factor Elk-1, which regulates Fos expression and thus contributes to the activity of the AP-1 TF, a complex of Jun/Fos proteins. PKC-θ regulates the assembly of the Carma1, Bcl10, and MALT1 (CBM) complex which acts as a signalosome and regulates the activation of NFkB. Upon initiation of TCR signaling, Carma1, and PKC-θ are recruited to the plasma membrane, where PKC-θ phosphorylates Carma1 and releases it from the autoinhibited state. Activated Carma1 induces the assembly of the CBM complex as it recruits Blc10 and MALT1. Even though

the detailed process is not well understood, it is thought that the CBM links to NFkB activation via activation of IkB kinase (IKK) which phosphorylates and targets for degradation of the IkB proteins. Degradation of IkB releases NFkB allowing for their translocation to the nucleus. Besides CBM signalosome mediated activation, NFkB can also be activated by the Akt signaling pathway as well[163,164].

Calcium-dependent Signaling Pathways

PLC-γ1 generated IP3 unleashes calcium mobilization following T cell activation. IP3 binds to IP3R on the endoplasmic reticulum (ER) which stimulates Ca^{2+} release from the ER to the cytoplasm. Depleting ER Ca^{2+} stores activate Ca^{2+} release activated Ca^{2+} (CRAC) channels on the plasma membrane, leading to further influx of Ca^{2+} into the cell cytoplasm. Intracellular Ca^{2+} binds to a small calcium-binding protein, calmodulin. Calmodulin regulates the calmodulin-dependent phosphatase, calcineurin, which in turn de-phosphorylates members of the NFAT family of proteins. Efficient de-phosphorylation of NFAT uncovers a nuclear localization signal that enables NFAT translocation from the cytosol into the nucleus. In the nucleus, NFAT proteins complex with other transcriptional regulators activated in response to TCR, co-stimulation or cytokine signaling. For example, the interaction of NFAT and AP-1, a Ras pathway regulated TF, leads to gene programs necessary for T cell activation and IL-2 production. In contrast, in the absence of AP-1 NFAT complexes with other proteins and can mediate anergic T cell programs[165,166].

T cell Co-signaling

Co-signaling receptors, including co-stimulatory and co-inhibitory, play a pivotal role in regulating T cell activation. The discovery of the proto-type co-stimulatory

receptor CD28[69] provided evidence for a two-signal model of T cell activation where functional T cells are generated only in the presence of both TCR and co-stimulatory signals. In contrast, lack of co-stimulation generates anergic or unresponsive T cells[167]. While TCR signaling is required to initiate the immune response, co-signaling is required to optimize and direct the outcome of the immune response. Because co-signaling can either be co-stimulatory and synergize with TCR signaling or co-inhibitory and antagonizes TCR signaling, tight spatiotemporal regulations exist, with receptor cell surface expression being the primary mode of regulation and differential patterns of receptor-ligand expression being the secondary mode of regulation. To understand the fluidic expression of co-signaling receptors, especially the dynamics between co-stimulatory and co-inhibitory receptors, the concept of the tidal model is proposed[168]. Based on this model, T cell activation is compared to an incoming tide where co-stimulatory receptors synergize with TCR signaling and drive the resting T cell towards a functional state. At peak tide, opposing molecular forces represented by a fine balance between co-expression of co-stimulatory and co-inhibitory receptors pull the T cell into distinct functional responsiveness. As the tide regresses, co-stimulatory receptors are replaced by co-inhibitory receptors, which antagonize TCR signaling and suppress the T cell activation[168]. Altogether, co-signaling receptors play a crucial role in fine-tuning the fate of T cells through regulation of the activation, differentiation, and proliferation of the T cells.

Co-stimulatory Receptors

CD28. CD28 is the prototype and most efficient co-stimulatory receptor required to initiate productive T cell activation upon engagement of the TCR[169,170]. Lack of CD28

signaling during T cell activation leads to T cell apoptosis or anergy, where T cells cannot proliferate or produce cytokines[171]. Unlike other co-stimulatory receptors, CD28 is constitutively expressed on ~80% of human T cells (~95% of CD4 and ~50% of CD8 T cells) and ~100% of mouse T cells[172]. CD28 engagement triggers a distinct signaling cascade than TCR[69]. However, CD28 signaling synergizes and augments the TCR signaling cascade and eventually leads to cytokine production, most significantly IL-2[173], because of increased transcription and stability of mRNA[174]. Furthermore, CD28 signaling leads to increased expression of the anti-apoptotic protein BCL-X_L, a member of the BCL-2 family, and thus promotes the survival of T cells[175,176]. CD28 signaling is thought to increase the sensitivity of T cells to respond to even suboptimal concentrations of antigen. In fact, one study suggests that for a T cell to initiate an antigen-specific response, a threshold of ~8000 TCRs has to be reached, whereas a threshold of ~1500 TCRs needs to be reached in the presence of CD28 co-stimulation[177]. To initiate the co-stimulatory signal transduction, CD28 uses an MYPPPY motif within the extracellular domain for binding to its ligand proteins, B7-1 (CD80) and B7-2 (CD86) expressed mainly on professional antigen-presenting cells[178–180]. CD28 mediates signaling through its short cytoplasmic tail, which lacks an enzymatic function but has several conserved motifs that serve as sites for recruitment of other proteins or sites for modifications that subsequently relay the signaling cascade[181]. However, even though CD28 is considered as the primary co-stimulatory receptor for T cell activation, CD28 alone is not enough to sustain a prolonged T cell response. Instead, the existence of alternative co-stimulatory pathways is required to further amplify and

sustain an effective T cell response and/or drive the development of memory T cell subsets[182].

ICOS. The inducible co-stimulatory molecule (ICOS) is a member of the CD28 family of receptors. Similarly to CD28, ICOS can greatly enhance the activation, proliferation, and effector functions of T cells in response to antigens. Unlike CD28 which is constitutively expressed on almost all T cells, ICOS expression is detected only in activated and memory T cells[183]. ICOS ligand (ICOSL) expression is mainly restricted to professional antigen-presenting cells, such as B cells[184,185], dendritic cells, and macrophages[186,187], but certain endothelial[188] and lung epithelial cells[189] can express it too. The most striking defect of ICOS deficient T cells is a failure to produce IL-4, a hallmark Th2 cytokine. Furthermore, ICOS or ICOSL knock-out mice fail to elicit a humoral immune response against various antigens. These data stress the importance of ICOS in allowing T cells to provide help in antibody production against foreign antigens. However, ICOS deficient mice are also more susceptible to the development of autoimmune inflammatory diseases, such as experimental autoimmune encephalomyelitis (EAE). Altogether, ICOS is a crucial co-stimulatory receptor that drives T cells toward induction of a humoral over inflammatory autoimmune response[190]. Even though ICOS and CD28 have a similar cytoplasmic tail, and mediate partially overlapping signaling cascades, key differences, especially in the PI3K and MAPK signaling, make the functional outcomes of these two co-stimulatory receptors different[191].

4-1BB. 4-1BB (CD137) is an inducible co-stimulatory receptor from the tumor necrosis factor receptor super family (TNFRSF) expressed in response to T cell

activation[192]. 4-1BB can also be expressed on NK cells, B cells, macrophages, monocytes, dendritic cells, and granulocytes[193]. Like other co-stimulatory molecules, activation of 4-1BB signaling results in robust proliferation, cytokine production and survival of T cells[194] 4-1BB is activated by 4-1BB-ligand (4-1BBL), expressed on antigen-presenting cells, such as activated B cells, macrophages, and dendritic cells[195]. Interestingly, even though both CD4 and CD8 T cells express 4-1BB upon activation, CD8 T cells preferentially respond to the proliferative and activating signals upon engagement of 4-1BB. *In vivo* studies have shown that activation of 4-1BB drives the proliferation of cytotoxic T cells in a graft versus host disease model, followed by robust enhancement of IFN-γ and IL-2 production[196]. Furthermore, mice deficient in 4-1BB cannot clear viral infections as their wild-type counterparts due to the lowered number of cytotoxic CD8 T cells, which also have reduced virus-specific cytotoxic response. 4-1BB deficiency does not affect the functionality of CD4 T cells *in vivo*[197]. 4-1BB lacks an enzymatic domain, and thus relies on adaptor proteins to mediate its co-stimulatory signals. Upon ligand binding, TRAF1 and TRAF2 proteins associate with the cytoplasmic tail of 4-1BB leading to the activation of NF-kB signaling pathway[198]. NF-kB signaling relays pro-survival signals by inducing the transcription of the anti-apoptotic genes Bcl-x_L and Bfll-1, in addition to inducing the production of cytokines such as IL-2, IFN-γ and IL-4[199–201]. Furthermore, cytokine production is also supported by the activation of MAPK signaling in response to 4-1BB receptor engagement[202,203].

OX40. OX40 (CD134) is another TNFRSF member which expression is highly dependent on the strength of the TCR signaling[204]. OX40 is only expressed on activated CD4 and CD8 T cells. OX40 activation prolongs the expansion phase and sustains the

effector functions of CD4 T cells, overall promoting the primary CD4 T cell response[205]. In CD8 T cells, OX40 doesn't play a role in the initial proliferation or differentiation into cytotoxic T cells. However, OX40 plays an important role in the effector functions of CD8 T cells, as OX40 deficient CD8 T cells fail to accumulate during an immune response[206]. Furthermore, OX40 plays an important role in memory formation for both CD4 and CD8 T cells. In CD4 T cells, OX40 drives the generation of effector memory populations and contributes to the production of Th1 and Th2 cytokines during recall. In contrast, OX40 doesn't play a role in the generation of central memory populations[207]. In CD8 T cells, OX40 promotes the expression of the anti-apoptotic gene Bcl-x_L and thus controls the survival and differentiation of primed CD8 T cells into memory subtypes[208]. Like 4-1BB, OX40 also signals through TRAF adaptor proteins (TRAF2 and TRAF5) to activate NF-kB dependent gene regulation[209].

Co-Inhibitory Receptors

CTLA-4. Identified in a murine cytolytic T cell cDNA library, CTLA-4 was initially thought to play a role in the cytotoxic response of T cells[210]. However, later it was found that CTLA-4 is a negative regulator of T cell activation, upregulated only after activation of conventional T cells but constitutively expressed by regulatory T cells. For its activation, CTLA-4 competes with the co-stimulatory receptor CD28 for binding to B7 ligands, B7-1 and B7-2[211] , but with much higher affinity[212–214]. Genetic deletion of CTLA-4 in mice further showed the importance of this inhibitory receptor, as mice deficient in CTLA-4 develop lethal autoimmune disease characterized with multiorgan inflammation due to massive lymphoproliferation and tissue destruction, followed by death by 3-4 weeks of age[215,216]. Deletion of CTLA-4 during adulthood also leads to

rapid immune activation, multiorgan inflammation and auto-antibody production; however, the disease severity is not lethal, implicating a role for the developmental stage of the organism. Interestingly, deletion of CTLA-4 during adulthood leads to an enhanced expression for other immunosuppressive molecules, such as IL-10, LAG-3, and PD-1, suggesting that compensatory inhibitory mechanisms might also account for the discrepancy between adult and congenital CTLA-4 deficiency[217,218]. One of the major mechanisms by which CTLA-4 regulates T cell activation is signaling independent and involves counteracting CD28 costimulatory signals by outcompeting for B7 ligands (B7-1 and B7-2) binding, along with active removal of these ligands from the surface of APCs[219]. Molecularly, CTLA-4 inactivates T cells by binding to TCRζ chain and reducing its phosphorylation via binding to the phosphatase SHP-2[220]. Furthermore, CTLA-4 can also inactivate Akt signaling via the serine/threonine phosphatase PP2A[221]. Another mechanism by which CTLA-4 regulates T cell activation is through the control of natural Treg cell functions. Treg-specific KO of CTLA-4 results in loss of Treg suppressive functions which subsequently leads to systemic lymphoproliferation of effector T cells and fatal autoimmunity on the one hand and a potent T cell-mediated tumor immunity on the other hand[222]. However, rather than serving as an inhibitory receptor that attenuates T cell activation only, CTLA-4 also provides negative co-stimulation that limits the range of phenotypes that T cells can differentiate into. In other words, CTLA-4 constrains T cells within specific phenotypes, as loss of CTLA-4 leads to the appearance of T cell phenotypes not observed in wild type counterparts. Furthermore, because the loss of CTLA-4 also leads to an increase in TCR clonality, it is hypothesized that CTLA-4 might serve as a restriction for tonic (i.e., promiscuous)

activation of T cells by raising the threshold for activation and thus preventing the expansion of self-reactive (i.e., promiscuous) T cell clones[223].

PD-1. The first description of the programmed death-1 (PD-1) gene suggests that PD-1 is a programmed cell death-specific inducer whose expression is increased when thymocyte cell death is induced by activation[224]. However, several years later, it was shown that PD-1 was transiently induced upon T cell stimulation through the TCR, but rather than being involved in the induction of programmed cell death, PD-1 acts as a negative regulator of T cells responses instead[225]. PD-1 is thought to play a critical role for maintaining peripheral tolerance, as deficiency of PD-1 leads to autoimmunity characterized with late-onset lupus-like proliferative arthritis and mild glomerulonephritis on the C57BL/6 background, and lethal autoimmune cardiomyopathy on the BALB/c background[226,227]. The severity of the autoimmune response is enhanced with age and is highly dependent on the genetic background of the mice. PD-1 signaling is initiated upon binding to its ligands, PDL-1 – widely expressed by both hematopoietic cells, such as T cells, B cells, DCs and macrophages, and nonhematopoietic cells, and PDL-2 – which expression is mainly restricted to antigen-presenting cells, such as B cells, DCs and macrophages. Not only is PD-1 expression inducible, but so is the expression of its ligand PDL-1 and PDL-2. While both ligands can be expressed on resting cells, their expression is robustly upregulated in response to inflammatory signals driven by cytokines produced by infiltrating T cells. This highlights the importance of PD-1 as a negative feedback mechanism to dampen ongoing T cell responses by inhibiting T cell proliferation and cytokine production. Even though PD-1 is a member of the CD28/CTLA-4/ICOS receptor family, it shares only ~20% sequence homology with the

other members, mainly in its extracellular domain, but has distinct features especially in the cytoplasmic domain[228]. PD-1's cytoplasmic tail contains two critical tyrosine residues located in the highly conserved immunoreceptor tyrosine-based motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM)[229]. The arrangement of the cytoplasmic tail of PD-1, and the spacing between the ITIM and ITSM is in direct resemblance to the CD33-related Siglec family of receptors expressed on most innate immune cells. Like PD-1, Siglec receptors also deliver negative signals and dampen the immune cell activation in an ITIM/ITSM dependent manner[230]. Engagement of the PD-1 receptor leads to phosphorylation of both tyrosine residues, in the ITIM and ITSM, mainly by Lck and/or Src kinases in T cells[231,232]. The phosphorylated tyrosine residues serve as docking sites for the recruitment of SH2 domain-containing phosphatases SHP2 and SHP1[229,233]. SHP recruitment then leads to inhibition of T cell receptor-induced phosphorylation of TCRζ/ZAP70 and downstream PKC-θ signaling[231,234], as well as PI3K/Akt signaling following CD28 activation[221], resulting in decreased proliferation and cytokine production. However, even though PD1 can inhibit both TCR and CD28 signaling cascades, quantitative studies show that CD28 is the preferred target over TCR for PD-1[232]. PD-1 is not expressed on naïve or resting memory T cells and is only upregulated upon antigen-driven stimulation via the TCR. Upon removal of the antigen, i.e., acute clearing, the levels of PD-1 decrease. However, if the presence of the antigen is prolonged, such as during chronic infections or cancer, PD-1 expression is sustained. Transient PD-1 expression, and the subsequent dampening of the T cell response serve as a major immunoregulatory mechanism that limits tissue-related immunopathology. On the other hand, sustained expression of PD-1 drives T

cell exhaustion and results in an inadequate immune response due to dysfunctional T cells. Notably, disrupting the PD-1 signaling axis, either by blocking PD-1 or its ligands, restores the ability of T cells to proliferate, secrete cytokines and kill altered or infected cells[235]. Targeting the PD-1 signaling axis is a promising immunotherapy strategy for treating chronic viral infections, such as HIV and HCV[236,237]. In the tumor microenvironment, T cells have sustained PD-1 expression due to chronic exposure to tumor antigens and PD-L1 upregulation on many cancer cells due to cancer cell-intrinsic or inflammation-mediated stimuli[238]. Targeting this inhibitory axis has proven as a successful immunotherapy in the treatment of several different cancers.

Section I

Checkpoint receptors play an important role in regulating the activation and effector functions of T cells. Activating checkpoint receptors sustain and enhance the effector functions of T cells. In contrast, inhibitory checkpoint receptors suppress and limit the effector functions of T cells. Because of their potent immunomodulatory functions, activating and inhibitory receptors can serve as targets for the development of immune-based therapies. Blockade of inhibitory checkpoint receptors, such as PD-1 and CTLA-4, have already proven successful in reinvigorating the effector functions of tumor infiltrating lymphocytes. However, such therapies are only successful in a very small fraction of patients due to development of resistance or activation of redundant inhibitory mechanisms. Identification of novel checkpoint receptors is imperative to allow for the development of successful combination treatments that can target multiple pathways and result in long term effective therapies.

Sialic Acids

For a long time, the major macroconstituents of the cell were assumed to be nucleic acids, proteins, and lipids. One major class of cellular components that was disregarded, but is gaining much attention nowadays, is sugar chains or glycans. There is a whole field of glycobiology now that focuses on understanding glycans' molecular, cellular, and physiological properties. It was initially thought that glycosylation happens only on molecules secreted on the extracellular layer of cell membranes. However, this dogma was proven wrong; instead, it was shown that many cytosolic and nuclear proteins are glycosylated as well[239,240]. Defects in genes involved in efficient and precise assembly or modification of glycans within consensus peptide sequences on proteins have broadly been categorized as congenital disorders of glycosylation. This group of disorders emphasizes the essential biological roles that glycosylation plays in the normal cellular functions[241]. In general, glycans' functions can be subdivided into structural and modulatory functions or the specific recognition of glycans by glycan-binding proteins and molecular mimicry of host glycans. Glycans in the cell often covalently bind with other macromolecules that make up the cells, more commonly known as glycoconjugates, either glycoproteins or glycolipids. The size of the glycan in the glycoconjugate can vary greatly, but often it is a substantial portion of the overall mass of the molecule. This results in a very dense array of sugars, generally called the glycocalyx. What makes glycans much more complicated than proteins is that unlike amino acids which form one primary type of linkage in the process of generating a polypeptide, the building blocks of glycans, monosaccharides, can generate either an

alpha or beta linkage to different positions of other monosaccharides in a chain, or molecules of other type[242].

Sialic acids are derivatives of the nine-carbon sugar neuraminic acid, usually attached to the terminal position of oligosaccharide chains on glycoconjugates. Some of the special structural features that make neuraminic acid derivatives diverse are the amine group found at position 5 and the carboxylic group found at position 1. These functional groups are negatively charged under physiological conditions, making neuraminic acid a strong organic acid and thus highly reactive. Neuraminic acid without any substitutions of the functional groups does not exist. Most frequently, the amino group is acetylated, giving rise to the most widespread form of sialic acids, N-acetylneuraminic acid (Neu5Ac). Further substitution of a hydrogen atom in the methyl residue of the acyl group with a hydroxyl group gives rise to another widespread sialic acid form N-glycosylneuraminic acid (Neu5Gc). However, Neu5Gc is not found in humans, except in pathologies, such as certain cancers. Furthermore, the hydroxyl groups at positions 7, 8 and 9 are subject to esterification reactions with acetic acids, which further contribute to the diversity of sialic acids. Other modifications that neuraminic acid can undergo include adding lactoyl groups at position 9, sulfate or methyl groups at position 8. It is important to mention that the modifications described so far can happen in more than one position simultaneously. The variability of sialic acids is further complemented by the type of linkage that attaches it to the underlaying glycan. Most frequently, sialic acids are attached to the position 3 or 6 of the penultimate sugar of a glycan chain via the C2, or position 8 of another sialic acid. The

expression of sialic acid derivatives depends on the tissue and developmental level of the species that express them[243–245].

Sialic acids are not uniformly distributed among all species; instead, they are found in higher vertebrates, such as echinoderms, and vertebrates. An exception are some protozoa, viruses, and bacteria. Generally, sialic acids are expressed as part of glycoconjugates on cell surfaces and intracellular membranes but can also be found as a part of the serum or mucus in higher animals. The characteristic biological structure and their unique distribution enable sialic acids to participate and fulfil important roles in maintaining the normal homeostasis of the organism. For example, being on the terminal end of glycoconjugates, sialic acids protect the underlying glycans and proteins from proteases in the case of pathogen invasion. The fact that Sialic acids are densely expressed on both the outer layer of cells and the interior of lysosomal compartments speaks of the vital role they play in stabilizing molecules expressed on the cell membranes and in modulating interactions with the cell surroundings. Sialic acids are negatively charged molecules, a property that makes them an important part of transporting ions and drugs, stabilizing the conformation of proteins, and increasing the viscosity of mucin layers which prevents the entry of pathogens[246,247].

Sialic Acids Promote Tolerance to Self by Activating Siglec Receptors

One of the most prominent characteristics of the immune system is its ability to recognize self against non-self or abnormal self. Carbohydrates can influence the immune system in two ways: by regulating the conformation and biological activity of their conjugates and by serving as recognition sites for different immune cell receptors.

As terminal moieties of glyacns, sialic acids are important recognition sites for a family of receptors known as sialic acid-binding immunoglobulin lectins (Siglecs). Siglec family members mediate inhibitory or activating signals upon binding to sialic acids expressed on normal tissues or antigens. The interaction between siglecs and sialic acids is one mechanism by which immune cells can mediate tolerance towards self and react against unhealthy or foreign antigens. For example, de-sialyation of cellular glycoconjugates or the lack of sialic acid expression on most microbes or virally infected and transformed cells can serve as a signal for missing or abnormal self. Subsequently this signal can direct immune cells to phagocytose or kill such cells and microbes.

Furthermore, sialic acids can also play a role in increasing the threshold for activation of certain cells[246–248]. For example, the sialic acid receptor Siglec-2, or CD22, counteracts the activating signals of the B cell receptor. It is postulated that by blocking BCR signaling, CD22 helps to prevent induction of unwanted antibody response against self-antigens[249]. Lastly, sialic acids also regulate the activation of the alternate complement cascade by binding to a protein called factor H. When factor H binds to sialic acids on normal cells, it prevents the formation of the C3 convertase and thus prevents complement activation[250,251].

Siglecs - Sialic Acid-Binding Immunoglobulin Superfamily Lectins

Siglecs are a family of immunoglobulin-type lectins that mediate protein-carbohydrate interactions specifically via sialic acids attached to glycoproteins or glycolipids[252–254]. Siglecs are single pass type I transmembrane proteins that share a high level of structural similarity. All Siglecs share a characteristic extracellular structure that contains one V-set domain, followed by a variable number of C2-set domains[253,255].

A conserved arginine residue within the V-set domain forms a salt bridge with the ionized carboxyl group of sialic acids, and thus is an essential part of the receptor-ligand binding[256–259]. Hydrophobic interactions between aromatic amino acids from the β-strands of the V-set domain with the N-acetyl and hydroxyl functional groups of Sialic acids also form important interactions that strengthen the receptor-ligand binding[257]. Furthermore, there is an unusual arrangement of cysteine residues in the V-set and adjacent C2-set domains that leads to the formation of an intra-sheet disulfide bond in the V-set as well as a disulfide bond between the two domains[260].

Most Siglecs mediate inhibitory signaling events and thus prevent immune cell activation. The inhibitory signals are mainly mediated by the characteristic cytoplasmic tail of SIglec receptors, consisting of conserved intracytoplasmic immune-receptor tyrosine-based inhibitory motifs (ITIM), defined by the consensus sequence I/LVxYxxL/V (x can be any amino acid)[261] and an immune-receptor tyrosine-based switch motif (ITSM), defined by the consensus sequence TIYxxI/V[262–264]. Some Siglecs, such as human Siglec 10, contain the consensus sequence YL/V/LNV/P, which binds the adaptor protein Grb2 and participates in the Ras signaling cascade[265]. Other Siglecs, such as Siglec 14, 15 and 16, lack a cytoplasmic signaling domain and instead pair up with the adaptor protein DAP12 via a positively charged lysine residue in the transmembrane region which have immune receptor tyrosine-based activating motifs (ITAMs), allowing them to participate in directly activating or enhancing signaling cascades[266–268].

Based on the evolutionary conservation among species, the Siglec family of receptors can be divided into two sub-groups. Siglecs common to all species are Sialo-

adhesin, or Siglec-1, CD22, or Siglec-2, and MAG, or Siglec-4a. In contrast, the CD33-related Siglecs are species-specific, rapidly evolving and include Siglec-3 and Siglec-5-11 and -14-16 in humans, and Siglec-3 and E-H in rodents. Among the CD33-related Siglecs, all but Siglec-14, -15 and -16, are characterized by having the conserved ITIMs in the cytoplasmic tails[266]. Sequence comparisons of the CD33rSiglecs in multiple mammalian species show that these Siglec genes have undergone rapid evolution by gene duplication, deletion and chimerism, as well as the selection of sequence domains that recognize only certain glycan structures[269].

Each Siglec is unique in that they have strong preferences to bind sialic acids based on the nature of the sialic acid, the type of glycosidic linkage, as well as the underlying glycan. Siglecs are mainly expressed on hematopoietic and immune cells. Most human and mouse immune cells express at least one type of Siglec, with others expressing multiple. Even though some Siglecs show unique cell expression, most have overlapping distribution across leucocytes, suggesting that they have specific and non-overlapping functional properties. Because each Siglec shows specificity for a unique pattern of sialylation, it is likely that each receptor has a unique function. Little to no expression has been reported for resting T lymphocytes of healthy humans, an exception being Siglec-7 and -9, which can be found in a small population of CD8+ T cells and mediate direct inhibition of TCR signaling[270,271]. Transfection studies of Siglec-7 and -9 into Jurkat T cells shows direct inhibition of T cell receptor signaling, suggesting that Siglec expression in T cells might regulate the cell activation[272]. In some pathologies though, Siglec expression in the T cell compartment can be altered. For example, a recent study shows that tumor-infiltrating lymphocytes from melanoma

patients have high expression of Siglec-9. The expression of Siglec-9 was inversely correlated with the clinical outcome and prognosis of disease[273]. Furthermore, HIV-infected patients have a greater frequency of circulating CD4+ T cells expressing Siglec-5 and -9, in contrast to healthy patients[274].

Siglec-5

Siglec-5 structure. Siglec-5 was cloned in 1998 by Paul R. Crocker's group from a human macrophage cDNA library. The newly discovered gene showed remarkable similarity with the human CD33 cDNA. Using in situ hybridization, the gene for Siglec-5 was mapped to chromosome 19q13.41-q13.43, a region where many other Ig superfamily members are also localized[275]. Siglec-5 is a 551 amino acids long type-1 transmembrane protein that belongs to the Immunoglobulin superfamily. Extracellularly, Siglec-5 comprises of a N-terminal V-set domain followed by three C2-set domains. Within the extracellular sequence, Siglec-5 carries eight potential N-linked glycosylation sites. It is hypothesized that glycosylation regulates Siglec-5 ability to discriminate sialic acids[276]. Like the other members of the Siglec family, the unique pattern of cysteine residues within the V-set domain is also conserved in Siglec-5. More specifically, the unusual intra-sheet disulfide bond between the B-E strands of the V-set domain, as well as an interdomain disulfide bond between Cys41 from the B strand of the V-set to Cys175 from the B-C loop of the C2-set domain are present in the Siglec-5 structure[260,277,278]. While this interdomain linkage is not directly involved in ligand binding, it is thought to impose flexibility limitations of the carbohydrate-binding domain. Siglec-5 has a single transmembrane domain that links the 4 extracellular Ig-like domains to an 89 amino acids long cytoplasmic tail that has two tyrosine-based

conserved motifs. The first motif, LHYAS/VL, is homologous to the consensus sequence for the immunoreceptor tyrosine-based inhibitory motif (ITIM), I/L/VxYxxL/V. The second motif, TEYSEI/V, resembles an immunoreceptor tyrosine-based switch motif (ITSM) that is also described in some immune cell proteins. Molecular characterizations of Siglec-5 showed that the protein exists as a homodimer on cell surfaces[277].

Siglec-5 ligands. Most Siglecs show a preference for one glycosidic linkages of sialic acids, but Siglec-5 is more promiscuous and binds equally well to both α2,3- and α2,6-linked, and to a lesser degree α2,8-linked sialic acids[277–279]. As reported for other Siglecs, the conserved Arginine residue in the G strand in the V-set domain plays a critical role in Sialic acid recognition; the Arginine binds the carboxyl functional group and neutralizes the negative charge of the Sialic acid, whereas the G strand forms a beta sheet-like hydrogen bonds with the glycerol side chains of Sialic acids. The reason for the variability of Siglecs recognizing different Sialic acid linkages arises from the interaction of Sialic acids with the highly different residues found in the GG' and CC' regions of the V-set domains[278].

A study by Rie Suematsu *et al.* identifies lipophilic ligands specific for Siglec-5, in addition to sialic acids. Here the authors show that Siglec-5 binds to lipids compounds, such as alkanes and triacylglycerols, made by the fungal species *Trichophyton*. Further, they identify several endogenous lipid-based ligands, among which most prominent are the mitochondrial lipid cardiolipin and the anti-inflammatory lipid 5-palmitic acid-hydroxysteric acid. Interestingly, lipid binding by Siglec-5 requires the hydrophobic N-terminal stretch, which is dispensable for Sialic acid binding[280].

Several protein ligands of Siglec-5 are also described in the literature. β-protein expressed in Group B Streptococcus[281,282], and Hsp70[283] mediate immunomodulatory effects via binding to Siglec-5.

Siglec-5 isoforms. A unique characteristic of Siglec-5 is that it has isoforms produced by alternative splicing of the extracellular and intracellular sequence. The original Siglec-5 sequence cloned by Paul Crocker's group is designated as the full length, or hSiglec-5 4-L, where 4 stands for having four Ig set domains, and L stands for the full-length of the cytoplasmic tail, including the ITIM and ITSM. The first isoform is identical to the Siglec-5 4-L in its extracellular sequence; however, it has a truncated cytoplasmic tauk of 38 amino acids which lacks the ITIM and ITIM-like motifs. This isoform was named Siglec-5 4-S, where S designates the short cytoplasmic tail. The second isoform has a variation in the extracellular domain and lacks one of the three C2-set domains. This isoform is designated Siglec-5 3-L. The third isoform has an un-spliced intron between the second and third C2-set domains, which introduces a premature stop codon in the extracellular sequence of Siglec-5. This isoform is predicted to encode a soluble truncated protein that has the extracellular sequence of Siglec-5 and is designated hSiglec-5-3C[284].

Tissue distribution. Tissue distribution of the full-length Siglec-5, and its Siglec-5-4S isoform, is mainly confined in immune tissues, such as bone marrow, spleen and peripheral blood, but at lower levels can also be found also in the lymph nodes, appendix, placenta, lung, thymus, pancreas, and fetal liver[277,284]. The variants Siglec-5-3L and Siglec-5-3C are also detected in immune-related tissues, but at much lower levels[284]. The functional significance of the different Siglec-5 isoforms is not clear, but

perhaps the differential tissue distribution might influence their function. Among leucocytes, Siglec-5 is expressed on monocytes, neutrophils, dendritic cells, subsets of tissue macrophages, and B cells, but not T cells or NK cells[277,285].

Function. Siglec-5 plays diverse physiological roles related to 1) signaling and recognizing pathogens and self-associated signals, 2) cell-cell interactions and 3) endocytosis of ligands.

Signaling and recognition of pathogens. Siglec-5 is an inhibitory receptor that blocks activating signals in innate immune cells, monocytes, neutrophils, and macrophages specifically. Engagement of Siglec-5 with its ligands results in the recruitment of tyrosine phosphatases SHP-1 and SHP-2 to the ITIM and ITSM within its cytoplasmic tail. Using transfected rat basophil leukemia (RBL) cells Avril, T. *et al.* first showed that the recruitment of SHP-1 and SHP-2 results in inhibitory signals leading to reduced calcium flux and serotonin release after co-ligation of the activating FCεRI receptor. Interestingly, inhibitory signaling events following Siglec-5 receptor engagement occur even in the absence of phosphorylation of the tyrosine residues within the ITIM and ITSM motifs. These observations suggest several hypotheses that need further testing: 1) that the mechanism by which Siglec-5 initiates inhibitory signals is independent of the ITIM/ITSM and protein-tyrosine phosphatase pathway; and 2) that weak interactions between SHP-1 and SHP-2 occur even with non-phosphorylated ITIM and ITSM motifs within Siglec-5, and are sufficient to trigger inhibitory signals via the phosphatase activity[286].

Most functional studies of the mechanism of Siglec-5 activation and signaling, as well as the biological outcomes, come from the assessment of Siglec-5 contribution to

bacterial pathogenesis. Pathogenic bacteria have evolved to use sialic acids as a molecular mimicry mechanism to avoid immune cell activation, by either escaping recognition, or activating of inhibitory immune cell receptors from the Siglec family. Several human pathogens, such as *Neisseria meningitidis*, *Campulobacter jejuni* and Group B *streptococci* (GBS) have been shown to engage Siglec-5, among other members of the Siglec family. These pathogens engage the SIglec-5 receptor mainly in a sialic acid dependent manner in order to suppress the activation of immune cells [287–289].

Interestingly, certain strains of GBS engage Siglec-5 in a Sialic acid-independent manner and instead use protein/protein interactions via the surface β-protein. Upon binding to β-protein, Siglec-5 accumulates at the point of contact with GBS and triggers a signaling cascade that begins with phosphorylation of the cytoplasmic tail and subsequent recruitment of SHP protein tyrosine phosphatases, SHP-1, and SHP-2. These events result in resistance to bacterial killing and phagocytosis by leucocytes. In neutrophils specifically, these events lead to impaired innate immune functions, such as weaker oxidative burst, decreased neutrophil extracellular traps release, and reduced production of cytokines such as IL-8[281,282]. Furthermore, even though GBS attaches on phagocytic cells the bacteria are not phagocytosed. It is thought that the recruitment of SHP1 and/or 2 to the cytoplasmic tail of Siglec-5 negatively regulates the phagocytosis pathway as well[290]. The role of SHP1 in the regulation of Fc-γ receptor-mediated phagocytosis has been well established, where SHP-1 is implicated in a direct blockade of phagocytosis activating signals mediated by the SRC family kinase Syk, the ubiquitin ligase Cbl, phosphatidyl inositol-3 (PI-3) kinase, and Rac[291]. Even though SHP-2 is the

phosphatase to be recruited more specifically after Siglec-5 activation, it is possible that in this scenario SHP-2 undertakes a similar signaling pathway as previously described for SHP-1 to block pathogen phagocytosis.

Signaling and recognition of self-associated signals. Several reports now show that Siglec-5 acts as a receptor for self-associated signals and thus regulates the inflammatory response. The heat-shock protein 70 kDa (Hsp70), a known intracellular chaperon, is described to modulate the immune response by engaging Siglec-5. It was shown that Siglec-5 interacts with intracellular Hsp70 from cell lysates as well as secreted Hsp70 from activated monocytic cell lines. Furthermore, Hsp70 also suppresses the inflammatory response of monocytic cells via activation of Siglec-5. Using Siglec-5 expressing THP1, a monocytic cell line, it was shown that LPS stimulation in the presence of Hsp70 leads to a decrease in inflammatory cytokine production, such as TNF-α and IL-8[283]. Other self-associated signals that suppress the immune response via binding to Siglec-5 are the mitochondrial protein cardiolipin and the endogenous lipids PAHSAs. Using reporter assays, these studies have shown that engagement of Siglec-5 by these lipophilic ligands contributes to an anti-inflammatory response mediated by innate immune cells such as monocytes[280].

Endocytosis of ligands. Like most CD33-related Siglecs, Siiglec-5 also serves as an endocytic receptor that clears sialylated antigens which could result in either enhancing or suppressing antigen presentation. While not completely understood, it is predicted that the endocytic functions of Siglec-5 are regulated via its tyrosine-based internalization signals[292] within the ITIM and ITSM. For example, sialylated *N. meningitidis* is internalized in a Siglec-5 dependent manner. However, whether uptake

of the bacteria is beneficial for clearance, or the internalization route simply allows for bacteria to survive and translocate within the phagocytes remains to be explored[289]. Another example of Siglec-5 mediated endocytosis is the internalization of coagulation factor VIII (FVIII) and von Willebrand factor (VWF), essential proteins for blood clotting. Clearance, and thus regulation of plasma levels of FVIII/VWF complexes depends on Siglec-5 mediated uptake, in addition to other well-recognized receptors such as LDL-receptor related protein-1, LDL-receptor and asialoglycoprotein[293]. More specifically, overexpression of Siglec-5 in cell lines, such as HEK293t, results in uptake of FVIII/VWF complex. *In vivo* studies using overexpression of Siglec-5 in murine hepatocytes also show the downregulation of FVIII/VWF circulating levels[294].

Cell-cell interactions. Cell-cell interactions refer to the interaction of molecules, ligands, and receptors on the surface of cells. This represents a mechanism of communication that cells use to respond to environmental signals. Some cell-cell interactions are permanent, such as the formation of tight junctions between epithelial cells; others are transient, such as immune cells being recruited to an inflammatory site. The migration of cells from one site to another is tightly regulated by the selective expression of adhesion and signaling molecules within the cells of the vascular systems. Different members of the selectin family of adhesion proteins mediate the tethering, rolling and extravasation of leukocytes. These processes are enabled by interactions of selectins with the counter-receptor carbohydrate ligands expressed on the surface of leucocytes. Siglec-5 regulates cell-cell interactions by binding to P-selectin glycoprotein-1 (PSLG-1). PSLG-1 is a transmembrane protein that plays a crucial role in the initial recruitment and rolling by interacting with P and E selectins

expressed on endothelial cells lining up the vasculature. Importantly, PSLG-1 is heavily glycosylated and the sialic acids expressed on the glycan termini are crucial for selectin interactions[295,296]. Siglec-5 directly binds to PSLG-1 in a sialic acid-dependent manner; *in vivo* studies confirm that soluble Siglec-5 reduces rolling and prevents recruitment of leukocytes at a sterile inflammation site[297]. It is possible that Siglec-5 binds to other P-selectin ligands as well, but more studies are needed to confirm this hypothesis. From a physiological standpoint, it is possible that Siglec-5 binds P selectin PSLG-1 to regulate excessive leukocyte rolling under homeostatic conditions. It is known that Siglecs interact with sialic acid-containing glycoconjugates under resting conditions and unmask such proteins or lipids upon cell stimulations. Thus, it could be that Siglec-5 binds to PSLG-1 or other P- and E-selectin ligands under resting leukocyte states but release the ligands upon stimulation to enhance leukocyte rolling. Furthermore, healthy human plasma contains about 75ng/ml of the soluble isoform of Siglec-5, Siglec-5-3S, but can increase 2-3-fold under pathological conditions, such as acute myeloid leukemia[298] or sepsis[299]. This could also suggest that increased levels of soluble Siglec-5 can contribute to pathologies by blocking leukocyte migration towards sites of infection.

In addition to regulating leucocyte rolling, soluble Siglec-5 binds and activates PSGL1 expressed on activated CD8 T cells. Subsequently, activation of PSGL-1 manifests with its well-known immune-checkpoint roles that promote CD8 T cell suppression and exhaustion. In a scenario such as sepsis, where soluble Siglec-5 levels are significantly increased, this could provide a mechanism for both reduced recruitment and functionality of leucocytes that leads to reduced pathogen clearance[299–301].

Siglec-5 and -14 – a Paired Receptor System

Siglec-14, another member of the CD33-related subgroup of Siglecs, has nearly identical ligand-binding domain with Siglec-5 but lacks the cytoplasmic signaling tail. Instead, Siglec-14 pairs with the adaptor protein DAP12 and mediates activation signals and is thought to be the pairing, activating receptor for Siglec-5[266]. Interestingly certain people lack Siglec-14 due to a fusion between the Siglec-14 and Siglec-5 genes, which gives rise to a new gene, Siglec-14/5. This fusion gene is identical to the coding sequence of Siglec-5; however, its expression is controlled under the Siglec-14 promoter. So there is a polymorphism within the human population, where people have both Siglec-5 and Siglec-14, or lack one or both alleles of Siglec-14 as it is replaced with the fusion gene Siglec-14/5[302]. Being an activating receptor, Siglec-14 enhances the responsiveness of immune cells to bacterial pathogens, and thus Siglec-14 polymorphism is advantageous for microbial survival and makes the host more susceptible to infections. The presence of pairing inhibitory and activating receptors in immune cells is not well understood but is thought to be a mechanism to fine-tune the immune response. Protein expression of Siglec-5 and Siglec-14 overlaps partially, and subpopulations of monocytes and neutrophils expressing one or the other exist as well. The fact that Siglec-14 counteracts the inhibitory effects of Siglec-5 in both monocytes and neutrophils is supporting evidence that the two are pairing receptors. In monocytes, inflammatory activation with LPS increases Siglec-14 mRNA levels at the expense of downregulating Siglec-5 mRNA. We can speculate that regulation at the level of receptor expression level maintains the balance between Siglec-5 and Siglec-14 activity, but further evidence is needed to prove it[303]. Evolutionarily, we can also

speculate that Siglec-14 emerged to compete with Siglec-5 binding ligands that generally would suppress the immune responses, thus balancing out the innate immune response to pathogens carrying such ligands.

However, Siglec-5 and Siglec-14 have unique sequences within their extracellular domains which might influence some of their ligand binding affinities or preferences. For example, a new study shows that Siglec-14 uniquely binds to sialylated glyco-RNA molecules expressed on cell surfaces, while Siglec-5 does not[304]. These observations suggest that even though Siglec-5 and Siglec-14 act as paired receptors and mediate opposing responses to the same ligand, they also have unique functions independent of each other.

Section II

Where, when, and how latent TGF-β is activated is not well understood. Several studies suggest that active TGF-β is expressed on cell surfaces[329,339]. However, the sequence of active TGF-β lacks a cell-binding domain, raising the question of how it is anchored to cell surfaces. Although several anti-TGF-β antibodies are commercially available, detection of cell surface-bound active TGF-β remains challenging and is not well documented. The most widely used, 1D11 antibody clone serves well for neutralizing TGF-β, but not for immunofluorescent detection purposes. Altogether, this technical hurdle leaves a knowledge gap in the TGF-β field.

Transforming Growth Factor-β (TGF-β)

In 1979, de Larco and Todaro described the presence of growth factors in supernatant fluids of sarcoma transformed mouse 3T3 cells that cause normal fibroblasts to become transformed by growing anchorage-independent colonies in soft

agar. These factors, termed transforming growth factors, were described to act as proximal effectors of transformation, as their removal reversed the phenotype[305]. By 1981, the transforming factor - β was purified and characterized by the teams of Harold Moses, and Michael Sporn and Anita Roberts[306,307]. Initially thought to be only produced by neoplastic cells, TGF-β than was also isolated from normal, non-neoplastic cells[308]. By 1982, Joan Massague provided evidence for a high-affinity receptor for TGF-β [309]. Using collaborative effort, it was established that there are three TGF-β receptors, now known as type I, type II and type III - a co-receptor also known as β glycan[310,311]. Unlike other described growth factors at that time, TGF-β was the first multifunctional growth factor. In addition to stimulating growth and transformation, TGF-β also inhibited the proliferation of primary and secondary cell cultures. Furthermore, TGF-β also induced cellular differentiation in some cell types while blocking it in others. Thus, it was apparent that the function of TGF-β must be evaluated based on the context of other signals present.

Latent TGF-β

TGF-β is secreted from cells in a biologically latent form as a part of high molecular weight protein complex that has to be activated to perform its biological effects[312]. Because the receptors for TGF-β are ubiquitously expressed, this is an important biological property, as it limits the cellular targets of TGF-β to those cells that can either directly or indirectly activate the latent form. Active TGF-β is a 25kDa homodimer linked by Cys77 disulfide bonds and derived by proteolytic cleavages of a larger precursor polypeptide[313,314]. Upon synthesis of the pre-pro-protein in the endoplasmic reticulum (ER) the signal peptide of the sequence is quickly removed, generating a pro-TGF-β

that undergoes N-glycosylation at predicted sites. Pro-TGF-β then translocates into the Golgi apparatus where it is processed by proteolytic digestion. Furin, an endopeptidase, cleaves the pro-protein at a consensus motif comprising of R-H-R-R sequences found at the junction between the pro-region and immediately before the N-terminal Ala residue of the mature growth factor[315]. This proteolytic event leads to two products, one a dimer of the N-terminal region, called latency-associated peptide (LAP), and two, a dimer of the C-terminal portion, the mature TGF-β. Despite the cleavage, the pro-region of TGF-β is essential for the folding and assembly of the mature dimer. More specifically, Cys 223 and 225 mediate disulfide linkage and assembly of dimeric LAP, which then non-covalently associates with the mature TGF-β dimer and generates the small latency complex (SLC)[316–318]. When part of the latent complex, TGF-β cannot bind to its receptors and thus is referred to as inactive. While in the ER, the dimeric pro-TGF-β is covalently linked to milieu proteins, such as members of the latent TGF-β binding protein (LTBP) family, LTBP1, 3 or 4. LTBPs are a family of proteins related to extracellular matrix (ECM) proteins and are characterized with unique 8-Cys-like repeats. A disulfide bond between the 3rd 8-Cys repeat of LTBPs and Cys 33 of LAP links LTBP to the SLC, giving rise to the large latent complex (LLC). The pro-TGF-β dimer that gives rise to the SLC can be secreted directly by some cell types; however, the covalent linkage with LTBP enhances the secretion, and thus is suggested to play a chaperone-like function in that it enhances the proper folding, secretion, and targeting to the ECM of the pro- TGF-β dimer[318,319]. While the milieu molecules LTBPs store pro-TGF-β in the ECM, another group of proteins, leucine-rich-repeat-containing proteins (LRRC) store the complex on the cell surface. Two LRRC proteins, LRRC32 and

LRRC33, have been described so far. LRRC32, also known as glycoprotein-A repetitions predominant protein (GARP), is expressed on platelets, endothelial cells and some immune cells, such as activated regulatory T cells and dendritic cells[320,321]. LRRC33 is specifically expressed in macrophages, especially in microglial cells[322]. Overall, LTBPs expression is ubiquitous, compared to the more cell-specific expression of GARP and LRRC33. The differential expression of milieu molecules that associate with latent TGF-β thus provides localization and selective activation in specific cells only. Based on the crystal structure solved by Tim Springer's group, latent TGF-β exist in a ring-like structure, where the arm domains of LAP connect at the elbows with crossed "forearms" formed by the dimer of mature TGF-β and the LAP "straitjacket" elements that surround each of the mature TGF-β monomers. LAP arms are connected at the neck via di-sulfide bonds and each shoulder carries and RGD motif, essential for integrin binding. On the opposite side, where the "straitjacketed" arms of mature TGF-β are crossed, LTBP binds to the straitjacket[323].

Conversion of latent to biologically active TGF-β. Regulation of the amount of active TGF-β is crucial for health in mammals. Too little or too much TGF-β is incompatible with life. Mice that lack either of the TGF-β isoforms do not survive past few days or weeks after birth. On the other hand, overexpression of TGF-β is embryonic or soon after birth lethal, resulting from organ-specific or generalized pathologies. Because TGF-β synthesis and receptor expression are ubiquitous, the major regulatory step of TGF-β activity occurs at the level of converting latent into active TGF-β.

Proteolytic activation. Several protease families are reported to activate latent TGF-β, including plasmin, MMP-2, and MMP-9. MMP9 and MMP2 are metalloproteases

that require cell surface localization to proteolytically activate latent TGF-β. In the case of MMP-9, cell surface localization is mediated by binding to CD44, a hyaluronan receptor, whereas MMP-2 is docked to the cell surface by αVβ3 integrin. Even though MMPs are noted as key players in tumor advancement and tissue remodeling via ECM proteolysis, their expression is also induced in normal stromal cells during tissue injury and repair. This could suggest that MMP-9 and MMP-2 are involved in activating TGF-β as a physiological mechanisms of tissue remodeling and this mechanism is adopted by cancer cells to promote their growth and invasion[324]. Plasmin, on the other hand, is a serine protease that activates latent TGF-β by proteolytic degradation of LAP[325,326]. However, mice that lack the genes involved in the proteolytic activation of TGF-β do not show any phenotype like TGF-β deficiency. This could result from a redundancy in activating enzymes or involvement of additional proteins or mechanisms of activation.

Activation by thrombosponding-1. Thrombospondin-1 (TSP1) is a disulfide-linked homotrimer secreted by many cell types shown to associate with both the LLC and SLC and activate TGF-β. The unique sequence within TSP-1, RFK, binds to the N-terminal region of LAP and causes a conformational change that makes the mature TGF-β peptide accessible to the receptors. According to this model, TGF-β is never released from the latent complex; instead, the conformational changes in the complex only expose the mature TGF-β upon binding to TSP-1. A strikingly similar pathology is observed between mice that lack TSP-1 or TGF-β1 in multiple organ systems, but especially the lungs and pancreas. These similarities are attributed to the lack of activation of TGF-β by TSP-1 and strengthen the conclusion that TSP-1 regulates TGF-β activation *in vivo*. It is thought that TSP-1 mediated activation of TGF-β is important

for maintaining the basal levels of active TGF-β needed for normal growth and development[327,328]. Furthermore, TSP-1 activity can be coupled with proteolytic enzymes to enhance the activation of latent TGF-β. It is demonstrated that the proteolytic efficiency of enzymes is enhanced if the protease-substrate complex is localized to the surface of the cell, and thus it is shown that surface anchoring of TSP-1 and latent TGF-β increase the levels of active TGF-β. TSP-1 is a natural ligand for CD36, a surface glycoprotein expressed by monocytes and macrophages. Besides being involved in platelet-monocyte/tumor adhesion, platelet aggregation and macrophage uptake of apoptotic cells, the interaction between TSP-1 and CD36 is also important for activating latent TGF-β. The proposed model for this type of latent TGF-β activation is that TSP-1-latent TGF-β complex localizes at the cell membrane by association with CD36. From here either TSP-1 proteolytic activity or cell-generated plasmin, a serine protease can then liberate active TGF-β to bind to its receptors[329].

Activation by integrins. Integrins are surface heterodimeric proteins composed of non-covalently associated α and β subunits involved in binding extracellular ligands to the cytoskeleton to mediate cell adhesion and migration. Among the 24 vertebrate integrins described, two integrins, αVβ6 and αVβ8, are specialized in efficiently activating latent TGF-β *in vivo*. Mice lacking either of αVβ6 or αVβ8 recapitulate all major phenotypes of TGF-β1 null mice[330]. αVβ6 and αVβ8 integrins interact with conserved RGD motifs of LAP and represent another major mechanism for TGF-β activation. Mice carrying an RGD to RGE mutation in the LAP sequence have a selective loss in integrin-mediated TGF-β activation, despite producing normal levels of latent TGF-β, and also recapitulate all major phenotypes of TGF-β1 null mice[331]. Even

though multiple mechanisms of TGF-β activation are described, integrin recognition of RDG motifs in LAP play a crucial role in TGF-β activation. However, different mechanisms of latent TGF-β1 activation have been proposed for the two integrins. In the case of αVβ6 integrin, the cytoplasmic domain of the β chain interacts with actin filaments which exerts tensile forces that lead to conformational changes in the latent complex. Whether this physical pulling only exposes or completely releases TGF-β is not well understood. Based on the crystal structure of latent TGF-β, it is proposed that TGF-β must be liberated. If integrin binding and physical force only change the conformation of the latent complex such that mature TGF-β is exposed, this is only enough for binding of the type II TGF-β receptor. Type I receptor binding overlaps with many of the interactions between TGF-β and the arm domains of LAP and thus a complete release from LAP would be required for receptor engagement[323]. αVβ6 is expressed at low levels on epithelial cells but in response to inflammation or injury the expression increases. Subsequently, the activation of TGF-β can mediate suppression of the inflammation[332]. αVβ8, on the other hand, mediates TGF-β activation in a protease-dependent manner. It is suggested that in this scenario, the integrin is necessary to concentrate latent TGF-β on the cell surface which allows proteases, such as the MMP membrane type 1 (MT1), to proteolytically degrade LAP and release active TGF-β [333,334]. αVβ8 is expressed by epithelial cells, fibroblasts, neurons, as well as immune cells such as glial cells, dendritic cells, and CD4+ T cells. One of the mechanisms human regulatory T cells use to induce tolerance and suppress inflammatory responses is TGF-β activation. Interestingly, even though latent TGF-β is

expressed at similar levels between both helper and regulatory T cells, the integrin αVβ8 is expressed at significantly higher levels in activated regulatory T cells, compared to helper T cells. Even though the proposed mechanism for TGF-β activation by αVβ8 involves proteolytic degradation of LAP, crystal structure analysis shows that the residues that β6 uses to bind to TGF-β, are conserved in β8 only, and no other integrin β subunits[335]. So it is possible that αVβ8 also uses pulling forces on LAP to liberate active TGF-β[336].

Activation by reactive oxygen species. The first evidence that reactive oxygen species (ROS) regulate TGF-β activation come from studies using irradiation of mammary gland cells. These studies show that irradiation induces a rapid shift from latent into active TGF-β. Ionizing radiation generates hydroxyl radicals and other ROS, which alter the conformation and stability of the latent complex and enable TGF-β activation. It is speculated that the mechanism of activation by ROS is by protein oxidation which results in loss of the non-covalent association between LAP and mature TGF-β [337,338].

TGF-β Signaling

Activation and regulation of receptors and Smad molecules. The mature TGF-β dimer, liberated from the latency complex, binds to a pre-formed homodimer of TβRII. TGF-β bound by TβRII is then recognized by TβRI homodimer, which is recruited to the complex and leads to the formation of a hetero-tetrameric TβRI-TβRII receptor complex[340,341]. Both TβRI and TβRII are dual-specificity kinases that contain a cytoplasmic serine/threonine and tyrosine kinase domain[342]. TβRII is constitutively

active and autophosphorylation of at least three critical serine residues plays a critical role in the regulation of the receptor kinase activity[343]. Ser213, located in the membrane-proximal region is phosphorylated via an inter-molecular mechanism and is suggested to cause conformational changes in the kinase domain of TβRII, resulting in kinase domain activity. Autophosphorylation of Ser409 and Ser416 requires dimerization of the TβRII and occurs via intra-molecular mechanisms. Interestingly, while phosphorylation of Ser409 leads to a stimulatory, phosphorylation of Ser416 has an inhibitory effect on the receptor kinase activity by regulating the phosphorylation state of the TβRI. Because the negative charge acquired during Ser409 phosphorylation is not enough to mediate TβRI phosphorylation, it is suggested that Ser409 phosphorylation changes the conformation of the kinase region, thus affecting its substrate binding sites. In contrast, phosphorylation of Ser416 is thought to cause a conformational change that is unfavorable for the kinase region activity[344]. Additionally, TβRII auto-phosphorylation on tyrosine residues Tyr259, 336 and 424 is also essential for receptor kinase activity[342]. However, TGF-β binding to TβRII is not enough to trigger receptor signaling, besides TβRII always being catalytically active; instead, it causes the recruitment of TβRI which is then trans-phosphorylated by TβRII kinase. Serine and threonine residues located in the glycine-serine (GS) rich domain preceding the kinase domain of TβRI are the major sites of phosphorylation by TβRII. Phosphorylation of the GS domain not only increases the TβRI kinase activity but also converts the GS domain from a binding site for the inhibitory protein FK506-binding protein 1A (FKBP12), to an efficient recruitment motif for its direct substrates, a family of intracellular signaling molecules known as Smads[345–349].

Canonical TGF-β signaling. TGF-β signaling mediates its effects by regulating gene expression via receptor-regulated activation of Smad proteins. Smads are a family of proteins that are ubiquitously expressed in all adult tissues. Functionally, Smads can be divided into three subfamilies: 1) receptor-activated Smads (R-Smads: Smad1, 2, 3, 5 and 8) get recruited to the activated TβRII- TβRI complex, and become phosphorylated by TβRI; common mediator Smads (Co-Smads: Smad4) which oligomerize with phosphorylated R-Smads; and inhibitory Smads (I-Smads: Smad 6 and 7), which compete with R-Smads for TβR binding but also mark the receptors for degradation. Structurally, Smad proteins have two conserved domains, an N-terminal Mad homology 1 (MH1) and C-terminal Mad homology 2 (MH2) domain, connected by a linker region of variable length and sequence. The MH1 domain is highly conserved among the R- and Co-Smads, but not I-Smads and regulates the import of Smads into the nucleus, as well as their transcriptional regulation via binding to DNA and nuclear proteins. MH2 on the other hand is highly conserved among all Smad sub-families and regulates Smad oligomerization, interaction with TβR, and cytoplasmic adaptor proteins. Inactive, cytoplasmic Smads are autoinhibited by an intramolecular interaction between the MH1 and MH2 domains[350]. Upon activation of the TβR complex, TβRI specifically recognizes and phosphorylates the R-Smads, Smad2 and Smad 3 at serine residues within the conserved SSXS C-terminal region. Recruitment of Smad proteins to the receptor complex is mediated by double zinc finger, or FYVE domain, containing proteins, one of them being Smad anchor for receptor activation (SARA). Through the FYVE domain, SARA binds to phosphatidylinositol 3-phosphate and thus organizes inactive Smad proteins to the plasma membrane or endosomal vesicles. Through the C-

terminus, SARA can bind to the activated TGF-β complex and thus can serve as a bridge between the R-Smads and the receptors[351]. Another FYVE domain protein, hepatic growth factor-regulated tyrosine kinase substrate (Hrs/Hgs), cooperates with SARA in the recruitment of Smad2 and Smad3 to the receptor complex and thus contributes to TGF-β signaling[352]. Once phosphorylated, R-Smads are ready for nuclear translocation, as nuclear localization signals (NLS) become exposed. In the case of Smad3, a conformation change exposes a five-residue NLS motif on the N-terminus, which is essential for a nuclear import via an importin-beta/Ran pathway[353,354]. On the other hand, the conformational change upon Smad2 phosphorylation reduces its affinity for the Smad binding protein SARA and thus unmasks its nuclear import activity[355]. Furthermore, upon phosphorylation of the R-Smads, the TβR – Smad interaction is weakened and leads to dissociation of the two protein complexes. In response to TGF-β, activated Smad2 and Smad3 then associate with the Co-Smad, Smad4, forming a functional oligomer that translocates to the nucleus[356–358]. In the nucleus, R-Smad/Co-Smad complexes regulate gene transcription directly by binding to the DNA sequence, or indirectly, by interacting with DNA binding proteins and transcriptional co-activators or co-repressors. One of the key molecules involved in the regulation and fine-tuning of TGF-β signaling is the inhibitory Smad, Smad7. At basal states, Smad7 resides in the nucleus, from where it translocates into the cytoplasm upon TGF-β stimulation[359]. Smad7 associates with TβRI and antagonizes TGF-β signaling via several different mechanisms, such as blocking R-Smad recruitment[360,361] to TβR, promoting de-phosphorylation of TβR[362], ubiquitination and degradation of TβR [363,364], as well as

blocking R-Smad/Co-Smad complexes from interacting with DNA target sequences in the nucleus[365].

Non-canonical TGF-β signaling. Non-canonical TGF-β signaling involves a non-Smad related signaling cascade that activates different branches of the mitogen-activated protein (MAP) kinase pathway, such as extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), p38 MAP kinase; Rho-like GTPase signaling pathways, and phosphatidylinositol-3-kinase/AKT pathways. Being dual-specificity kinases, upon tyrosine phosphorylation, the TGF-β receptors can serve as sites for recruitment of Src homology 2 (SH2) domain-containing proteins. Recruitment of signaling mediators is thought to enable TGF-β receptors to trigger non-Smad-related signaling pathways[366].

TGF-β in Cancer

Most advanced human cancers overexpress TGF-β. During the early stages of tumorigenesis, TGF-β has an anti-proliferative effect on the tumor cells, thus restricting tumor growth. However, later, tumor cells become refractory to TGF-β cytostatic effects and re-purpose the TGF-β signals for tumor progression pathways. Additionally, by overproducing TGF-β, cancer cells exploit TGF-β's immune regulatory role to subvert the anti-tumor immune response. TGF-β mediates escape from T cell immune-surveillance by directly repressing the T cell cytotoxic program[367,368], favoring regulatory T cell differentiation[369], as well as restricting trafficking of anti-tumor T cells into the tumor microenvironment (TME)[370,371]. Because of its pro-tumorigenic effects, TGF-β is a prime candidate for therapeutic targeting. Currently, several therapeutic approaches, including TGF-β antibodies, antisense oligonucleotides, and receptor kinase inhibitors,

are being evaluated for cancer treatment. However, because TGF-β is involved in the maintenance of many normal physiological processes, all current strategies face the fact that blocking TGF-β signaling might have adverse off-target effects. In fact, because of the anti-proliferative effect that TGF-β has on pre-malignant cells, anti-TGF-β1 blocking antibodies has been shown to stimulate the growth of preneoplastic lesions[372]. Subsequently, in one phase 1 clinical trial that evaluated anti- TGF-β therapeutic potential in melanoma patients, the development of squamous cell carcinomas was observed[373]. Thus, the development of therapies that can target TGF-β in the TME specifically is of utmost importance.

CHAPTER II: MATERIAL AND METHODS

Cell Culture

Complete RPMI used for suspension cell cultures was prepared with RPMI-1640 (HyClone, Logan, UT) and supplemented with 10% fetal calf serum (Gemini Bioproducts, West Sacramento, CA), essential amino acids (Corning, Corning, NY), non-essential amino acids (Gibco, Waltham, MA), 1mM sodium pyruvate (Corning, Corning, NY), 50mM 2ME (Fisher Scientific, Waltham, MA), and 0.1M Hepes (Corning, Corning, NY) and Penicillin/Streptomycin (HyClone, Logan, UT). Adherent cells were maintained in DMEM supplemented with 10% FCS (Gemini Bioproducts, West Sacramento, CA) and Penicillin/Streptomycin (HyClone, Logan, UT). All cells were maintained with 5% CO_2 at 37C.

Human lymphocytes were obtained from adult or cord blood. Adult peripheral blood mononuclear cells (PBMCs), a kind gift from Dr. Michael Nishimura, were purchased from Key Biologics (Memphis, TN) or Zen Bio (Research Triangle Park, NC) and came from de-identified adult healthy donors. Cord blood mononuclear cells were isolated from whole umbilical cord blood kindly donated from Loyola University Medical center from healthy donors (exclusion criteria: 1. autoimmunity; 2. malignancy; 3. use of immunosuppressive medication; 4. hyper or hypothyroidism). Heparinized blood was separated using Lymphopure density gradient medium (Biolegend, San Diego, CA) according to the manufacturer's instructions. Whole PBMCs were seeded at 1-2x10^6

cells per well in 48-well plates in the presence of 200ng/ml soluble anti-CD3 (OKT3 clone, Biolegend, San Diego, CA) and 10ng/ml IL-2 (Peprotech, Rocky Hill, NJ). For conditions extending past 3 days, cells were split 1:2 and fresh media supplemented with 10ng/ml IL-2 was added back.

Simian virus 40 large T antigen transfected Jurkat cells, a kind gift from Dr. Art Weiss (UCSF, San Francisco, CA), were maintained in complete RPMI, and were used for all transfection experiments.

MEL624 (ATCC, Manassas, VA) were maintained in DMEM (HyClone|Cytiva, Logan, UT) supplemented with 10% FCS and 10% Penicillin/Streptomycin.

T Cell Isolation and Culture

CD3+, naïve CD4+ and naïve CD8+ T cells were isolated from mononuclear cells from healthy adult or cord blood via negative selection using the MojoSort CD3+, naïve CD4+ or naive CD8+ T cell enrichment kit (Biolegend, San Diego, CA). Cell purity for all enrichments ranged between 90-98%. In all assays using enriched cells, cells were cultured at 0.5-1x10^6 cells/ml in plates coated with anti-CD3 (OKT3, 5ug/ml) and anti-CD28 (5ug/ml) and complete RPMI media supplemented with 10ng/ml IL2.

Flow Cytometry

Antibodies used for the flow cytometry analysis were anti-CD4, -CD8, -Siglec 5 (1A5), -Siglec-3, -Siglec-7, -Siglec-9, -Siglec-10, -CD137, -PD-1, -CD69, -Granzyme B, -CD25, -OX40, -ICOS, -CTLA-4, -IFN-γ, -TNF-α, -IL-2, -CD107a, -CD14, -CD36 (Biolegend, San Diego, CA), or in lab prepared phage particles or recombinant Fc fusion proteins. Cells were blocked with human Fc receptor blocking solution (Human TruStain FcX, Biolegend) for 10 minutes on ice. For detection of cell surface markers,

cells were stained with antibody cocktail for 30 minutes at 4C. Intracellular markers were analyzed after fixing and perming with 4% paraformaldehyde and permeabilization buffer (50mM NaCl, 0.02% NaN3, 5mM EDTA, 0.5% TritonX, pH7.5), respectively. Data was collected using BD FACSCanto or BD LSRFortessa and analyzed using FlowJo v.10 software.

Western Blot and Immunoprecipitation Analysis

For Western blot analysis, cells were lysed in non-reducing SDS sample buffer (2% SDS, 0.05% bromophenol blue, 62.5mM Tris-HCl, pH 6.8, 10% glycerol, 10mM Iodoacetamide (IAM)) and boiled 2 x 5min. Equal amount of proteins, based on cell number, was loaded and separated on SDS-PAGE gel. PVDF membrane transferred proteins were probed with antibodies against anti-Siglec 5/14 (clone 1A5, Biolegend, San Diego, CA).

For immunoprecipitation of Siglec-5, cord blood mononuclear cells were stimulated using soluble anti-CD3 (200ng/ml) and IL2 (10ng/ml) for 2-3 days. 50-60x10^6 cells were lysed in 1ml of lysing buffer containing 0.5% NP-40, 0.15M NaCl, 5mM EDTA, and protease and phosphatase inhibitors. The cell lysates were pre-cleared with Protein G-Sepharose pre-incubated with mIgG1 isotype antibody for 2hrs and immunoprecipitated with CNBr-activated Sepharose conjugated with anti-Siglec5/14 antibody (clone 1A5) for 2hrs. After washing 3 times with lysis buffer, the immunoprecipitants were either boiled in 1x SDS buffer (2% SDS, 0.05% bromophenol blue, 62.5mM Tris-HCl, pH 6.8, 10% glycerol, 125mM DTT) or further subjected to de-glycosylation with PNGase-F (NEB, Ipswich, MA) following the manufacturer's instructions. De-glycosilated samples were also boiled in reducing SDS buffer. Samples

were run on SDS-PAGE gel, and proteins transferred on PVDF membrane. Membranes were probed with antibodies against anti-Siglec5/14 (polyclonal antibody, R&D, Minneapolis, MN).

Reporter and Expression Constructs

cDNA sequences of Siglec-5 (SinoBiological) and Siglec-14 (IDT, inc.) were subcloned into a pME vector using PCR based cloning. To generate a truncated version of Siglec-5 (pME-tSiglec-5) the ITIM and ITSM were excised from the pME-Siglec 5 plasmid with the restriction enzymes BbsI/MfeI (NEB, Ipswich, MA) and blunt ends were generated with DNA Polymerase I, Large (Klenow) Fragment (Thermo Fisher, Walthman, MA). The blunt ended plasmid was then ligated using T4 DNA polymerase (Thermo Fisher, Walthman, MA). NFAT-luciferase and AP-1 luciferase were previously described[374]. All plasmid DNA was prepared using CsCl purification method.

Clone 6FN3/WT FN3 – Fc constructs were generated using PCR based cloning. Sequences were cloned into a pME vector.

Luciferase Assay

Jurkat Tag cells (2x10^6) were transfected using electroporation (0.8uF and 0.260mV) with 10ug of NFAT or AP1 reporter DNA, along with 1ug of cytomegalovirus promoter-driven expression vector for *Renilla* luciferase and 30ug of the expression vectors for pME-Siglec-5 or pME-tSiglec 5. 48hrs post transfection equal number of cells were plated on anti-CD3 coated wells in 96-well plates. 4hrs post stimulation cells were harvested, and luciferase activity was measured using a bioluminescent reporter

assay kit (Promega, Madison, WI) and a luminometer. For each transfection condition, the relative luciferase activity was adjusted based on *Renilla* luciferase activity.

B6N::sfGFP and sfGFP Production

The B6N region (aa 1-154) of the Group B Streptococcus β-protein was cloned as a fusion protein with GFP into a pET-15b vector (MilliporeSigma, Burlington, MA). This gave us the B6N::sfGFP protein.GFP alone was cloned as a control, giving rise to sfGFP protein. Plasmids were expressed in *lysY/I^q E. coli* under inducible conditions. After overnight culture, bacteria were pelleted and lysed by sonication. Cell free supernatants were run on a Nickel column and eluted fractions were pulled together and run on FPLC. FPLC fractions were verified by Coomassie, and proteins were concentrated and stored in 40% glycerol in -80C.

B6N::sfGFP ELISA

Purified B6N::sfGFP or sfGFP were immobilized on Nunc Maxisorp 96-well plate (Thermo Fisher Scientific, Waltham, MA) for 2 hours at RT. Target coated plates were washed 3x with PBST (0.005% Tween-20) and then blocked with 3% BSA for 1 hour at RT. After washing 3x with PBST, recombinant Siglec 5-Fc chimeric protein (Biolegend, San Diego, CA) was added and incubated for 2hrs at RT, followed by 3x washes and incubation with anti-human IgG-HRP antibody for 1hrs. After 3xPBST washes, 1-Step Ultra TMB-ELISA (Thermo Fisher Sciebtific, Waltham, MA) was added and absorbance was determined at 450nm with a microtiter plate spectrophotometer (BioTech).

T Cell Stimulation with B6N::sfGFP

Naïve CD4+ or naïve CD8+ T cells were cultured with plate bound anti-CD3 (5ug/ml) and anti-CD28 (5ug/ml) stimulation in the presence of IL2 (10ng/ml) for 3 days.

Cells were than harvested, washed and re-stimulated with plate-bound anti-CD3 (5ug/ml), anti-CD28 (5ug/ml) and B6N::sfGFP or sfGFP (166nM) for additional 3 days. To reverse the inhibitory effects B6N::sfGFP has during T cell stimulation, equimolar amount of B6N::sfGFP or sfGFP were pre-incubated with recombinant hIgG1 Fc or Siglec-5-Fc proteins for 1hr at 4C with rotation. Mixed proteins were than coated on 96 well non-tissue cultured plates along with anti-CD3 and anti-CD28, before adding 3 days stimulated CD4 T cells. Supernatants from 3-day long cultures were collected and used for cytokine bead array (CBA) analysis for the expression of IL-5, IL-13, IL-2, IL-6, IL-9, IL-10, IFN-γ, TNF-α, IL-17A, IL-17F, and IL-22 using the LEGENDplex Human Th Cytokine (Biolegend, San Diego, CA). Cells from 3-day long cultures were used for evaluating expression of: CD4, CD8, PD-1, CD137, CD69, CD25, Siglec-5 and Granzyme B.

Evaluation of Putative Siglec-5 Ligand Expression in Cell Lines

Cell lines: MEL624, Jurkat, Raji, THP1, U937, T2, SNU-475, HEK293t, A549 were blocked with human Fc receptor blocking solution (Human TruStain FcX, Biolegend) for 10 minutes on ice. Cells were than stained with Siglec-5 Fc chimeric protein pre-incubated with anti-human IgG Fc fluorescent antibody. As a negative control, cells were stained with anti-human IgG Fc antibody alone.

Human 1383i T Cell Stimulation

Human 1383i T cells are tyrosinase specific and HLA-A2 restricted T cells that are generated by transduction of activated primary human T cells with the 1383i T Cell receptor (isolated from tumor infiltrating lymphocytes from a melanoma patient). Cells or 1383i TCR constructs were a generous gift from Dr. Michael Nishimura at Loyola

University Chicago. To stimulate the 1383i T cells, T2 (1x10^6 cells/ml) (ATCC, Manassas, VA) cells were pulsed with tyrosinase peptide 368-376 (YMDGTMSQV) (10ug/ml) for 2hrs at 37C. T2 pulsed with tyrosinase were than irradiated with 4425cGy and used for culturing with 1383i T cells at a ratio of 3:1 (1383i T cells : T2). After 2 days of stimulation, 1383i T cells were harvested and cell activation and expression of Siglec-5 were verified. Cells were than used for re-stimulated with MEL624 (ATCC, Manassas, VA) at 1:1 ratio. Before co-culture, MEL624 was pre-treated with hIgG1-Fc (10ug/ml) (Biolegend, San Diego, CA) or Siglec-5-Fc chimeric protein (1 or 10ug/ml) for 30mins. Cell culture was carried in the presence of 1x Monensin and 1x BrefeldinA (Biolegend, San Diego, CA). At 12hrs post-stimulation, expression of CD107a, IFN-γ, TNF-α and IL-2 was evaluated in the CD4 and CD8 compartments of 1383i T cells.

Preparation of Phage Particles

To prepare phage particles displaying clone 6 FN3 (Ph6) or non-selected FN3 (LBR), bacteria were grown in 2XYT media (Thermo Fisher Scientific, Waltham, MA) at 37C with shaking at 250rpm. Once the bacteria reached density of OD_{600}=0.5, bacteriophages were rescued by adding M13KO7 helper phage (MOI=10) (NEB, Ipswich, MA) for 1hr at 37C. Bacteria were than sub-cultured into fresh 2XYT media supplemented with 50ug/ml carbenicillin and 100ug/ml kanamycin and grown overnight at 30C with shaking at 250rpm. Phage particles were precipitated from bacterial supernatants by 6% PEG 800, 300mM NaCL for 1hr at 4C. Phages were pelleted by spinning down at 12,000rpm for 20mins. Phage were resuspended in phosphate buffered saline (PBS) and stored at 4C for short term usage.

Labeling Phage Particles

100 μl of PEG-precipitated phages re-suspended in sterile PBS (OD 7-10) were mixed with 100 μl of 0.2mM Sodium Carbonate buffer. 3.3μl of 10mM Alexa Fluor™ 488 or 594 NHS ester (Molecular Probes, Eugene, OR) was added and the mix was incubated at RT and in the dark for 1hour with vortexing every 15 min. Phages were purified from free fluorochrome by two PEG-precipitations and suspended in PBS. Freshly labeled phages were used for flow cytometry staining at a concentration of 10OD.

TGF-β ELISA

Recombinant active TGF-β1 was immobilized on Nunc Maxisorp 96-well plates (Thermo Fisher Scientific, Waltham, MA) for 2 hours at RT. Target coated plates were washed 3x with PBST (0.005% Tween-20) and were than blocked with 3% BSA for 1 hour at RT. After washing 3xPBST, phage particles (Ph6 or LBR) or recombinant FN3 based Fc fusion proteins were added and incubated for 2hrs at RT, followed by 3x washes. Phage binding were detected using anti-P8 M13-HRP antibody. Recombinant proteins were detected using anti-human IgG-HRP antibody. 1-Step Ultra TMB-ELISA (Thermo Fisher Sciebtific, Waltham, MA) was used to quantify binding by measuring absorbance at 450nm with a microtiter plate spectrophotometer (BioTech).

6/WTFN3-Fc Protein Production and Labeling

pME-6/WTFN3-Fc constructs were transfected into CHO cells (ATCC, Manassas, VA), using PEI as a transfection agent. Stably transfected cells were selected by growing the cells in Puromycin selection media (10mg/ml). For protein production CHO cells were cultured in DMEM prepared with 10% FCS previously stripped from immunoglobulins by running FCS on a protein G column. 1x10^6 CHO

cells were plated in 30ml media in a T175 flask (Thermo Fisher, Hampton, NH) and grown for 7 days. Cell culture supernatants were collected and filtered using 0.45uM filters. Proteins were purified using pre-packed protein G column (GE Healthcare, Chicago, IL). Fc based proteins were labeled using a One-Step Antibody Biotinylation Kit (Miltenyi, Beeergisch Gladback, Germany). Biotinylation, as well as specificity of 6FN3-Fc for TGF-β was verified using an ELISA by detecting 6FN3-Fc binding to TGF-β with Streptavidin-HRP.

CHAPTER III: RESULTS

Section I

Characterization of the Siglec-5 Receptor in T cells

CD33rSiglec Expression in T cells

CD33rSiglecs are expressed mainly on immune cells. Most human and mouse immune cells express at least one type of Siglec, with others expressing multiple. Because each Siglec shows specificity for a unique pattern on sialyation, it is likely that each receptor has a unique function. Little to no expression has been reported for resting T lymphocytes of healthy humans, an exception being Siglec-7 and -9, which can be found in small populations of CD8+ T cells and mediate direct inhibition of TCR signaling[277,285]. However, in some pathologies such as cancer or chronic viral infections, Siglec expression in the T cell compartment can be altered. For example, HIV-infected patients have a greater proportion of circulating CD4+ T cells expressing the inhibitory receptors Siglec-5 and Siglec-9, than uninfected healthy donors. Furthermore, expression of the inhibitory Siglecs correlates with resistance to excessive immune activation and subsequent HIV-induced cell death[274]. Similarly, it has been reported that tumor infiltrating lymphocytes from melanoma patients have high expression of Siglec-9, which negatively correlates with the prognosis and survival of patients[273].

Because both cancers and chronic viral infections provide constant T cell stimulation, we hypothesized that Siglecs expression might be dependent on the

activation state of the T cell. Using T cells from healthy adult donors we performed a screen for Siglec receptor expression (Siglec-3, -5, -7, -8, -9 and -10) in resting versus stimulated cells (up to 7 days of stimulation). As a positive control for the staining, we confirmed the expression of each Siglec in Monocytes (size gated and CD4low) from unstimulated adult PBMCs (Figure 1C). Looking into CD4 (Figure 1A) and CD8 (Figure 1B) T cells specifically, we observed minimal levels of expression for Siglec-6, -8, -9, and -10. Low levels of expression of Siglec-3 were detected in both CD4 and CD8, but Siglec-3 expression was not associated with the activation state of the T cells. As previously reported, we observed that a small population of CD8 T cells expresses Siglec-7, but the expression was also not associated with the activation state of the T cells. Unexpectedly, only one of the Siglecs, Siglec-5, had a unique, and activation associated pattern of expression.

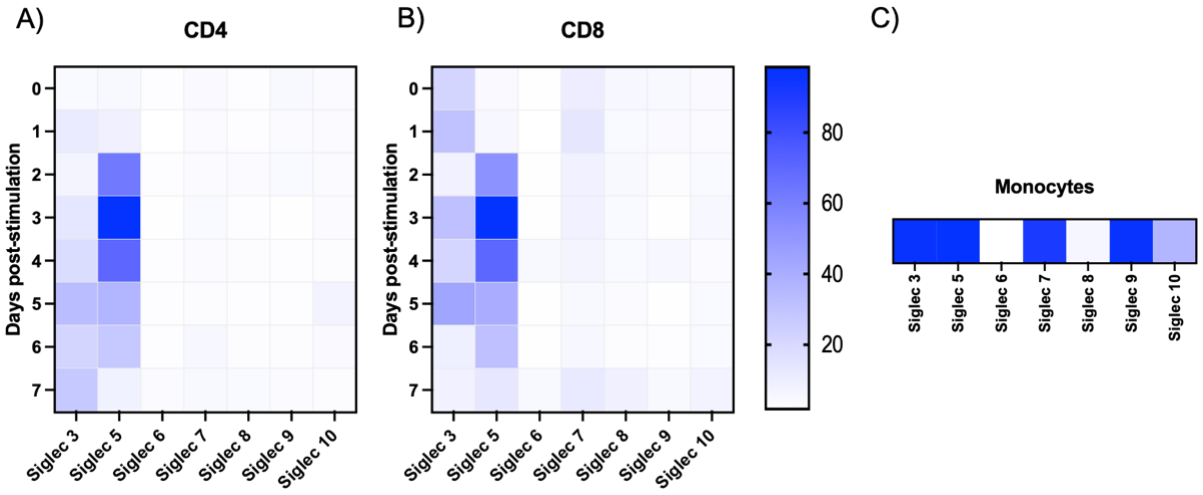


Figure 1: CD33rSiglec Expression in Resting and Activated T cells. Adult PBMCs were subjected to *in vitro* activation using soluble anti-CD3 (200ng/ml) and IL-2 (10ng/ml) for up to 7 days. Cells were split every 2-3 days. Expression of Siglecs-3, -5, -6, -7, -8, -9 and -10 was evaluated in **A)** CD4 and **B)** CD8 T cells at each time point. **C)** Monocyte expression of Siglecs-3, -5, -6, -7, -8, -9 and -10. Experimental donors n=3.

Siglec-5 Expression in T cells

Surface expression of Siglec-5 begins at 48 hours post-activation in both adult and cord blood T cells. In adult T cells, Siglec-5 expression peaks at 72hrs post-activation, upon which, it begins to decrease gradually and is entirely lost by day 7 post-activation (Figure 2A and 2B). In cord blood T cells however, surface expression of Siglec-5 peaks at 48hrs post stimulation, and then begins to decrease gradually (Figure 2C and 2D). Due to convenient availability of cord blood, most further experiments were performed using cord blood T cells.

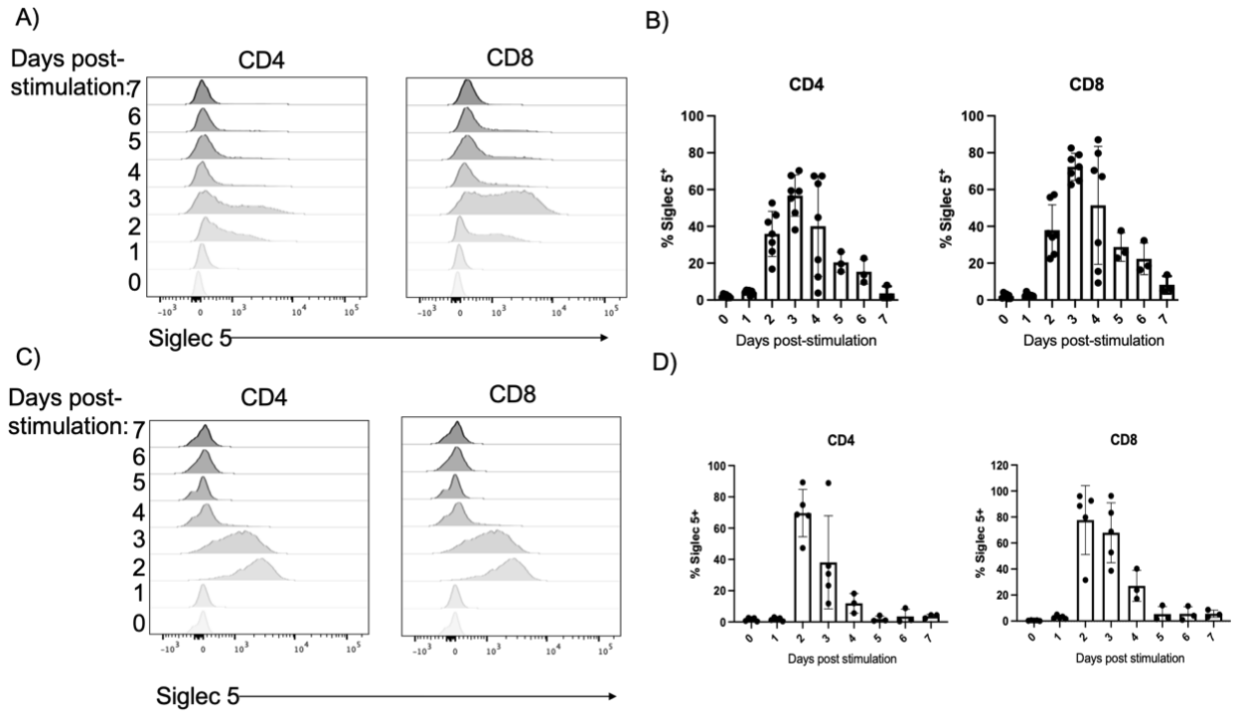


Figure 2: Siglec-5 Kinetics of Expression. **A)** Representative plot and **B)** summary for multiple donors of Siglec-5 expression within CD4 and CD8 from adult PBMCs subjected to *in vitro* stimulation using soluble anti-CD3 and IL2 for up to 7 days. **C)** Representative plot and **D)** summary for multiple donors of Siglec-5 expression within CD4 and CD8 from Cord blood mononuclear cells subjected to in vitro stimulation using soluble anti-CD3 and IL2 for up to 7 days.

Interestingly, even though the surface expression of Siglec-5 does not appear until 48hrs post-stimulation (Figure 2), using Western blotting we can detect Siglec-5 protein as early as 24hrs post-stimulation (Figure 3). This data suggests a regulatory mechanism between translation and trafficking of the protein to the cell surface.

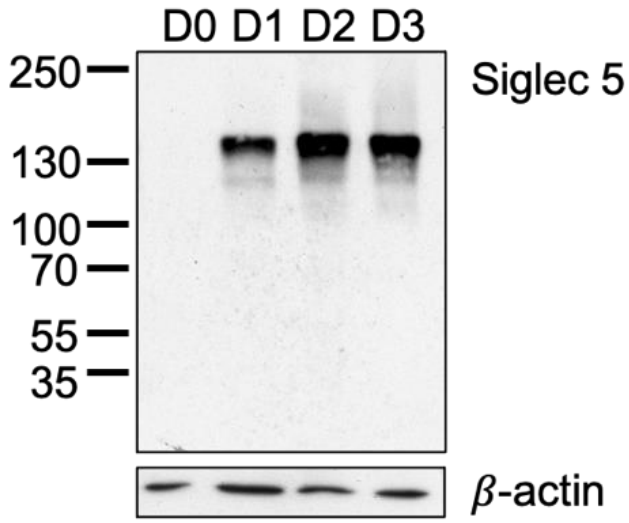


Figure 3: Siglec-5 Expression Analysis in T cells Using Western Blot. CD3+ T cells enriched from adult PBMCs were cultured with plate-bound anti-CD3 and anti-CD28 stimulation with IL-2 for 0, 1, 2, 3. At each time point cell lysates were prepared using denaturing, non-reducing conditions (Representative blot of 3 donors).

mRNA studies of Siglec-5 showed a basal level of expression even in resting T cells, though the levels peak at 24hrs post-stimulation (Figure 4).

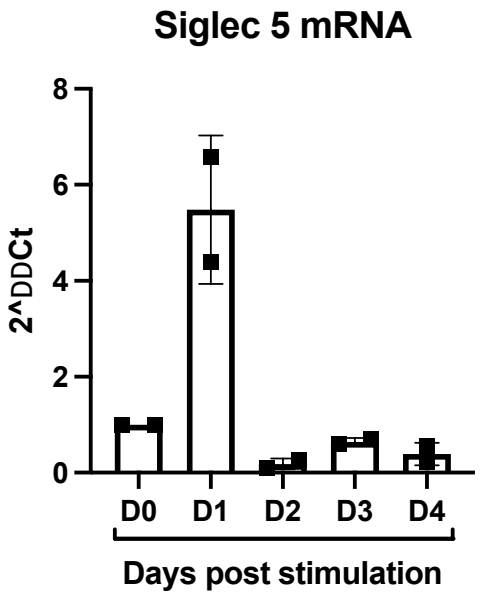


Figure 4: Siglec-5 mRNA Expression in T cells. CD3+ T cells were enriched from adult PBMCs and cultured with plate-bound anti-CD3 and anti-CD28 stimulation with IL-2 for 0, 1, 2, 3 or 4 days. mRNA was prepared from each time point and Siglec-5 mRNA was detected using qPCR.

Within its extracellular and ligand-binding domain Siglec-5 shares high sequence homology with its paired receptor Siglec-14. Because most commercially available antibodies recognize Siglec-5 within its extracellular domain, we cannot rely on this method of detection of the protein to claim Siglec-5 expression. Instead, a distinction or co-expression with Siglec-14 must be confirmed by Western blot analysis because of the distinct molecular weights the two receptors have. Using Western blot analysis of immunoprecipitated proteins, we sought to determine whether activated T cells express Siglec-5, Siglec-14, or both. If the protein observed by flow cytometry is Siglec-5, we expect to see a band of 68kDa, versus a band of 42kDa for Siglec-14. Compared to Jurkat T cells overexpressing Siglec-5 or Siglec-14 cDNA, we observed that T cells express Siglec-5, with minimal to no expression of Siglec-14 (Figure 5A). However, the bands we observed for Siglec-5 in this immunoprecipitation assay did not match the

predicted 68kDa. Because Siglec-5 has at least 8 described N-based glycosylation sites, we hypothesized that removal of the core glycosylation will give us a more accurate assessment of which protein, Siglec-5, or Siglec-14, is expressed in activated T cells. Using PNGase F treatment of Immunoprecipitated proteins, we removed all N-linked glycosylation and observed that the IP-ed protein samples from activated T cells correspond to Siglec-5. Minimal expression of Siglec-14 was observed (Figure 5B).

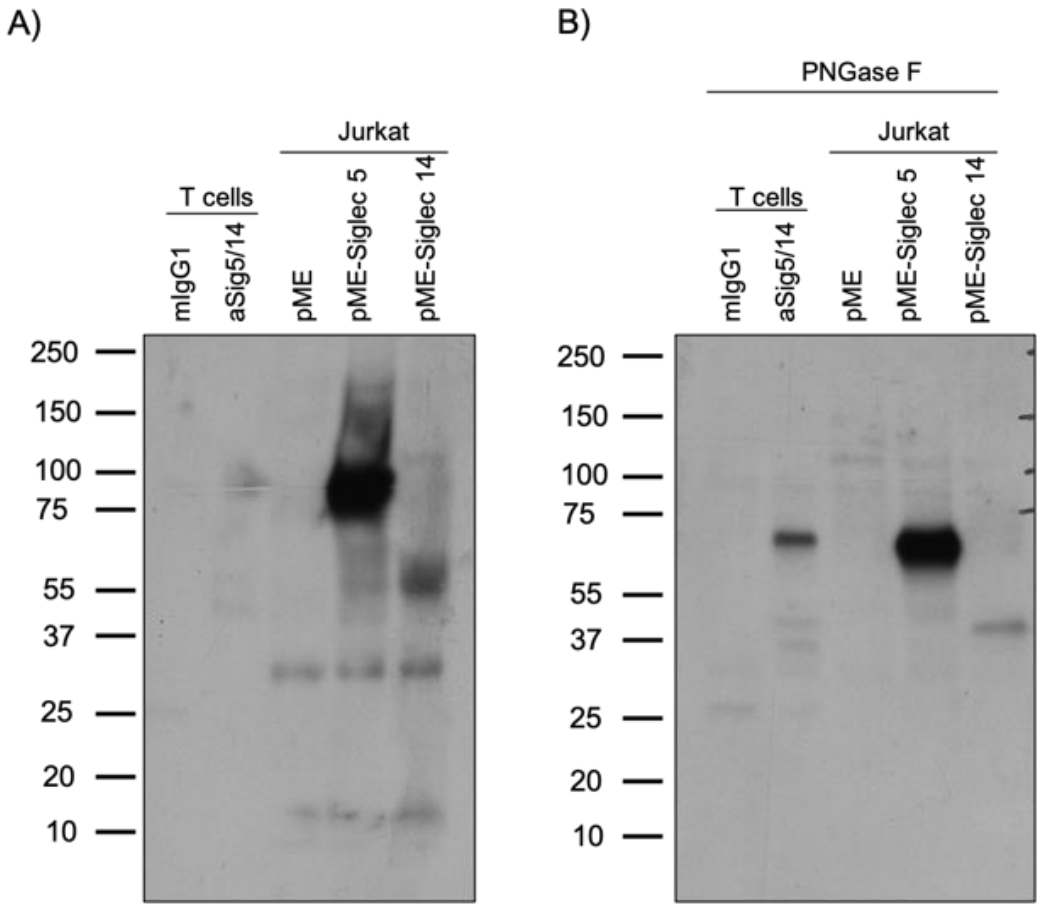


Figure 5: Immunoprecipitation of Siglec-5 from T cells. Cord blood T cells were stimulated with soluble anti-CD3 and IL2 for 2 days. Cells were lysed using mild detergent (0.5% NP-40) and pre-cleared with Protein G-Sepharose pre-incubated with mIgG1 isotype antibody. Proteins were immunoprecipitated by incubating with anti-Siglec-5/14 mAb (clone 1A5) or mIgG1 isotype CNBr conjugated beads. Where indicated, immunoprecipitated proteins were treated with PNGase F to remove N-linked glycosylations. As controls, Jurkat T cells were transfected with Siglec-5, Siglec-14 or an empty vector and whole-cell lysates were prepared. All samples were run on SDS-PAGE and transferred to PVDF membranes. Membranes were blotted with anti-Siglec5/14 polyclonal antibodies.

Siglec-5 Follows Expression Kinetics of Co-inhibitory and Co-stimulatory Checkpoint Receptors in T cells

Checkpoint receptors, co-stimulatory and co-inhibitory, play a crucial role in fine-tuning the fate of T cell activation, proliferation, and differentiation. These receptors are

expressed only upon T cell activation and the balance between stimulating and inhibitory receptors drives T cells into distinct functional states. Co-stimulating receptors, such as OX-40, ICOS, 4-1BB synergize with TCR signaling and help T cells develop an effective functional response. On the other hand, co-inhibitory checkpoints, such as PD-1 and CTLA-4, antagonize TCR signaling and suppress T cell activation, thus preventing excessive inflammatory responses and the development of autoimmune diseases. Specific activation of checkpoint receptors depends on the availability of their distinct ligands, which provides another level of regulation of their activity.

Because Siglec-5 has activation associated pattern of expression (Figure 2), we asked if it follows the expression kinetics of other, well-described checkpoint receptors. Comparing Siglec-5 expression with co-stimulatory checkpoint receptors, we observe a close to overlapping expression pattern with OX-40 (Figure 6B). In the case of ICOS (Figure 6A) and 4-1BB (Figure 6C), we see that T cells upregulate these molecules at day 1 post-stimulation versus day 2 post-stimulation for Siglec-5. 4-1BB is predominantly expressed by activated CD8 T cells and not CD4 T cells. On the other hand, Siglec-5 almost completely overlaps the expression kinetics of the co-inhibitory checkpoint receptor PD-1 (Figure 7A). Siglec-5 kinetics of expression closely resembles CTLA-4 expression too, but CTLA-4 precedes and first appears at day 1 post-stimulation (Figure 7B). Altogether, these data show that Siglec-5 follows the expression kinetics of other checkpoint receptors in T cells.

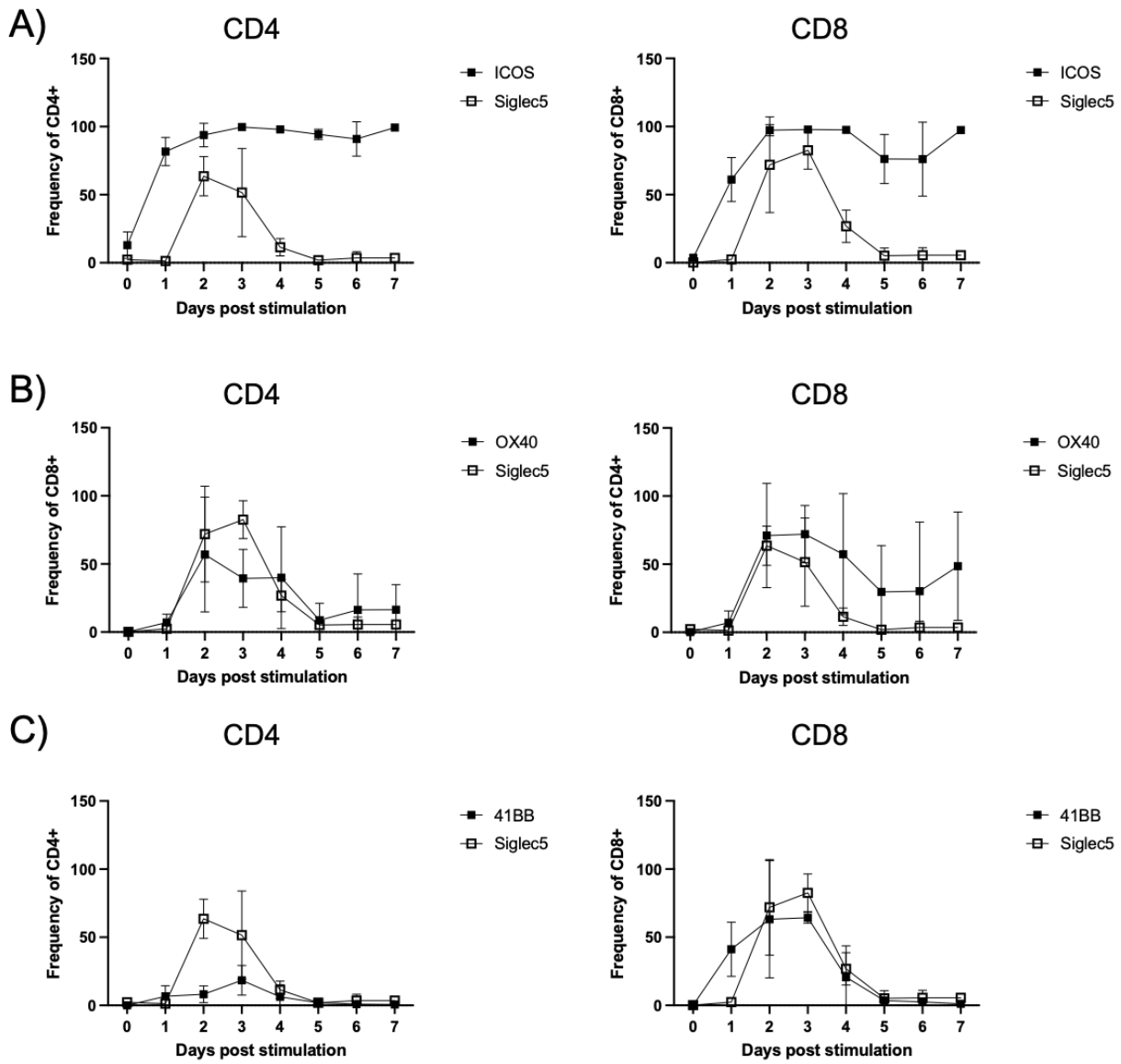


Figure 6: Expression Kinetics of Siglec-5 and Stimulatory Checkpoint Receptors OX-40, ICOS and 4-1BB in T cells. Cord blood mononuclear cells were stimulated with soluble anti-CD3 (0.2ug/ml) and IL2 (10ng/ml) for up to 7 days. Cells were split every 2-3 days. At each time point, cells were stained and analyzed for expression of Siglec-5 and **A)** OX40, **B)** ICOS or **C)** 41BB within the CD4 or CD8 T cell compartments. Data is a summary for n=3 individual donors.

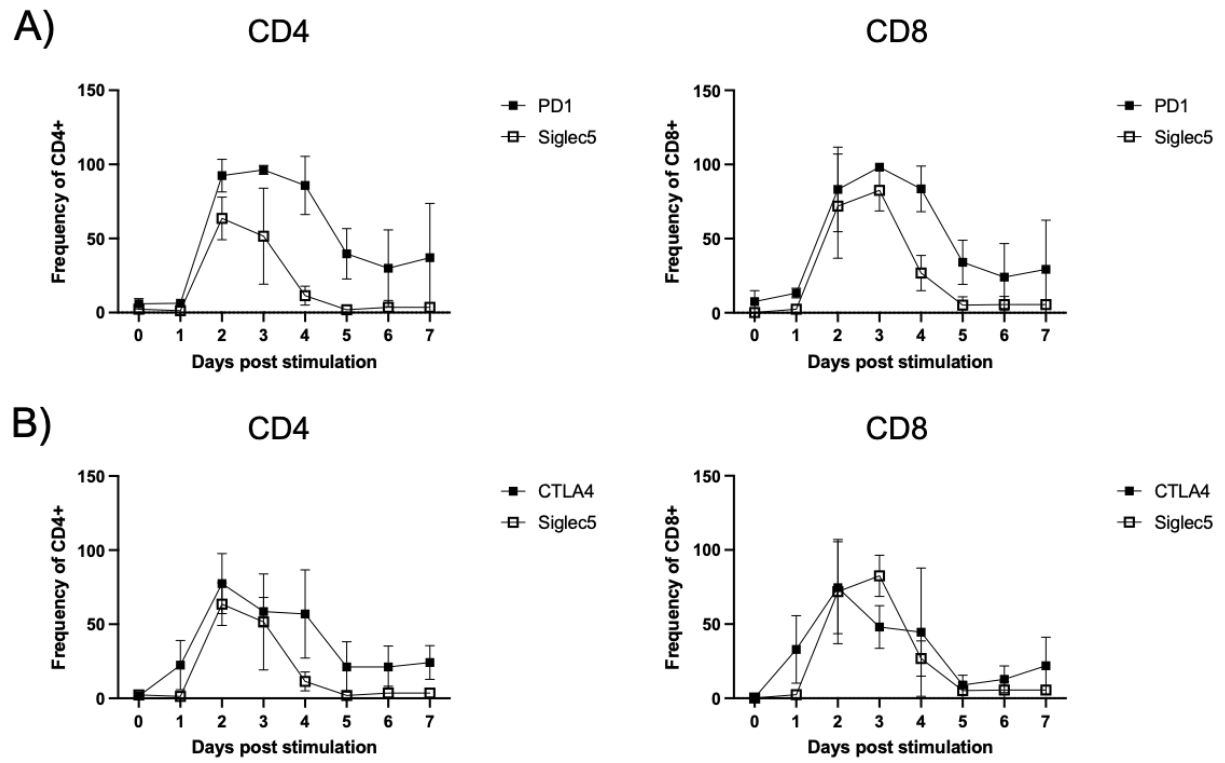


Figure 7: Expression Kinetics of Siglec-5 and Inhibitory Checkpoint Receptors PD-1 and CTLA-4 in T cells. Cord blood mononuclear cells were stimulated with soluble anti-CD3 (0.2ug/ml) and IL2 (10ng/ml) for up to 7 days. Cells were split every 2-3 days. At each time point, cells were stained and analyzed for expression of Siglec-5 and **A)** PD1 or **B)** CTLA-4 within the CD4 or CD8 T cell compartments. Data is a summary for n=3 individual donors.

The Function of Siglec-5 in T cells

Studies evaluating Siglec-5 expression in innate immune cells such as monocytes and neutrophils show that Siglec-5 is an inhibitory receptor that mediates its function through the recruitment of Shp1 and Shp2 phosphatases to the ITIM and ITSM motifs. These phosphatases de-phosphorylate molecules involved in signaling cascades and thus suppress immune cell activation[286]. Furthermore, having observed that Siglec-5 follows the expression kinetics of the well-known inhibitory checkpoint PD-1 (Figure 7A) we looked at whether the two receptors share any other features. Even

though PD-1 belongs to the CD28 family of receptors, its cytoplasmic domain is more like the CD33rSiglec family. When we compared the cytoplasmic and thus signaling domain of PD-1 and Siglec-5, we saw that they both have the inhibitory ITIM and ITSM (Figure 8) which they use to mediate inhibitory functions.

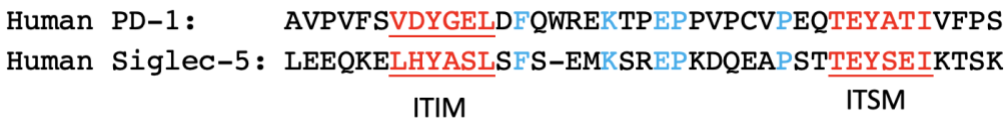


Figure 8: Similarity within the Cytoplasmic Tail of Siglec-5 and PD-1. The cytoplasmic sequence of Siglec-5 was compared to the cytoplasmic sequence of PD-1. Shared protein motifs are highlighted in red, and conserved amino acids are highlighted in blue.

Based on our knowledge about Siglec-5 from innate immune cells and its similarity with PD-1, we hypothesized that Siglec-5 is a negative regulator of T cell activation. To test this hypothesis, we used an overexpression system. We co-transfected Siglec-5 expression vector with reporter vectors for NFAT and AP-1 and measured the luciferase activity. If Siglec-5 is an inhibitory receptor, we expect to see reduced NFAT and AP-1 activity. Compared to control cells, overexpression of Siglec-5 leads to a significant reduction in NFAT and AP-1 activity upon anti-CD3 stimulation (Figure 9).

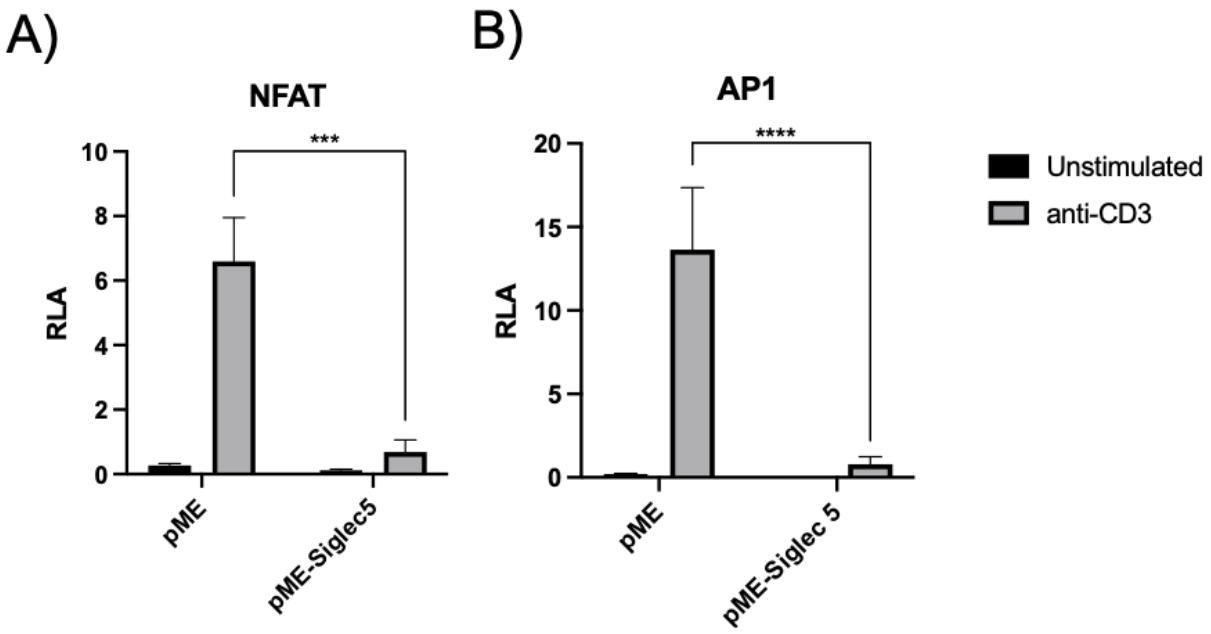


Figure 9. The Function of Siglec-5 in T cells. Jurkat T ag cells were transiently transfected with empty or Siglec-5 expression vector, along with NFAT and AP-1 luciferase reporter vectors. 4hrs post anti-CD3 stimulation, **A)** NFAT and **B)** AP-1 luciferase activity was measure. Relative luciferase activity was calculated based on Renilla luciferase activity. Statistical analysis: 2wayANOVA, Tukey's multiple comparison test, *** $p<0.001$, **** $p<0.0001$. Representative figures from 3 separate experiments, each performed in triplicate.

To understand how Siglec-5 blocks T cell activation, we tested if the ITIM and ITSM are required. For this, we mutated the Siglec-5 receptor such that we generated a cytoplasmic truncation by excising both the ITIM and ITSM. If ITIM and ITSM are the only domains of Siglec-5 responsible for its inhibitory effects in T cells, we expected to see complete restoration of NFAT and AP1 activity. However, we only observed partial restoration for both reporters (Figure 10).

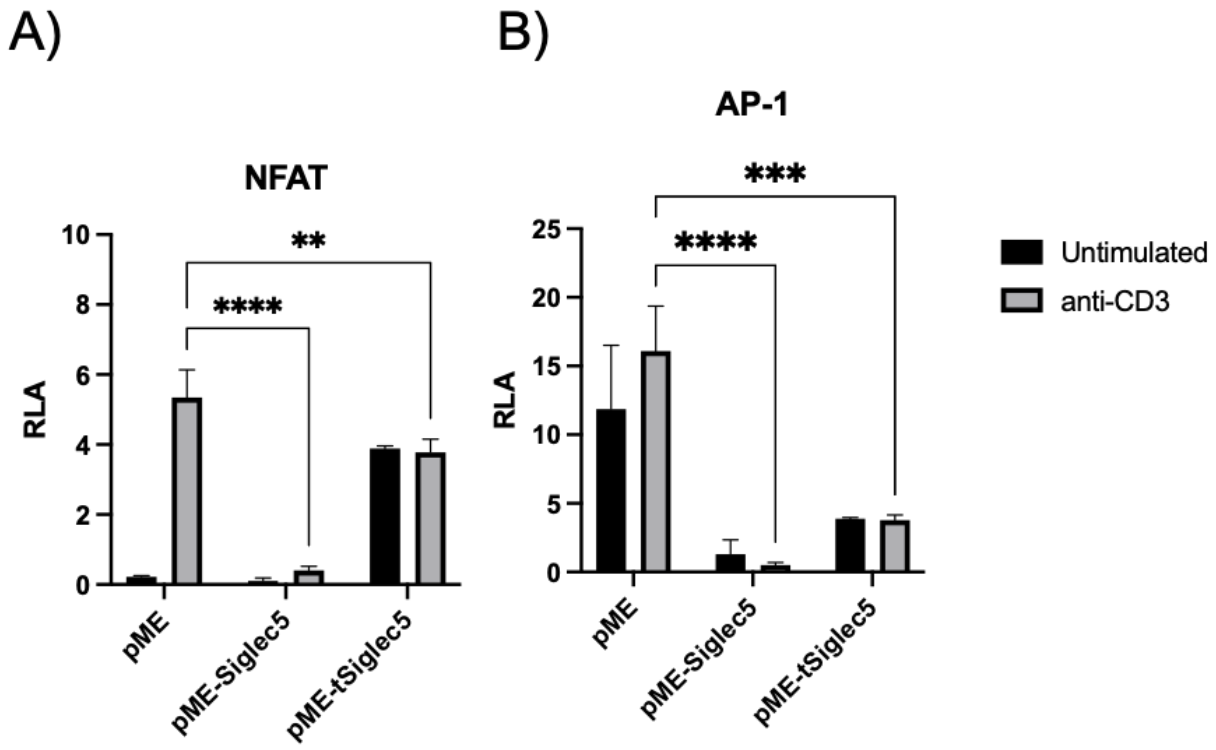


Figure 10: ITIM and ITSM are Partially Required for Siglec-5 Mediated Inhibition of T cells: Jurkat T ag cells were transiently transfected with empty, full-length Siglec-5, or a truncated Siglec-5 (tSiglec5) lacking both the ITIM and ITSM motifs. 4hrs post-anti-CD3 stimulation, **A)** NFAT and **B)** AP-1 luciferase activity was measure. Relative luciferase activity was calculated based on Renilla luciferase activity. Statistical analysis: 2way ANOVA, Tukey's multiple comparisons test, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Representative figures from 3 separate experiments, each performed in triplicate.

β-protein Mediated Regulation of T cell Activation

Pathogens have evolved to evade the immune responses by utilizing immunomodulatory mechanisms that generally serve to keep immune cells from over-activation. Group B Streptococcus (GBS), a major pathogen of newborns, expresses a surface protein, β-protein, that suppresses different arms of the immune response, including binding and activating the Siglec-5 receptor in innate immune cells. More specifically, it was narrowed down that the B6N domain (aa1-152) of the β-protein was

responsible for engaging and activating the Siglec-5 receptor. β-protein/Siglec-5 interactions result in inhibition of phagocytosis and suppression of proinflammatory cytokine production by neutrophils and monocytes in response to GBS[281,282]. Using the B6N region of the β-protein, we asked whether engaging Siglec-5 with a specific ligand will affect the activation of primary T cells. From our overexpression studies (Figure 9) we know that Siglec-5 is an inhibitory receptor in T cells, and now we hypothesized that direct activation of Siglec-5 with B6N will suppress T cell activation. To begin testing our hypothesis, we first generated the B6N protein as a fusion with GFP (B6N::sfGFP). As a control, we expressed GFP alone (sfGFP). Using an indirect ELISA system where B6N::sfGFP and sfGFP were immobilized on the plate, we verified Siglec-5 specificity for B6N::sfGFP, but not sfGFP, using a recombinant Siglec-5-Fc chimeric protein for detection (Figure 11).

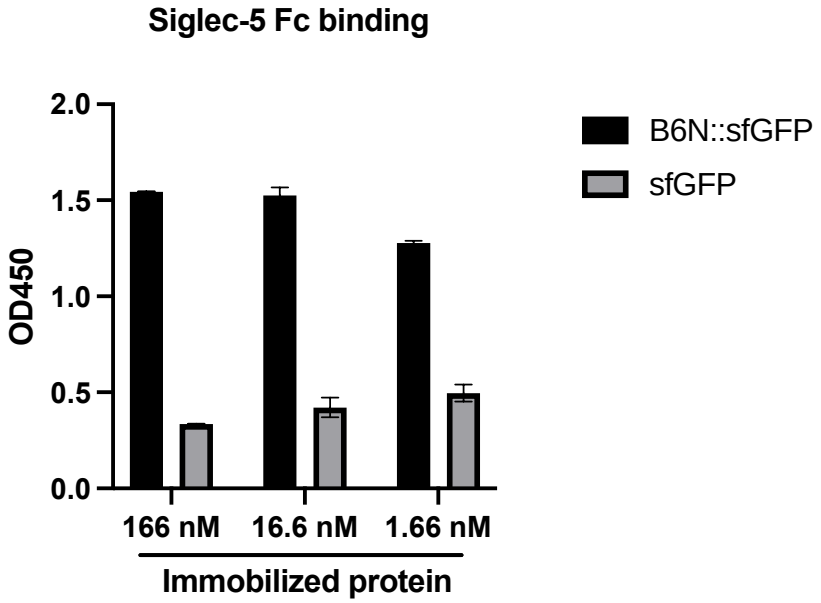


Figure 11: Siglec-5 Binds B6N::sfGFP, but not sfGFP. B6N::sfGFP and sfGFP were immobilized on ELISA plates. Unconjugated recombinant Siglec-5-Fc chimeric protein was used as a detection reagent. HRP-conjugated anti-hIgG was used for the detection of Siglec-5-Fc binding.

To test our hypothesis that activation of Siglec-5 with B6N::ssGFP will suppress T cell activation we set up an assay where naïve CD4 or CD8 T cells were stimulated for 3 days. From our kinetics studies (Figure 2), at day 3 post-stimulation Siglec-5 expression is high and majority of cells express it. We used the 3 day stimulated T cells for a re-stimulation assay, using plate-bound anti-CD3, anti-CD28 along with B6N::sfGFP or sfGFP. We cultured the cells for additional 3 days and then looked at cytokine production and expression of activation associated molecules. We observed that CD4 T cells stimulated in the presence of B6N::sfGFP had reduced production of the proinflammatory cytokines IFN-γ and IL-22 (Figure 11A). In contrast, we observed an increase in Th2 associated cytokines such as IL-4, IL-5, IL-13 (Figure 11B). Furthermore, effector molecules, such as Granzyme B were also significantly reduced in the presence B6N::sfGFP (Figure 12).

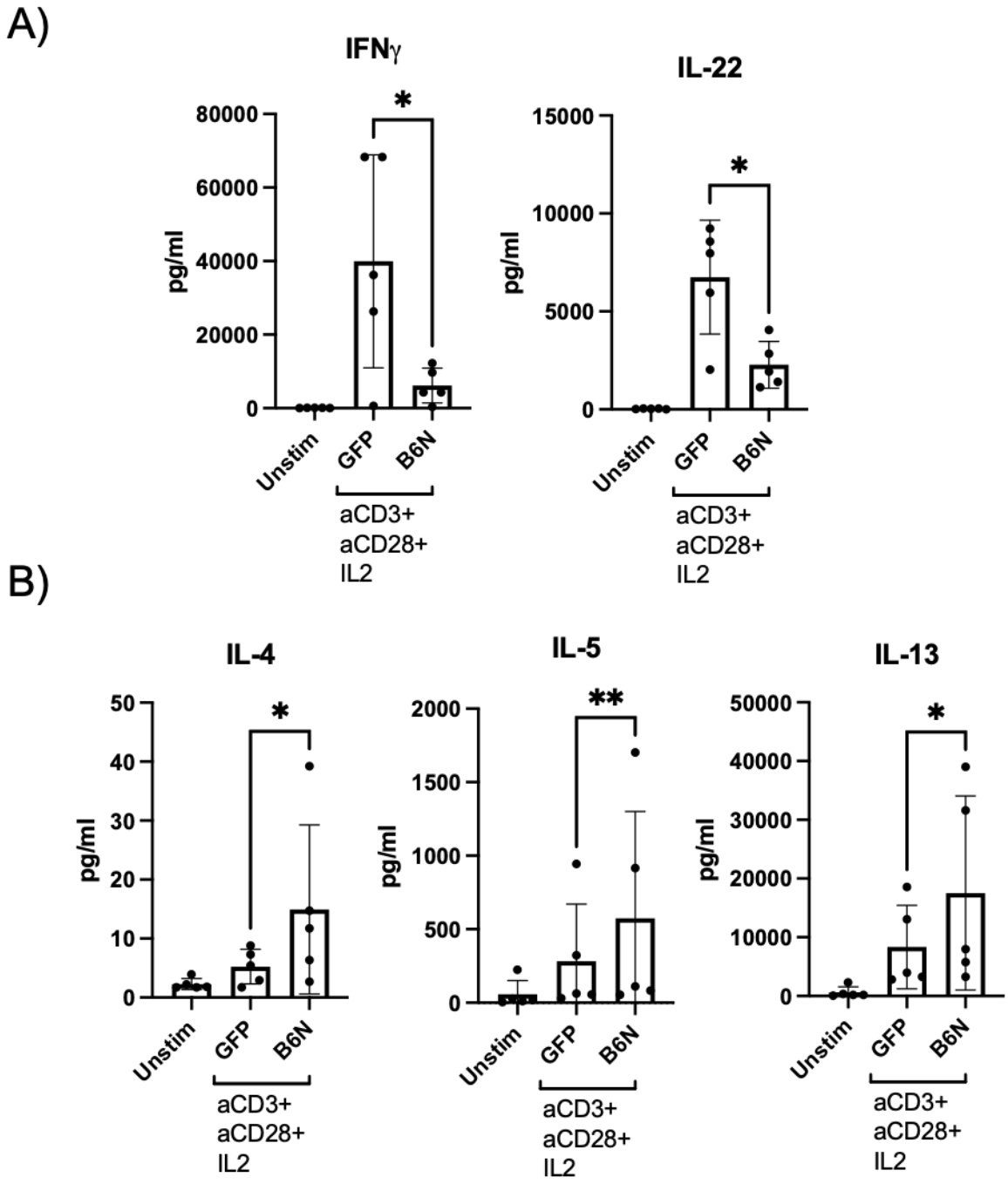


Figure 12: B6N::sfGFP Suppresses Inflammatory Cytokine Production in CD4 T cells. Naïve CD4 T cells were cultured with plate-bound anti-CD3 and anti-CD28 stimulation in the presence of IL-2 for 3 days. Cells were then harvested and re-stimulated with plate-bound anti-CD3, anti-CD28 and B6N::sfGFP or sfGFP. Supernatants from the 3 days cultures were collected and used for cytokine bead array analysis. Summary for A) IFNy IL-22 and B) IL-4, IL5 and IL13 levels from multiple donors. Statistical analysis: Ratio paired two-tailed t-test; * $p<0.05$; ** $p<0.01$; ***.

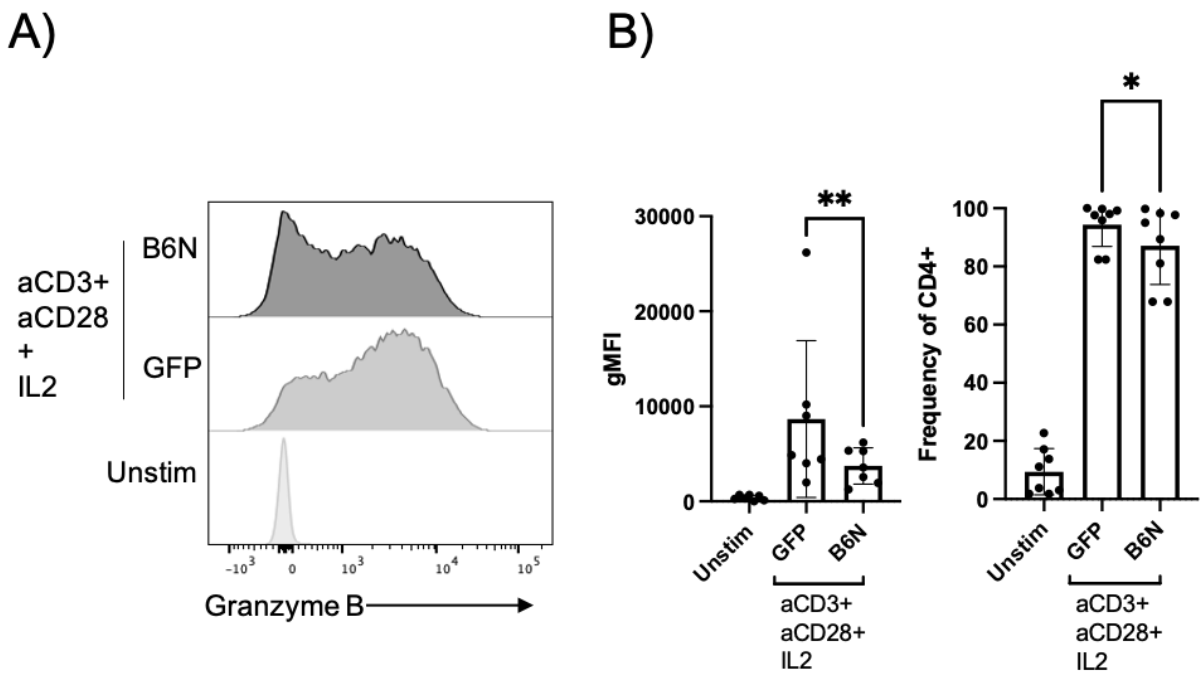


Figure 13: B6N::sfGFP Decreases Granzyme B Expression in CD4 T cells. Naïve CD4 T cells were cultured with plate-bound anti-CD3 and anti-CD28 stimulation in the presence of IL2 for 3 days. Cells were then harvested and re-stimulated with plate-bound anti-CD3, anti-CD28 and B6N::sfGFP or sfGFP. At day 3 post re-stimulation intracellular stain for Granzyme B was performed. **A)** Representative plot and **B)** summary for gMFI and frequency for multiple donors. Statistical analysis: Ratio paired two-tailed t-test; * $p<0.05$; ** $p<0.01$.

Following the reduction in function-associated markers, B6N::sfGFP also suppressed the expression of activation-associated molecules in CD4 T cells, such as CD69, CD137 and PD1.

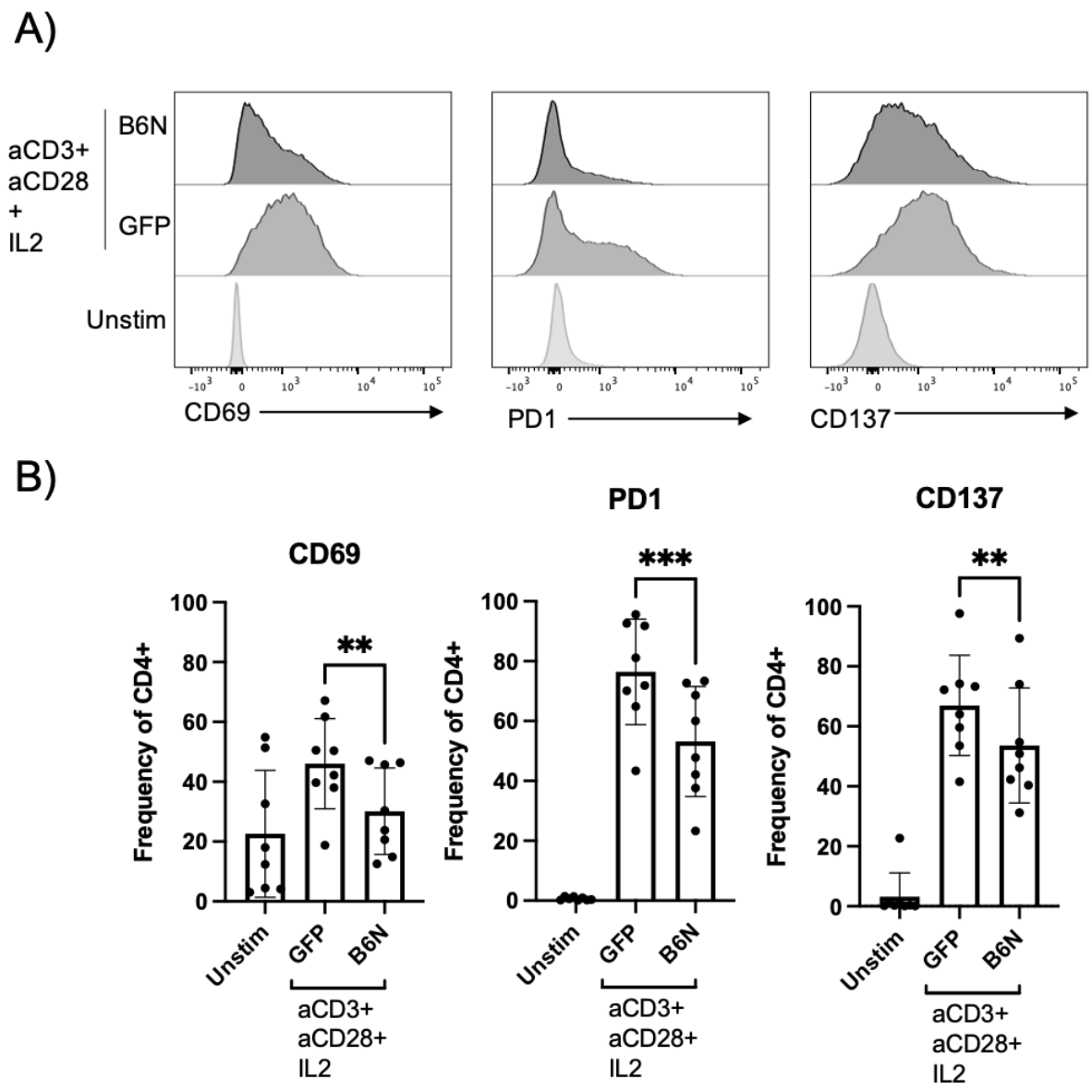


Figure 14: B6N::sfGFP Suppresses Expression of Activation Associated Molecules in CD4 T cells. Naïve CD4 T cells were cultured with plate-bound anti-CD3 and anti-CD28 stimulation in the presence of IL2 for 3 days. Cells were then harvested and re-stimulated with plate-bound anti-CD3, anti-CD28 and B6N::sfGFP or sfGFP. On day 3 post-re-stimulation cells were stained for surface marker expression. **A)** Representative plots and **B)** summary for CD69, PD1 and CD137 expressing cells. Statistical analysis: Ratio paired two-tailed t-test; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

In CD8 T cells stimulation in the presence of the B6N protein led to reduced production of the proinflammatory cytokine IFN-γ (Figure 15). Like CD4 T cells, B6N

also caused a reduction in Granzyme B production by CD8 T cells; however, no significance was reached (Figure 16). Activation markers, such as CD137 and PD-1, but not CD69 were also significantly reduced in CD8 T cells stimulated in the presence of the B6N protein (Figure 17).

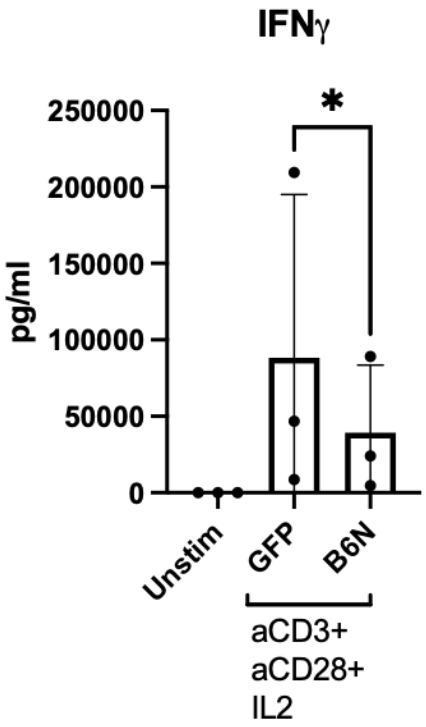


Figure 15: B6N::sfGFP Suppresses Inflammatory Cytokine Production in CD8 T cells. Naïve CD8 T cells were cultured with plate bound anti-CD3 and anti-CD28 stimulation in the presence of IL-2 for 3 days. Cells were than harvested and re-stimulated with plate bound anti-CD3, anti-CD28 and B6N::sfGFP or sfGFP. Supernatants from the 3 days cultures were collected and used for cytokine bead array analysis. Statistical analysis: Ratio paired two-tailed t-test; *, $p<0.05$

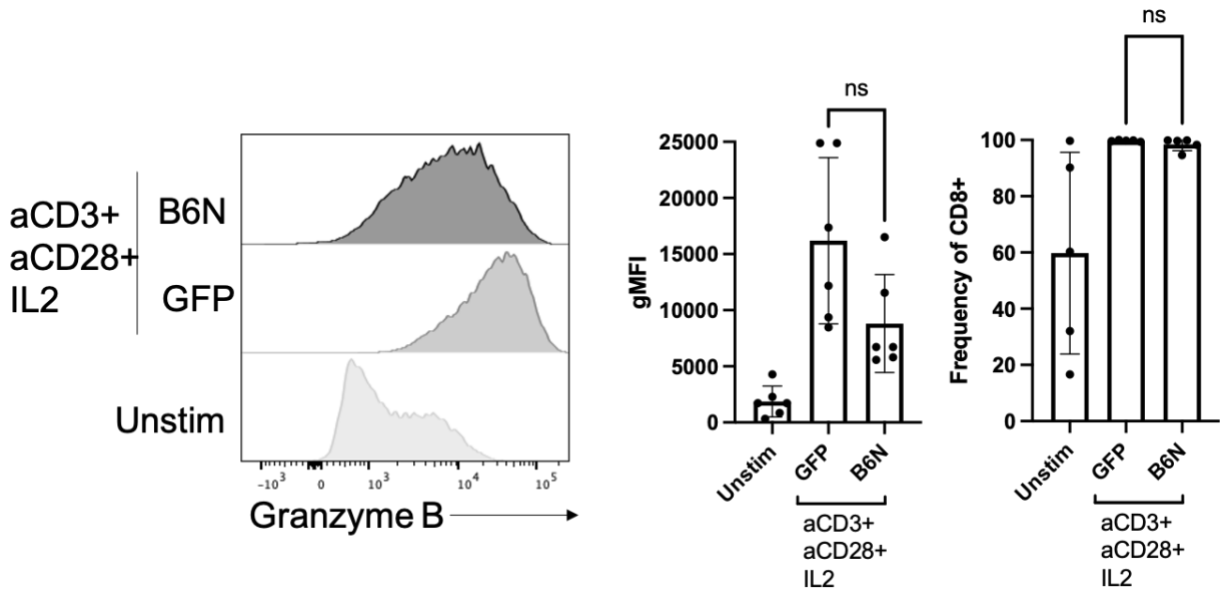


Figure 16: B6N::sfGFP Reduces Granzyme B Expression in CD8 T cells. Naïve CD8 T cells were cultured with plate-bound anti-CD3 and anti-CD28 stimulation in the presence of IL2 for 3 days. Cells were then harvested and re-stimulated with plate-bound anti-CD3, anti-CD28 and B6N::sfGFP or sfGFP. On day 3 post-re-stimulation intracellular stain for GranzymeB was performed. **A)** Representative plot and **B)** summary for gMFI and frequency for multiple donors. Statistical analysis: Ratio paired two-tailed t-test; ns, $p>0.05$

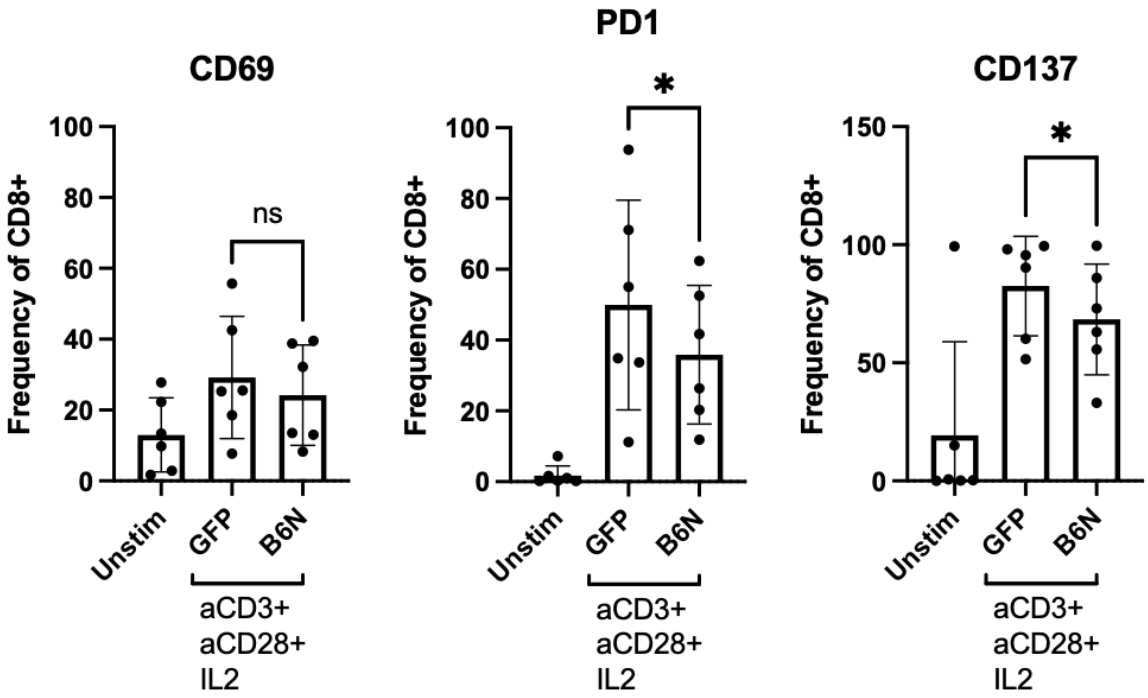


Figure 17: B6N::sfGFP Reduces Expression of Activation Associated Molecules in CD8 T cells. Naïve CD8 T cells were cultured with plate-bound anti-CD3 and anti-CD28 stimulation in the presence of IL2 for 3 days. Cells were then harvested and re-stimulated with plate-bound anti-CD3, anti-CD28 and B6N::sfGFP or sfGFP. At day 3 post re-stimulation cells were stained for surface marker expression. Data summary for CD69, PD1 and CD137 expressing cells in multiple donors. Statistical analysis: Ratio paired two-tailed t-test; ns, $p>0.05$, * $p<0.05$

β-protein Mediates Inhibition of T cell Activation via Engagement of Siglec-5

From the literature, as well as our ELISA data (Figure 11), we know that Siglec-5 binds to B6N region of the β-protein. However, whether in our assay B6N engages Siglec-5 to mediate its inhibitory effects is unclear (Figures 12-17). To understand this, we set up an assay where we pre-incubated B6N with soluble Siglec-5 Fc chimeric protein before coating it on plates, along with anti-CD3 and anti-CD28. We hypothesize that during the pre-incubation the Siglec-5 Fc will bind to B6N, and thus when coated onto plates B6N will no longer be available for binding to the endogenous Siglec-5 receptor on T cell.

Using this setting, we observed that B6N did not suppress the activation of T cells, as measured by the level of expression of the effector molecule Granzyme B (Figure 18).

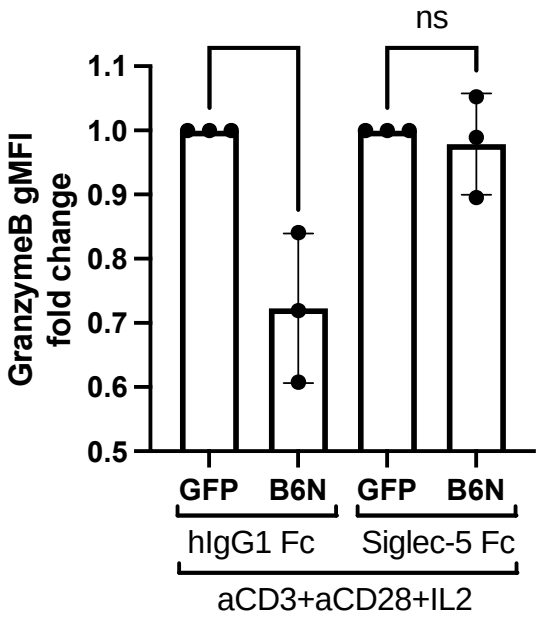


Figure 18: Limiting the Availability of B6N::sfGFP by Pre-incubation with Soluble Siglec-5 Protein, Rescues the Expression of Granzyme B in CD4 T cells. Naïve CD4 T cells were cultured with plate-bound anti-CD3 and anti-CD28 stimulation in the presence of IL-2 for 3 days. Cells were then harvested and re-stimulated with plate bound anti-CD3, anti-CD28 and B6N::sfGFP or sfGFP pre-incubated with hIgG1 Fc or Siglec-5 Fc at equimolar ratios. On day 3 post-re-stimulation intracellular stain for Granzyme B was performed. Data represents a summary for fold change in gMFI for multiple donors. Statistical analysis: one-way ANOVA, Tukey's multiple comparisons test, ns $p>0.05$, ** $p<0.01$.

Siglec-5 Engagement Suppresses the T cell-specific Anti-tumor Response *in vitro*

The data so far suggest that Siglec-5 is a checkpoint-like receptor that inhibits T cell activation. Considering the immunosuppressive functions that inhibitory checkpoint receptors, such as PD-1 and CTLA-4, play in regulating the anti-tumor T cell response, we asked if Siglec-5 provides a a mechanism by which cancers mediate T cell immune suppression. Siglec-5 ligands are generally sialic acids attached to glycoproteins and glycolipids. While we know the exact patterns of sialyation that Siglec-5 prefers, the

underlying proteins or lipids are poorly characterized. To obtain a broad idea if cancer cells express any putative ligands for Siglec-5, we stained different human cancer cell lines with a recombinant Siglec-5-Fc chimera protein. We found that several of the tested cell lines have varying levels of Siglec-5 binding, suggesting the presence of putative ligands expressed by the cancer cell lines (Figure 19).

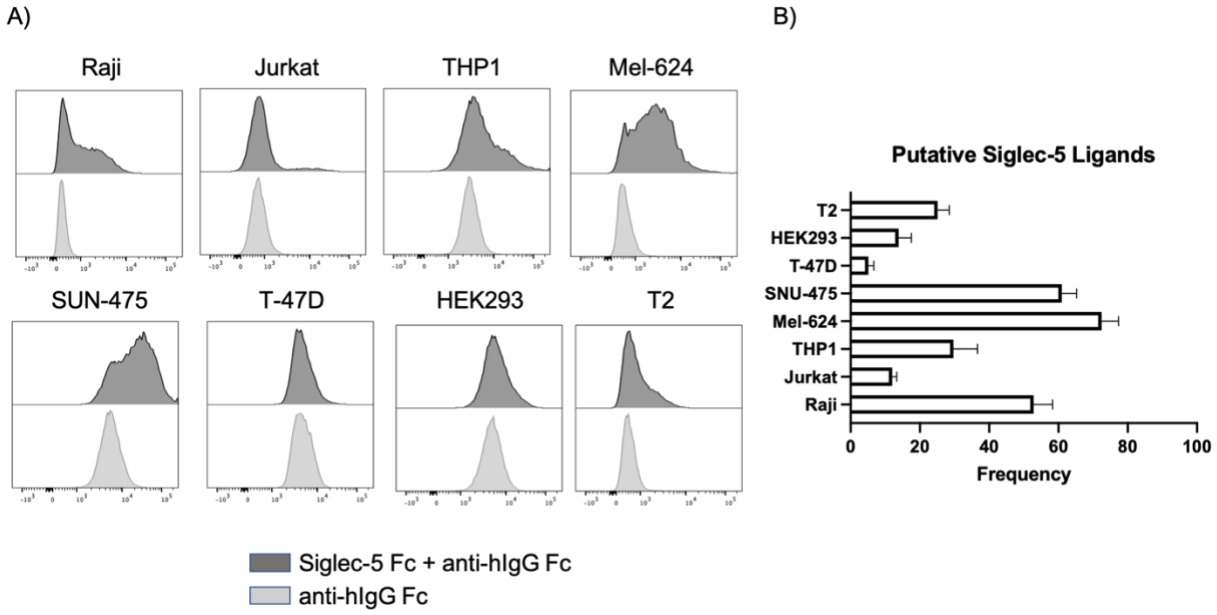


Figure 19: Putative Siglec-5 Ligands are Expressed by Cancer Cell Lines. A) Representative plots of human cancer cell lines stained with recombinant Siglec-5 Fc chimeric protein and anti-human IgG1 Fc fluorescent antibody. B) Summary of multiple staining.

Because cancer cells express putative ligands, we hypothesized that Siglec-5 is a mechanism by which cancers evade the anti-tumor T cell response. To test this hypothesis, we used adult T cells transduced with a TCR (1383i TCR) specific for the melanoma antigen tyrosinase (TCR constructs, or 1383i TCR T cells provided by Dr. Michael Nishimura's lab). We first tested and confirmed that the 1383i TCR T cells stimulated in an antigen-specific manner also follow the expression kinetics of Siglec-5 like primary T cells. In other words, resting 1383i TCR T cells have low Siglec-5

expression. Upon stimulation with T2 cells pulsed with tyrosinase peptide we see activation-dependent increase of Siglec-5 (Figure 20).

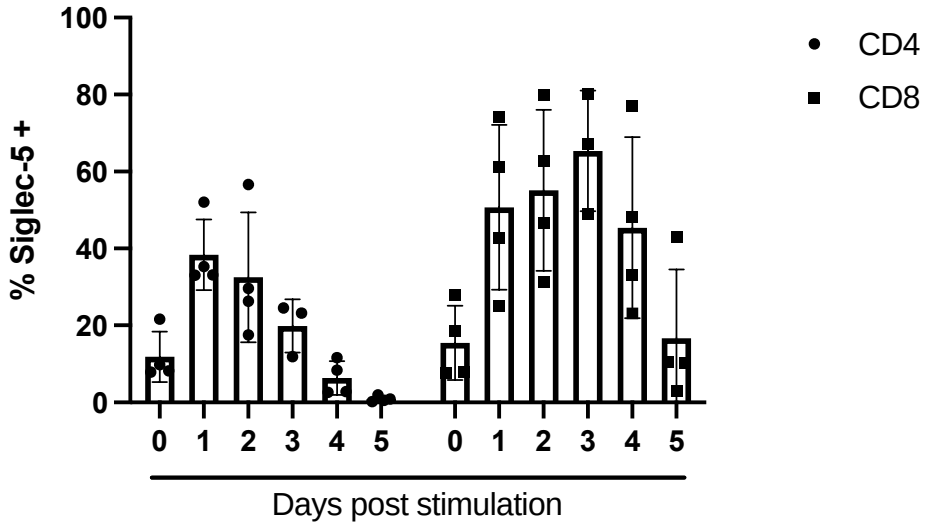


Figure 20: Tyrosinase Specific T cells Upregulate Expression of Siglec-5 After Stimulation. Adult T cells were transduced with the 1383i TCR specific for the melanoma antigen tyrosinase. Transduced T cells were enriched using the CD34t selection marker, which is expressed in-frame with the tyrosinase specific 1383i TCR and denotes 1:1 expression ratio. 1383i TCR T cells were then stimulated with a 1:3 ratio of T cells to irradiated T2 pulsed with tyrosinase, and cultured for 0, 1, 2, 3, 4, and 5 days. Siglec-5 expression within the CD4 and CD8 compartment was evaluated at each time point.

We established an assay where 1383i TCR T cells were stimulated in an antigen-specific manner for 2 days. When the expression of Siglec-5 is high, we re-stimulated the 1383i TCR T cells with the MEL624 cancer cell line, which carries putative Siglec-5 ligands, in the presence of recombinant Siglec-5 Fc chimeric protein. We hypothesize that the Siglec-5 Fc will bind and limit the availability of the putative Siglec-5 ligands expressed by MEL624 and in that way will disrupt the activation of SIglec-5 signaling in the T cells. Because Siglec-5 signaling blocks T cell activation, we expect that disrupting the Siglec-5 signaling axis will reinvigorate the T cell-specific anti-tumor response. A marker of functionality of T cells is their ability to produce cytokines and

release cytotoxic granules. The higher the frequency of such cells, the stronger the response is. In a dose dependent manner, Siglec-5 Fc treatment led to increase in the frequency of IFN-γ, TNF-α, IL-2 (Figure 21) and CD107a (Figure 22) producing CD4 T cells.

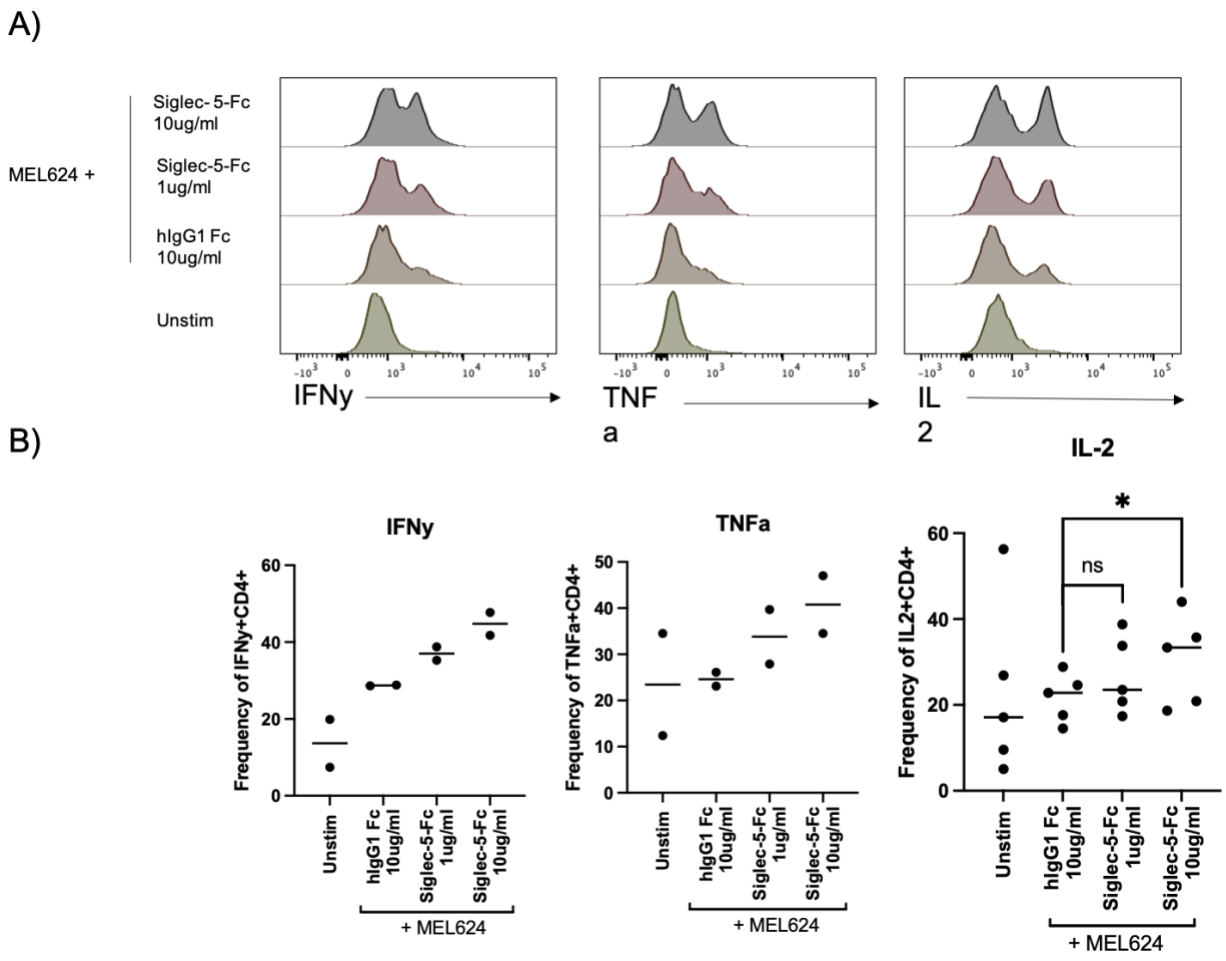


Figure 21: Disrupting the Sigle-5 Signaling Axis Re-invigorates T cell Specific Cytokine Production in Response to Cancer Cells. 1383i T cells were stimulated with T2 pulsed with tyrosinase peptide. 2 days post-stimulation, Siglec-5 expression was verified, and cells were used for stimulation with the Siglec-5 ligand carrying MEL624 cancer cell line in the presence of Siglec-5-Fc or control hIgG1-Fc protein. The assay was carried in the presence of Brefeldin A and Monensin. **A)** Representative plots and **B)** summary of multiple donors of frequency of CD4 T cells expressing IFN-γ, TNF-α and IL-2 at 12hrs post-stimulation. Statistical analysis: Ratio paired two-tailed t-test; *, $p<0.05$

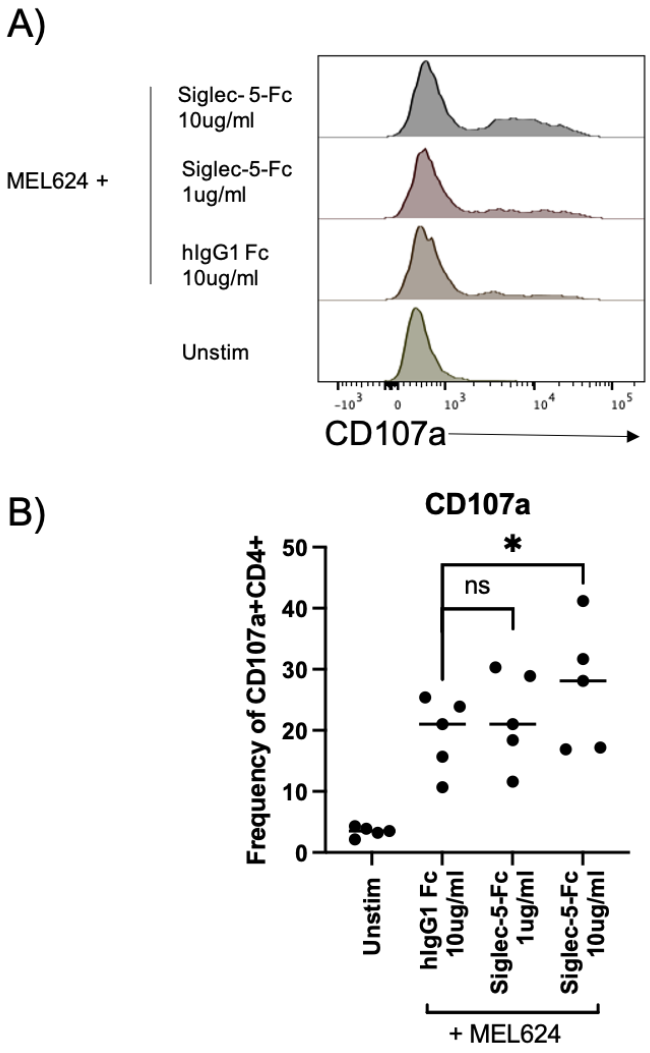


Figure 22. Disrupting the Siglec-5 Signaling Axis Re-invigorates T cell Expression of the Cytotoxic Marker CD107a. 1383i T cells were stimulated with T2 pulsed with tyrosinase peptide. 2 days post-stimulation, Siglec-5 expression was verified, and cells were used for stimulation with the Siglec-5 ligand carrying MEL624 cancer cell line in the presence of Siglec-5-Fc or control hIgG1-Fc protein. Assay was carried in the presence of BrefeldinA and Monensin. **A)** Representative plots and **B)** summary of multiple donors of frequency of CD4 T cells expressing CD107a. Statistical analysis: Ratio paired two-tailed t-test; *, $p<0.05$

Section II

Development of Novel Tools to Identify TGF-β Producing Cells

Development and Identification of Active TGF-β Specific Reagent

To develop a reagent selective for active TGF-β, a previous member of our lab, Veronica Volgina, PhD, generated a fibronectin type III (FN3) domain-based phage display library by mutating the BC, DE, and FG loops within the FN3. Phage display technology is a powerful *in vitro* technique for the development of high affinity reagents. The three loops within the FN3 domain structurally resemble the immunoglobulin complementary determining regions and serve as sites for diversification and target recognition. The library of proteins displayed onto coat protein III of M13 bacteriophages was used for several selection rounds. First, clones specific for latency-associated protein (LAP) were removed using positive selection with LAP-coated beads. Next, negatively selected phages were screened for binding to active TGF-β (Ala 279-Ser290) coated beads. Out of the 400 clones screened, 16 clones bound to TGF-β with minimal cross-reactivity to LAP (Figure 23A). Because of its highest binding affinity, the phage clone 6 (Ph6) was used for further characterization.

Ph6 was next shown to not only bind but also neutralize TGF-β biological effects. Looking at phosphorylated SMAD2/3 as a readout for TGF-β signaling, it was shown that Ph6 blocks SMAD2/3 phosphorylation in Jurkats treated with TGF-β (Figure 23B). Furthermore, it was also shown that Ph6 blocks epithelium to mesenchymal transition (EMT), a well-described process upon treatment of NMuMG cells with TGF-β. Blockade of EMT in this assay was assessed by measuring the levels of E-cadherin, a marker of epithelial, but not mesenchymal cells (Figure 23C).

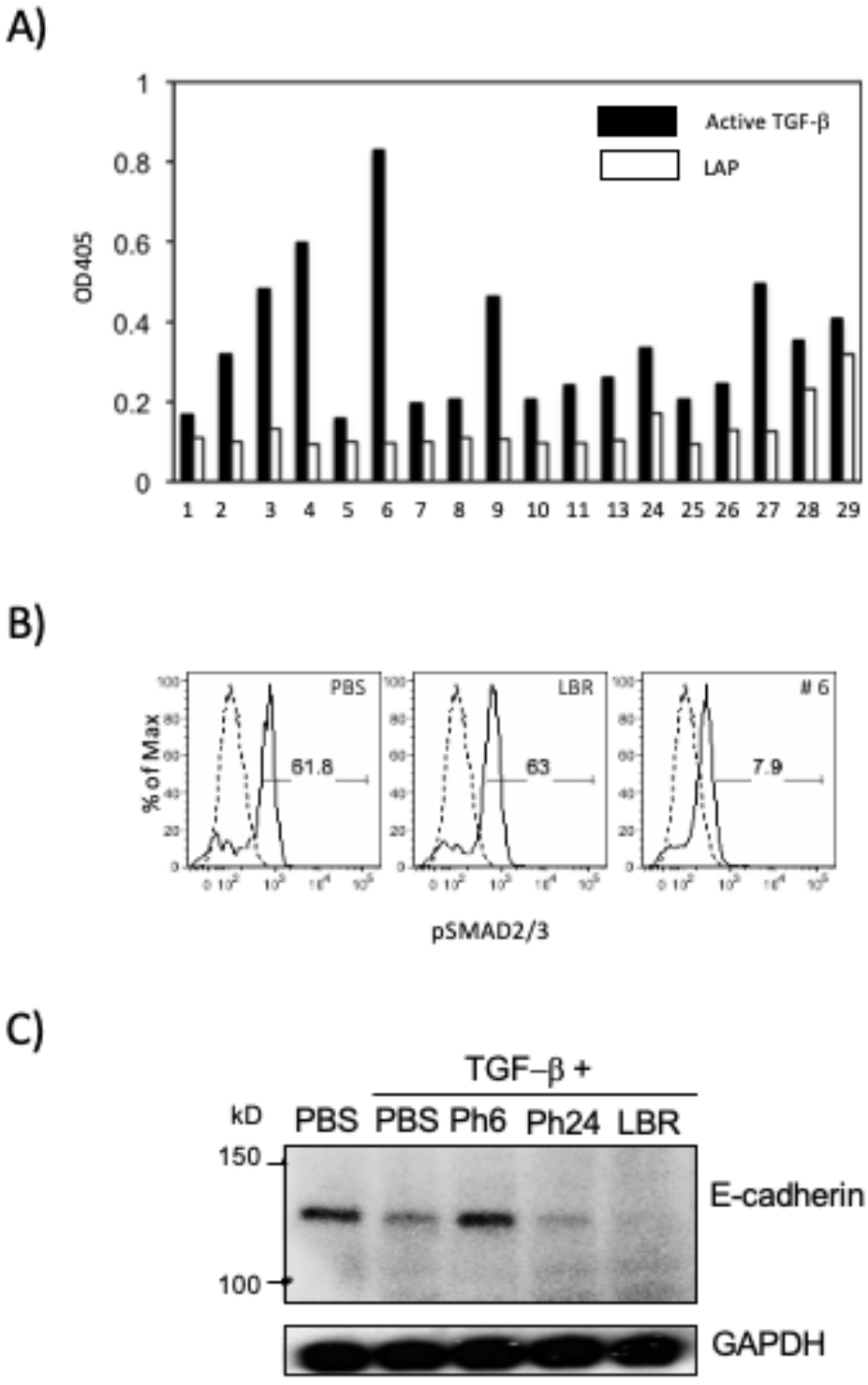


Figure 23: Development and Characterization of an Affinity Reagent Specific for Active TGF-β. A) Phage clones binding to active TGF-β -b or LAP evaluated via ELISA. The detection reagent used for detecting phage is an anti-P8 phage protein antibody. X-axis denotes each clone tested. **B)** Jurkat T cells were incubated overnight with TGF-β

(10ng/ml) preincubated with Ph6 or unselected library (LBR). Phosphorylation of SMAD2/3 was assessed by flow cytometry. **C)** NMuMG cells were stimulated for 48hrs with TGF-β (10ng/ml) or TGF-β pre-incubated with 1 OD concentration of phage clones 6, 24 or LBR. Cells were analyzed by Western blot for expression levels of E-Cadherin, a marker of epithelial, but not mesenchymal cells (Data generated by Veronica Volgina, PhD)

Detection of Membrane-bound TGF-β uUsing Ph6

Although several anti- TGF-β antibodies are commercially available, detecting of cell surface-bound active TGF-β remains challenging. To develop an alternative approach, we tested if phage clones 6 (Ph6) can be used to detect active TGF- β via flow cytometry. We prepared phages and verified Ph6 specificity for TGF-β via ELISA (Figure 24). We then labeled the phages with Alexa Fluor 488 or 594 NHS ester (Molecular Probe) and used them for staining cells for flow cytometry analysis.

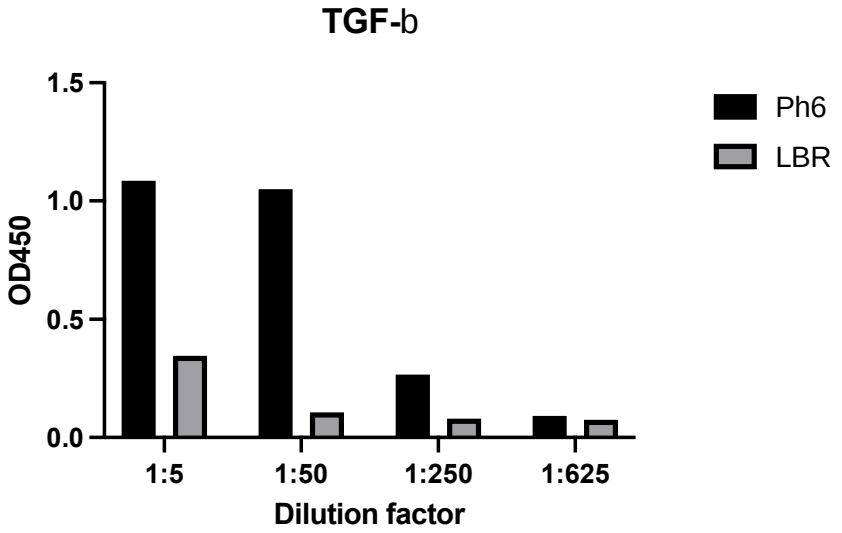


Figure 24: Verification of Ph6 Binding to TGF-β via ELISA. Phage particles were precipitated from bacterial supernatants (bacteria grown overnight) by 6% PEG 800, 300mM NaCl for 1hr at 4C. Phages were pelleted by spinning down at 12,000rpm for 20mins. Isolated phages at ssDNA OD = 9 were used for testing their ability to bind TGF-β via ELISA. Phages were detected using an anti-P8 M13-HRP antibody.

Our lab has shown that monocytes provide TGF-β to naïve CD4 T cells for regulatory T cell induction in a cell-contact dependent manner. This suggests that monocytes carry active TGF-β on their surface[375]. We hypothesized that if monocytes provide active TGF-β to naïve T cells, then we will detect active TGF-β on monocyte surfaces. Using human umbilical cord blood mononuclear cells, we found that a subpopulation of $CD14^{+}CD36^{hi}$ monocytes expresses active TGF-β (Figure 25).

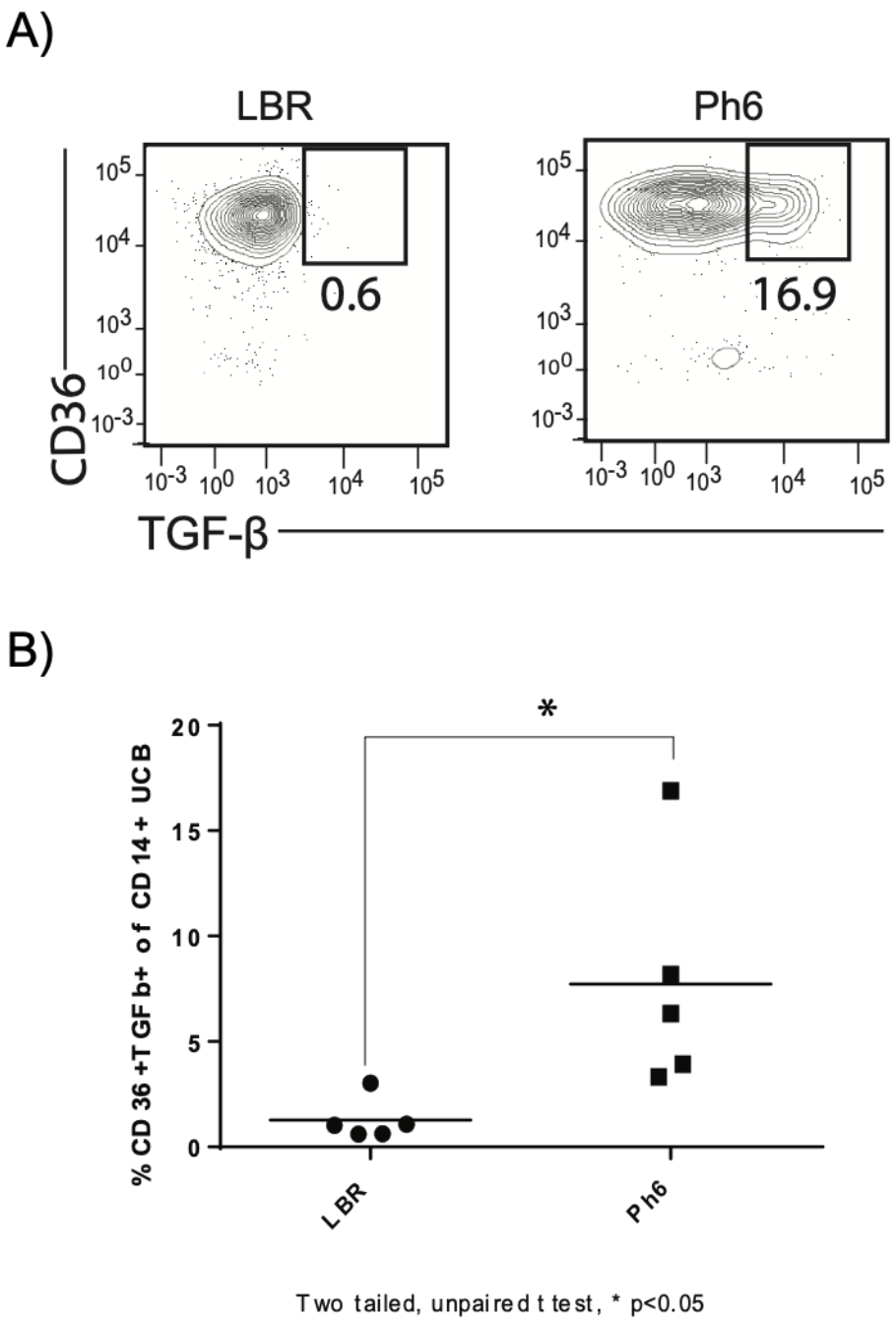


Figure 25: Surface Expression of Active TGF-β on Human CD14+CD36+ Monocytes. Flow cytometry analysis of human monocytes stained with Ph6 or LBR directly conjugated to Alexa Fluorochromes. Mononuclear cells were isolated from human umbilical cord blood and stained with antibodies against CD14 and CD36, in addition to phage particles, LBR or Ph6, labeled with AF488 or A594. Cells were stained for 1 h at +4C and then analyzed using flow cytometry **A)** Representative plots and **B)** summary of CD36+TGF-β+ within CD14+ monocytes.

Generation of Recombinant Clone 6 FN3 Based Proteins

Monobodies displayed on the phage surface can be used as affinity reagents, however, a soluble form of the antigen-binding protein might be more robust for *in vitro* and *in vivo* applications. We generated a soluble protein by using a mammalian expression system consisting of the FN3 domain fused to the constant regions (CH2 and CH3) of human IgG1, separated by the hinge region. (Figure 26). The construct was trasnfected into CHO cell line. Upon establishment of a stably producing cell line using drug selection, cells were grown and suppernatants were collected for protein purification using a protein G column.

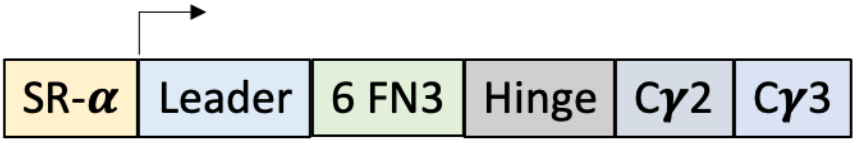


Figure 26: Schematic of 6/WT FN3-Fc Chimeric Protein. Clone 6 or WT FN3 was cloned as a fusion with CH2 and CH3 domains of human IgG1. The hinge region of human IgG1 was used as a spacer between FN3 and Fc regions. The protein was expressed under the control of a constitutively active SR-a promoter.

Recombinant 6FN3-Fc or control WTFN3-Fc affinity for TGF-β was verified using an ELISA (Figure 27). This data suggest that we have successfully expressed a recombinant protein specific for active TGF-β.

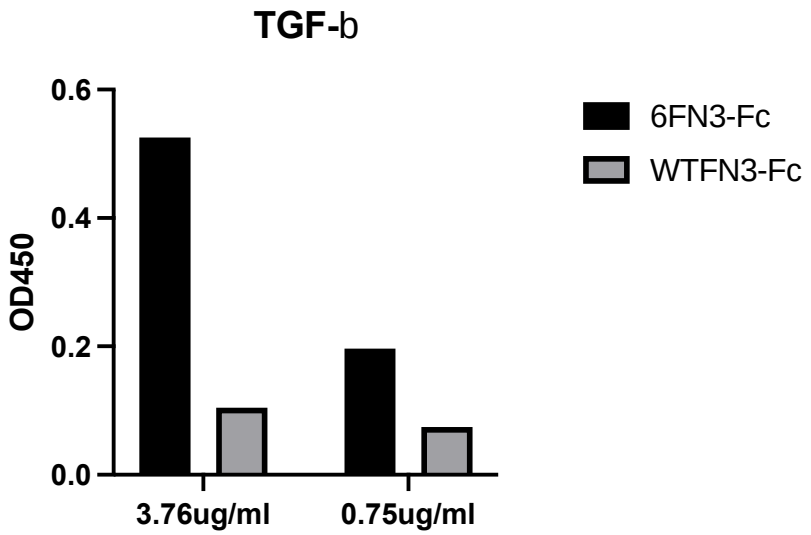


Figure 27: Verification of 6FN3-Fc Protein Binding for TGF-β. 15ng/ml of active TGF-β was immobilized on ELISA plate and 3.75ug/ml or 0.75ug/ml of 6FN3-Fc or WTFN3-Fc proteins were used as detection reagents. Anti-human IgG-HRP antibody was used to detect Fc protein binding to TGF-β.

Detection of Surface Bound TGF-β Using 6FN3-Fc

To use the proteins for analysis of TGF-β expressing cells via flow cytometry, we biotinylated the 6FN3-Fc protein. Due to poor production yield for WTFN3-Fc, as a control, we biotinylated a hIgG1 Fc protein. We verified the biotinylation and binding to TGF-β using ELISA and Streptavidin-HRP as a detection reagent (Figure 28).

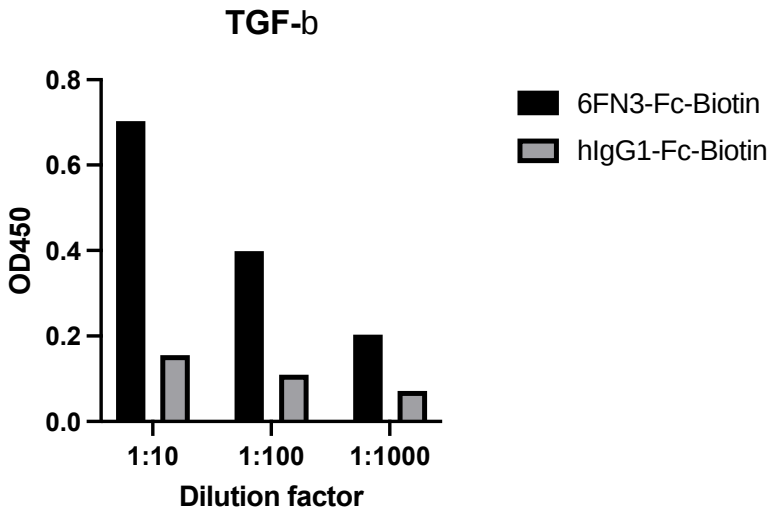


Figure 28: Verification of Biotinylated 6FN3-Fc Protein Binding to TGF-β. 6FN3-Fc and hIgG1 Fc proteins were biotinylated using a one-step biotinylation kit (Miltenyi Biotech). Successful biotinylation was confirmed by the ability of 6FN3-Fc to bind to TGF-β and its detection using Streptavidin-HRP.

However, even though 6FN3-Fc retains its ability to bind TGF-β via ELISA, it did not bind to live cells, like the Ph6 clone did (Figure 29).

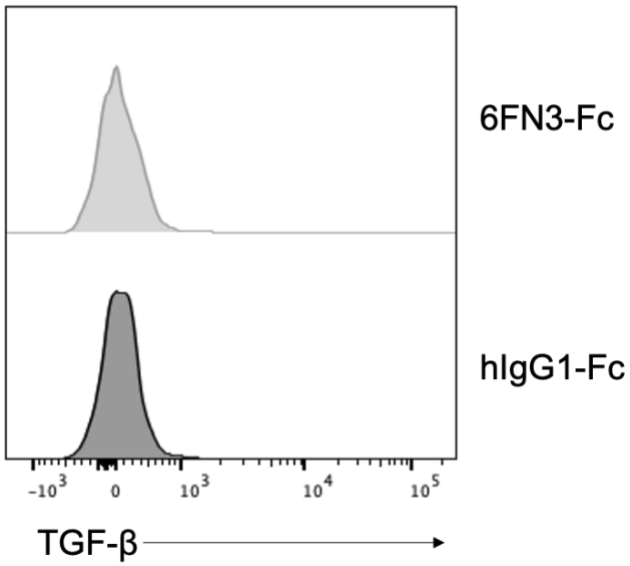


Figure 29: Recombinant 6N3-Fc Protein is not Suitable for Detection of Active TGF-β Expressed on Cell Surfaces. Mononuclear cells were isolated from human umbilical cord blood and stained with antibodies against CD14 and CD36, in addition to biotinylated 6FN3-Fc or hIgG1 Fc proteins.

CHAPTER FOUR: DISCUSSION

Section I

Siglec-5 – a Novel Checkpoint Receptor in T cells

Siglec-5 Expression in T cells is Activation Dependent

To prevent inappropriate immune activation and subsequent immune injuries, the immune system has evolved strategies to help discriminate self from non-self, or healthy from altered and infected. These strategies relay on activating different receptors that recognize molecular patterns specific for self and healthy cells. One such family of receptors is the CD33rSiglecs, a family of C-type lectins specific for sialic acids. Sialic acids are derivatives of the sugar neuraminic acid and are attached to the terminal position of glycoproteins or glycolipids. Because of their convenient location, sialic acids can easily interact and engage Siglec receptors and thus regulate host homeostasis. Most Siglecs have inhibitory functions, and their recognition of sialic acids is one mechanism by which the immune system mediates tolerance towards self. The interaction of sialic acids with Siglecs can also play a role in increasing the activation threshold of immune cells, as is the case of Siglec-2 (CD22) in inhibiting B cell receptor signaling and subsequent prevention of unwanted antibody responses against self-antigens[249]. However, in some pathologies, such as bacterial and viral infections or cancer, normal sialyation can be altered in favor of the pathogen or altered cells and result in an inappropriate immune response.

Reports from the literature suggest that human T cells have evolutionally lost the expression of most CD33rSiglecs under healthy conditions[270]. However, in pathologies such as cancer or chronic viral infections the expression of some Siglecs is upregulated[274,376]. Because both cancer and chronic viral infections provide chronic antigen stimulation of T cells, we asked if CD33rSiglec expression changes during T cell activation. We screened activated T cells for Siglecs -3, -5, -6, -7, -8, -9, and -10 and found that among all, only Siglec-5 had an activation associated pattern of expression. We detected Siglec-7 expression in small fraction of CD8 T cells, as well as Siglec-3 in both CD4 and CD8 T cells, but their expression was not associated with activation (Figure 1). We investigated the kinetics and found that Siglec-5 expression is only detectable after 48hrs of stimulation, a unique trait for most checkpoint receptors which expression is upregulated only after T cell activation (Figure 2). Interestingly, in adult T cells, the peak expression of Siglec-5 was at 72hrs post-stimulation (Figure 2A and 2B), but in perinatal T cells (from cord blood) the peak was at 48hrs (Figure 2C and 2D). Following peak expression in both adult and perinatal T cells, the levels of Siglec-5 gradually decrease and by day 7 post-stimulation the expression is completely lost. The discrepancy between the peaks of Siglec-5 expression might be coming from the different ratios of naïve versus antigen-experienced T cells, with perinatal T cells having higher frequency of naïve T cells than adult. Alternatively, these differences might be due to intrinsic differences between adult and perinatal T cells[377]. Using Western blotting, we can detect Siglec-5 protein expression as early as 24hrs post-stimulation, in contrast to its surface expression at 48hrs post-stimulation (Figure 3). These data

suggest that localization and translocation of Siglec-5 to the surface are tightly controlled, and distinct signals or mechanisms might be regulating these two processes. Further studies are needed to elucidate the delay between translation and trafficking of Siglec-5 to the cell surface.

Siglec-5 and Siglec-14 share high sequence homology within their ligand-binding domains and are described as paired receptors with opposing functions due to differences in their intracellular and thus signaling domains[303]. While Siglec-14 promotes activation of innate cells such as neutrophils and monocytes, Siglec-5 blocks it. The balance between Siglec-5 and Siglec-14 signaling is thought to fine-tune the innate immune response. Even though Siglec-5 and Siglec-14 have unique domains, most of the commercially available antibodies bind within the shared sequences and don't distinguish between the two. To determine whether T cells express both Siglec-5 and Siglec-14, or either one of the two proteins, we performed Western blot analysis on immunoprecipitated proteins from primary T cells (Figure 5). Our data suggest that the dominant protein expressed in T cells is Siglec-5. We don't exclude the possibility that small amounts of Siglec-14 are also expressed, but further verification is needed. To confirm the presence of Siglec-14 we could perform mass spectrometry on the immunoprecipitated proteins. To further strengthen our data that Siglec-5 is the dominant protein expressed in activated T cells, we also performed mRNA studies to detect Siglec-5 (Figure 4), but not Siglec-14 mRNA (Data not shown).

Siglec-5 is an Inhibitory Receptor in T cells

Siglec-5 is an inhibitory receptor that blocks the activation of innate immune cells, such as monocytes, neutrophils, and macrophages. The cytoplasmic tail of Siglec-5

contains two inhibitory domains, ITIM and ITSM, which are well conserved among other inhibitory receptors as well. Upon ligand engagement, the ITIM and ITSM of Siglec-5 get phosphorylated and serve as recruitment sites for phosphatases such as Shp1 and Shp2[281,282]. These phosphatases de-phosphorylate proteins involved in signaling cascades that activate immune cells and, in that way, prevent the activation of the immune cell. For example, it has been reported that Siglecs impair the function of other activating receptors, such as TLRs, in innate immune cells. Siglec-5 and Siglec-9 in particular bind most strongly and cross-react with all TLR receptors. Siglecs bind to TLRs in a sialic acid-dependent manner and mediate dampening of the TLR-dependent activation of the immune cell in response to pattern- or danger-associated molecular patterns (PAMPs or DAMPs) via the recruitment of the Shp phosphatases. Upon activation TLR4 for example can upregulated the expression and surface localization of neuraminidase, Neu1, that cleave the sialic acids and disrupt the Siglec binding. Subsequently the Siglec mediated restrain of TLR4 activation can be released[378,379].

Biochemically, Siglec-5 mediates its inhibitory signaling using the exact inhibitory domains, ITIM and ITSM, that the well-known checkpoint PD-1 does. PD-1 is a well-characterized inhibitory receptor that mediates suppression of T cell activation. PD-1 engagement with its ligands PD-L1 and PD-L2 results in phosphorylation of the tyrosine residues within the ITIM and ITSM domains of its cytoplasmic tail. The phosphorylated tyrosine residues then recruit Shp1/2 phosphatases. Shp1/2 mediate T cell inhibition by reducing the phosphorylation of TCRζ/ZAP70 and downstream PKC-θ signaling[231,234], as well as PI3K/Akt signaling following CD28 activation[221]. Altogether, these leads to decrease in T cell proliferation, cytokine production and cytolysis.

Based on its similarity with PD-1 (Figure 8), as well as what we know about it from innate immune cells, we hypothesized that the function of Siglec-5 is to inhibit T cell activation. To test this hypothesis, we used an over-expression system where we co-expressed Siglec-5 with NFAT or AP-1 reporters in Jurkat T cells. Our data show that Siglec-5 strongly inhibits the activity of both NFAT and AP-1 upon activation of the cells (Figure 9). NFAT and AP-1 are key transcription factors which activity regulates the expression of genes involved in the activation and effector functions of T cells. However, we did not provide additional signals that activate the Siglec-5 receptor, which raises the question how Siglec-5 is activated and how it mediates the inhibitory effects in this system. Using soluble Siglec-5 receptor we stained and found that Siglec-5 binds to ~ 20-30% of Jurkat T cells (Figure 19). These data suggest that Jurkat T cells have putative endogenous ligands that can bind and activate the Siglec-5 receptor.

Most inhibitory receptors with ITIM and ITSM motifs mediate their function through these domains. We looked at whether in T cells Siglec-5 also mediates its inhibition via ITIM and ITSM by generating a truncated mutant that lacks these domains. Interestingly, we observed only a partial restoration of NFAT and AP-1 activity (Figure 10). These data might suggest that ITIM and ITSM domains are only one of the mechanisms how Siglec-5 mediates its inhibition, and perhaps other domains of Siglec-5 are also involved in its functional outcomes. However, we also do not exclude the possibility that the lack of rescue in NFAT and AP-1 activity (Figure 10) using the truncated SIglec-5 is just an artifact of the system. Jurkat T cells can express endogenous Siglec-5 under conditions we don't yet understand. Since Siglec-5 is a

dimer, it is possible that pairing of endogenous and truncated Siglec-5 happens and leads to suboptimal Siglec-5 signaling.

Siglec-5 Follows the Expression Kinetics of Other Checkpoints in T cells

Our data show that Siglec-5 is an inhibitory receptor, and its expression is strongly dependent on the activation of T cells. This type of kinetics is well described in the literature for the expression of different checkpoint receptors in T cells. Checkpoint receptors, including co-stimulatory and co-inhibitory, play a crucial role in regulating the activation of T cells. Co-stimulatory receptors synergize with TCR signaling and contribute toward the T cell functional outcome. At peak activation however, opposing, co-inhibitory receptors begin to be expressed. Co-inhibitory receptors antagonize TCR signaling and prevent exuberant activation of T cells. Signaling through inhibitory receptors is a mechanism for mediating tolerance and modulation of the length of T cell effector function to minimize the collateral damage in the surrounding tissues. The opposing forces coming from the different checkpoint receptors, co-stimulatory and co-inhibitory, which activation depends on their ligand availability, define the fate of T cells in terms of activation, differentiation, and proliferation. When we compared Siglec-5 expression to other checkpoints we observed an overlap with PD-1 and OX40. Other checkpoints, such as CTLA-4, 41BB, and ICOS, are expressed by T cells sooner than Siglec-5, but still share a similar peak expression and downregulation (Figures 6 and 7).

Altogether, our data on the expression kinetics similarity with other checkpoints, supported with the inhibitory function that it plays in T cells, suggesting that Siglec-5 is a previously unrecognized inhibitory checkpoint receptor in T cells.

So why do T cells express multiple different checkpoint receptors upon activation? One explanation is that the ligands for these receptors are expressed at different spaciotemporal points and dictate which receptor will be activated. Furthermore, each checkpoint receptor has a distinct signaling cascade that targets a specific pathway following T cell activation. In this way, different checkpoint receptors can augment or suppress specific arms of the T cell effector functions. Finally, it is possible that T cells need so many checkpoint receptors to successfully lower the threshold for activation in response to low affinity or low abundance antigens or decrease the activation state in response to strong stimuli.

Siglec-5 Receptor Activation Using Specific Ligands Suppresses the Activation of Primary T cells

The overexpression system we used to test the functionality of Siglec-5 confirmed our hypothesis that SIglec-5 is a negative regulator of T cell activation. To test whether this holds true in primary T cells expressing the endogenous receptor, we developed a system to activate Siglec-5 using a previously described ligand, β-protein. β-protein is a membrane protein and a virulence factor expressed by certain serotypes of the Group B *Streptococcus* (GBS) bacteria. This protein encodes several distinct domains, each capable of inhibiting different arms of the immune system, such as neutralization of IgA antibodies, inactivation of the complement system and activation of the inhibitory Siglec-5 receptor. Altogether, β-protein mediates immune evasion and persistence of GBS in the host[380]. We cloned the most N-terminal region of the β-protein, B6N (aa1-152), previously described as the domain engaging and activating the Siglec-5 receptor[380]. We conjugated the B6N domain to GFP, and then expressed GFP

as a control. Using ELISA, we verified that the recombinant B6N::sfGFP protein, but not control sfGFP, binds to Siglec-5 (Figure 11). To test our hypothesis that direct activation of Siglec-5 antagonizes T cell activation, we used naïve CD4 or CD8 T cells, previously activated *in vitro* for 3 days using plate-bound anti-CD3 and anti-CD28. We used this time point to set up re-stimulation of the T cells because at day 3 all T cells express high levels of Siglec-5 (Figure 2). Re-stimulation of CD4 T cells, along with activation of Siglec-5 led to suppressed production of proinflammatory cytokines, IFN-γ and IL22 (Figure 12A), but an increase in Th2 cytokines, such as IL-4, IL-5, and IL-13 (Figure 12B). Similarly, we also observed reduced IFN-γ production from CD8 T cells re-stimulated in the presence of the of Siglec-5 activating signal. IFN-γ plays a crucial role in the clearance of GBS in neonates. CD4 depletion during GBS infection decreases the levels of IFN-γ produced and leads to an increase in the mortality of infected neonatal animals[381–383]. These data stress the importance of CD4-mediated IFN-γ production. Our data support the reported observations and provides a potential mechanism for how GBS can evade the adaptive immune response by activating Siglec-5 signaling and suppressing cytokine production. The increase in Th2 cytokines produced by CD4 T cells that we observed in this experimental set up could be justified with two potential explanations. One is that Siglec-5 signaling directly affects the differentiation, or maintenance of Th2 cells. To test this hypothesis, we would perform stimulation of naïve CD4 T cells under Th2 polarizing conditions in the presence of signals that also activate Siglec-5. If Siglec-5 signaling directly contributes towards Th2 differentiation, we expect to see increased frequency of Th2 cells. A second explanation is that the increase in Th2 cytokines is simply due to the reduced production of IFN-γ. IFN-γ and IL-4 mediate

antagonizing physiological responses against each other. IFN-γ expression is triggered by APCs-derived IL-12 which can only signal into activated T cells. IL-12 signaling in T cells leads to more IFN-γ production. IFN-γ creates a positive feedback loop that markedly increases both IFNγ by T cells and IL-12 by APCs. Furthermore, IFN-γ also triggers the expression of the key transcription factor for the Th1 lineage, Tbet. Tbet itself activates more IFN-γ production and IL12 receptor expression, but importantly directly restricts the polarization of T cells into the Th2 lineage[75,384,385]. Similarly, IL-4, the key Th2 cytokine is responsible for induction of GATA3 expression, a transcription factor responsible for the development of the Th2 lineage. Together with GATA3, IL-4 silences the expression of IFN-γ in T cells, and counters the activity of Tbet[76,77]. It is possible that in our system, Siglec-5 targets and suppresses the production of IFN-γ in T cells. Subsequently, the production of Th2 cytokines increases due to reduced IFN-γ levels that can counter Th2 cytokine production. Alternatively, the decrease in IFNγ and increase in Th2 cytokines might be coming from changes in TCR signaling strength in response to Siglec 5 signaling. Siglec-5 mediates its effects by recruiting Shp phosphatases to phosphorylated tyrosine residues within the ITIM and ITSM of its cytoplasmic tail. Engagement of Siglec-5 could lead to de-phosphorylating vital proteins involved in the TCR signaling cascade, thus decreasing TCR mediated stimulatory signals. While development of Th1 and Th2 lineages is driven primarily by the specific cytokines, the strength of the TCR signal in response to antigen plays a role too. Antigens that bind to TCR with high affinity or are present in abundance induce strong TCR signaling cascades and trigger the differentiation of Th1 cells. In contrast, antigens with weak affinity for TCR or low abundance trigger Th2 cell differentiation[386,387]. By

decreasing the strength of the TCR signaling, Siglec-5 might be favoring the production of Th2 cytokines over the Th1 cytokine IFN-γ.

Furthermore, in the presence of the β-protein signal that activates Siglec-5, CD4 T cells have reduced expression of Granzyme B. Granzyme B is a serine protease most widely known for its lytic activity towards target cells. Both CD4 and CD8 T cells produce Granzyme B in response to altered or infected cells. Besides its role in mediating cell killing, Granzyme B also plays other, intrinsic roles in CD4 T cells, such as controlling activation-induced cell death of specific subsets. Suppression or deficiency of Granzyme B production for example, reduces Th2 cell death and increases animals' susceptibility to allergen-induced asthma[388]. Furthermore, one study reported that Granzyme B is also involved in the differentiation of CD4 T cells, with Granzyme B sufficient Th1 cells producing more IFNγ, compared to Granzyme B deficient ones[389]. Considering this information, we could speculate that Siglec-5 targets Granzyme B production in CD4 T cells, and its reduced expression might play a role in the lowered levels of IFN-γ produced. Furthermore, lower Granzyme B could also be allowing for increased survival of Th2 cells, responsible for the increase in Th2 cytokines that we also observe. Upon recognition of infected cells, killer cells deliver cytotoxic granules containing granzymes to induce apoptosis. Not only do granzymes target the infected host cell, but also the intracellular bacteria. Through degrading critical proteins in their electron transport chain complex, Granzymes increase the oxidative stress by generating reactive oxygen species that kill the bacteria[390]. However, Granzyme B released from immune cells can trigger a multistep cell death program in both intracellular and extracellular pathogens. By cleaving a conserved set of proteins

among many bacteria, Granzyme B can directly disrupt key biosynthetic and metabolic pathways that are vital for bacterial survival[391]. Since GBS expresses the ligand for activating the Siglec-5 receptor, we can hypothesize that during an infection with this pathogen, T cells are less likely to directly kill both infected host cells, but also GBS itself in a Granzyme B dependent manner. Granzyme B production by CD8 T cells did not reach statistically significant reduction by Siglec-5 receptor engagement. Pro-inflammatory effector functions in CD4 T cells are regulated by the transcription factor Tbet, as T cells deficient for Tbet are defective in their ability to make IFN-γ. In contrast, in CD8 T cells T-bet deficient cells still exhibit normal IFNγ production and cytolytic activity. T-bet independent CD8 T cells functionality is due to the complementary functions of another transcription factor, Eomes[392,393]. Based on the slightly different response we get from CD4 and CD8 T cell-mediated Granzyme B production upon engagement of the Siglec-5 receptor, we can hypothesize that Siglec-5 signaling affects and reduces Tbet expression or function. In such a scenario, the reduction of Tbet affects CD4 T cell production of Granzyme B because Tbet is the master regulator of the effector functions. However, in CD8 T cells, reduced Tbet expression/function doesn't influence Granzyme B production as much, because of Eomes, which compensates for the lack of Tbet. To test this hypothesis, we would need to look directly into the expression and/or function of Tbet in T cells stimulated in the presence of Siglec-5 activating signals.

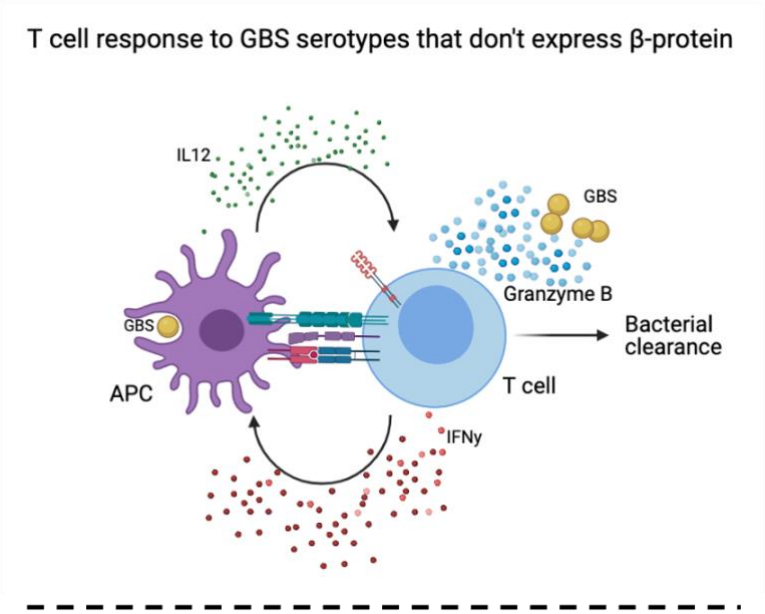
We confirmed that B6N mediates its inhibitory effects via Siglec-5 by performing an assay where B6N was pre-incubated with soluble Siglec-5 Fc protein before coating the pre-incubated mix on the plate. This way, the B6N is not available for binding by the

endogenous Siglec-5 receptor once the T cells are added. Using this system, we observed that we could restore the B6N mediated suppression of Granzyme B production in CD4 T cells (Figure 18). These data suggest that direct activation of Siglec-5 antagonizes the activation of T cells and subsequent effector functions.

Altogether, our data from studying T cell effector functions in the presence of GBS-derived signals that activate the Siglec-5 receptor allow us to propose a model where GBS can use different mechanisms to not only prevent activation of the innate immune response as described previously, but also the adaptive immune response. We propose that during infection, GBS drives suboptimal activation resulting in reduced production of IFN-γ as well as reduced expression of Granzyme B by CD4 and CD8 T cells. By reducing the levels of IFN-γ produced, GBS not only decreases the pro-inflammatory responses that IFN-γ itself promotes, but also the anti-microbial effects that IFN-γ can stimulate in antigen-presenting cells[394]. Furthermore, by suppressing Granzyme B production, GBS could decrease targeted killing of infected cells or extracellular bacteria. Reduction in Granzyme B can potentially directly affect the differentiation of IFN-γ producing T cells as well. By exploiting the inhibitory functions of Siglec-5, GBS can successfully evade the T cell-specific immune response and reduce the overall bacterial clearance (Figure 30). Using this knowledge, we can suggest that immune therapies targeting Siglec-5 and preventing its activation could be beneficial for the treatment of GBS. Checkpoint immune therapies for the treatment of infectious diseases are currently a topic of investigation, as checkpoint-mediated inhibition of T cell activation can contribute significantly to the pathogenesis of infectious agents. Such

therapies would be to reverse and improve the functional state of exhausted T cells during chronic infections such as HIV, malaria, and hepatitis B virus[395].

A)



B)

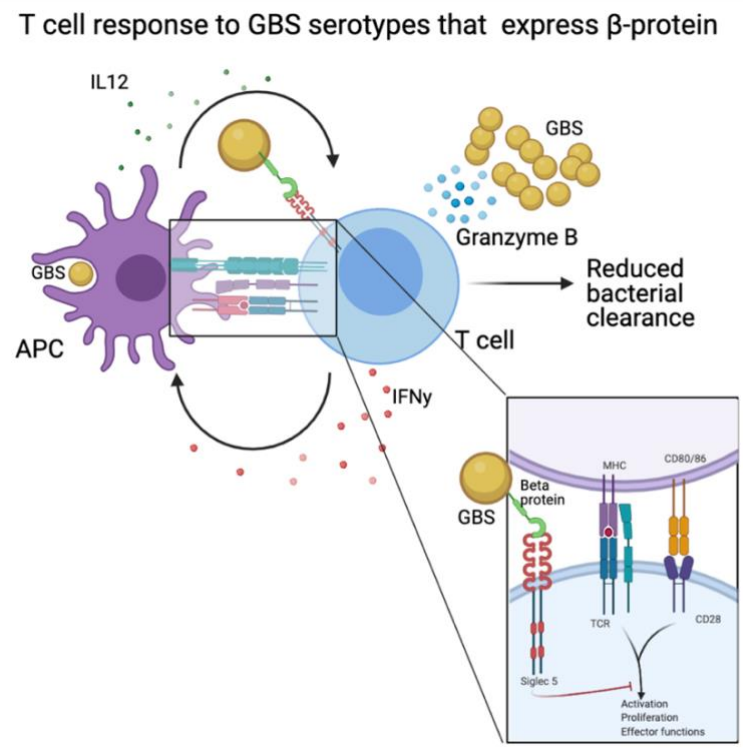


Figure 30: GBS Serotypes That Express β-protein Suppress the Adaptive Immune Response and Decrease Overall Bacterial Clearance. A) GBS serotypes that don't express the β-protein virulence factor are cleared by the robust innate and adaptive immune response in response to the pathogen **B)** GBS serotypes that express the β-protein virulence factor suppress the immune response by activating Siglec-5 in T cells thus decreasing their IFN-γ and Granzyme B production leading to decreased IFNγ mediated anti-microbial responses coming from APCs as well as decreased Granzyme B killing of both bacteria and bacteria-infected APCs.

Siglec-5 Signaling Axis is a Mechanism by which Cancers Suppress the Anti-tumor T cell Response

To escape detection by the immune system, cancers adopt mechanisms for evasion. One such mechanism is alteration and increase in glycosylated proteins and lipids, with the addition of sialic acid glycan moieties being one of the most common modifications. Altered glycosylation can increased the branching of N-glycan structures, which affects cell-cell adhesions and allows cancer cells to dissociate and metastasize. Changes in glycosylation of growth factor receptors can alter their signal transduction pathways and result in modulated cancer cell growth. Increased glycosylation can also cloak the growing tumor, preventing the recognition of tumor specific-antigens and subsequent targeted immune response[396,397]. Finally, cancers can alter their glycosylation pattern in a way to engage different receptors from the lectin family which regulate the inflammatory and immune response against the cancers. Tumor-derived sialic acids are well described to engage Siglec receptors on innate immune cells in the tumor microenvironment. For example, the activation of Siglec-7 and Siglec-9 suppress the inflammatory response in monocytes and macrophages while increasing the expression of immune suppressive cytokines, such as IL-10[398]. Altogether, altered glycosylation, and sialyation in particular, play an essential role in mediating

suppression of the immune response and thus evasion of cancer recognition and elimination.

Our data suggest that Siglec-5 is a negative regulator of T cell activation. Knowing from the literature that sialic acids, the ligands of Siglecs, are often upregulated and used by cancers to evade the immune response, we asked if the SIglec-5 signaling axis plays a role in cancer evasion. We stained cancer cell lines from different tissues using a soluble Siglec-5 receptor and found that Siglec-5 can bind to varying degrees (Figure 19). While we don't know what precisely soluble Siglec-5 is binding to, these data suggest that cancer cell lines express putative ligands that can bind and activate Siglec-5. Our observations show that cancer cell lines have distinct subpopulations that Siglec-5 can or cannot bind to. In data not shown, we observed that the degree of binding of Siglec-5 to the cancer cells depends on the confluency of the cells. This observation could suggest that Siglec-5 binding depends on different metabolic, cell cycle, or stress-related states of the cells. Even though Sialic acids are the main described ligands for Siglec-5, protein ligands such as β-protein do exist. Further studies are needed to evaluate what Siglec-5 is binding to on the different cancer cells. We hypothesized that disrupting the Siglec-5 receptor/putative ligands interaction with cancer cells could re-invigorate the anti-tumor T cell response. One of the cancer cell lines with the highest levels of Siglec-5 binding was the melanoma cell line MEL624. To test our hypothesis, we used engineered melanoma-specific T cells. These cells are transduced with a T cell receptor specific for the tyrosinase tumor antigen and can be activated in an antigen-specific manner. If cancer cells use Siglec-5 to suppress the T cell response, then blocking the ligand availability, using soluble

Siglec-5-Fc chimeric protein, will reinvigorate the T cells' effector functions, such as cytotoxic granule release and cytokine production. We found that in a dose dependent manner, blocking Siglec-5 putative ligand availability on cancer cells leads to increased frequency of CD4 T cells producing IL-2, IFN-γ and TNF-α. Cytokine production by tumor-specific cells is an essential arm of the anti-tumor T cell response, as it represents the effector functions of the T cells that eventually lead to the killing and regression of the tumors. IFN-γ, for example, is a pleiotropic cytokine that overall coordinates immune surveillance and establishes an effective adaptive immune response. By enhancing antigen presentation, through upregulating MHC molecule expression, and the whole antigen processing and presentation machinery[399,400], IFN-γ initiates antigen exposure and subsequent triggering of a targeted immune response. As the major cytokine produced by pro-inflammatory Th1 T cells and cytotoxic CD8 T cells, IFN-γ not only orchestrates the effector response but also contributes to the maintenance and differentiation of these cells. Finally, IFN-γ can directly induce tumor cell killing through the activation of various mechanisms such NADPH-dependent cell killing, production of NO, depletion of tryptophan, as well as upregulation of lysosomal enzymes[401]. The role of TNFα in cancer is controversial, with some studies reporting anti- and others pro-tumorigenic effects. TNFα can be produced by cancer cells to promote their growth, metastasis, and angiogenesis. However, TNFα produced by T cells contributes to cancer cell death, both directly, but also through the activation of cytotoxic programs in other immune cells. It has been reported that TNFα plays an essential role during priming, proliferation, and recruitment of T cells to the cancer site. Furthermore, this necessity is only true under suboptimal T cell activation conditions, as

is cancer, where co-stimulation and cytokines are limited[402]. IL-2 is the critical cytokine required for the growth of T cells[403]. IL-2 is predominantly secreted by CD4 T cells, but CD8, NK and dendritic cells can also produce some levels too[404,405]. Not only is IL-2 essential for the survival and growth of T cells, but also for the differentiation of naïve CD4 T cells into different subsets[406–408], enhancement of the cytotoxic program in CD8 T cells[409], and the suppressive functions of regulatory T cells[408,410]. Because of its importance in sustaining T cell survival as well as functionality, IL-2 has been a candidate for cancer immunotherapy since its discovery in 1976, and first received FDA approval for treatments in 1992. Nowadays, IL-2 is used in monotherapies, or in combination with other therapies such as chemo, immune checkpoint blockade and adoptive cell transfer. Besides cytokines, we also see increased frequency of cells expressing CD107a, a marker for cytotoxic degranulation. CD107a, also known as LAMP-1, is a lysosome-associated molecule that marks cells that have released cytotoxic granules (CGs). CGs are specialized lysosomes that comprise of granzymes and perforins. Perforins can bind target cells and create membrane holes through which granzymes can be delivered into the cytoplasm where they induce cell apoptosis[411–413].

Altogether, the increased frequency of cytokine-producing cells, as well as cells that have undergone cytotoxic granule release suggest that blocking the Siglec-5 signaling axis reinvigorates the effector functions of tumor-specific cells. Based on these in vitro results, we hypothesize that *in vivo*, countering the activation of Siglec-5 would enhance the T cell response against the cancer. The increased production of IL-2 will support the growth, differentiation, and maintenance of the tumor-specific T cells. The increased IFN-γ and TNF-α production will lead to enhanced direct killing of the cancer

cells, as well as increased recruitment and functionality of the T cells, followed by increased antigen presentation and targeted cancer cell killing by cytotoxic granule release. We did not measure direct tumor cell killing; however, based on the increased cytokine production and granule release, we hypothesize that cytolysis is increased as well (Figure 31).

In the assay system used here, T cells are initially stimulated under optimal conditions. We used the APC-like cell line T2 pulsed with tyrosinase to activate and induce Siglec-5 expression, before re-stimulating the cells with the cancer cell line MEL624 to measure the effects of disrupting the Siglec-5 signaling axis. Cancer cells often drive the development of anergic T cells, which don't produce cytokines with maximum capacity. In cancer, anergic T cells result from repeated antigen stimulation or stimulation under suboptimal conditions where co-stimulatory signals are lacking. We hypothesize that this is the reason why CD8 T cells do not produce any cytokines during the restimulation with the cancer cell lines. However, the assay system of re-stimulation mimics the repeated stimulation of T cells in the tumor microenvironment, and the reinvigoration of CD4 T cell functionality with blocking of Siglec-5 signaling could have a great therapeutic implication.

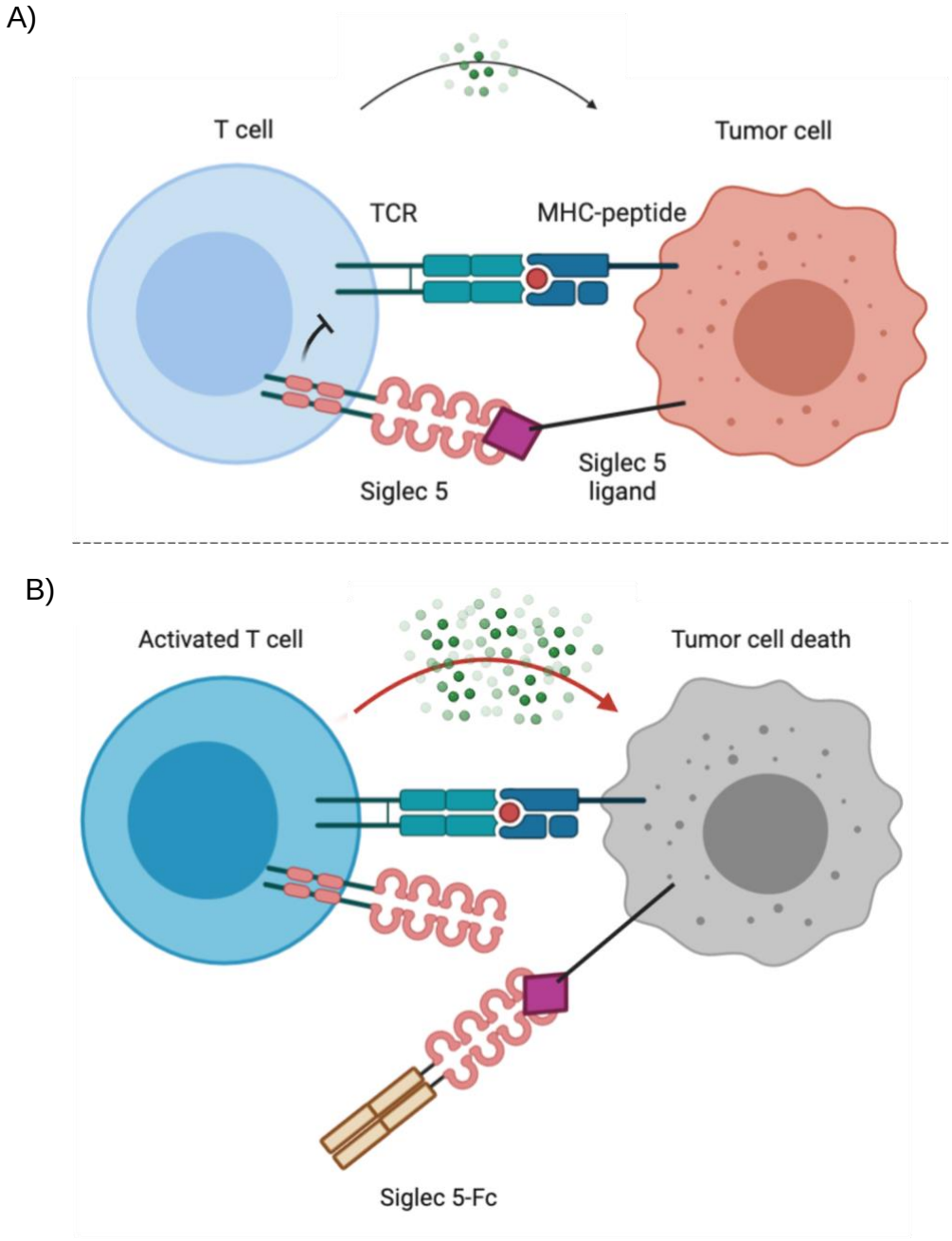


Figure 31: Siglec-5 Signaling Axis is a Mechanism by which Cancers Evade the T cell-specific Immune Response. A) Cancers increase their surface sialyation and, in that way, can engage the Siglec-5 receptor expressed on activated T cells. Subsequently, Siglec-5 dampens the anti-tumor T cell response, allowing for cancers to progress. B) Blocking ligand availability using soluble Siglec-5 receptor disrupts the Siglec-5 signaling axis and reinvigorates the T cell response to cancers.

Siglec-5 as a Target for Cancer Immune Checkpoint Therapy Development

Checkpoint receptors have become a major target for designing cancer immunotherapies. Immune checkpoint blockade therapies work by releasing the breaks and allowing T cells to mount a robust immune response. To date, across 14 different malignancies, the FDA has approved 7 different drugs, targeting 3 different inhibitory checkpoints, namely PD-1, PD-L1 and CTLA-4. The approval of these therapies has revolutionized the way malignancies are treated nowadays and has brought hope and commodity for many patients. However, based on a 2018 study, even though ~43.6 % of cancer patients in the US are predicted to respond to immune checkpoint therapies, the percentage of patients that respond is ~ 12.5%[414]. This low response rate is due to either primary or acquired resistance and suggests that blocking one inhibitory pathway is not enough to rescue the T cell response, as compensatory mechanisms by other checkpoint receptors are upregulated to prevent T cell activation. As a result, extensive research has been focusing on identifying new checkpoint receptors. Currently, emerging immune checkpoint targets such as lymphocyte activation gene-3 (LAG-3)[415], T cell immunoglobulin and ITIM domain (TIGIT)[416], T cell immunoglobulin and mucin-domain containing-3 (TIM-3)[417], V-domain Ig suppressor of T cell activation (VISTA)[418], B and T cell lymphocyte attenuator (BTLA)[419] and B7 homolog 3 protein (B7-H3)[420] are all under investigation in pre-clinical or clinical trials. Our data show that Siglec-5 is a previously unidentified checkpoint receptor, that mediates strong inhibitory effects leading to suppressed T cell responses against tumor cells, similarly to all other inhibitory checkpoints described to date. The identification of yet another checkpoint is of great importance as it can serve as a target for the development of novel checkpoint

inhibitor therapies. From our work we see that the single agent blockade of Siglec-5 strongly reinvigorates the T cell response. Alone, or in combination with other checkpoint targets, blockade of Siglec-5 can serve as a strategy to prevent cancer immune evasion.

Siglec-5 as a Target for Immune Therapies to Treat Autoimmune Disorders

Cancer and autoimmunity are on the opposite sides of the balance that maintains immunological homeostasis. In cancer, the immune response is suppressed and therapies such as checkpoint inhibitors work well to restore the immune response. However, a major side effect of checkpoint inhibitor therapies for cancer is the appearance of immune-related adverse events (IRAEs), which manifest as autoimmune phenotypes in a wide range of organs such as the skin, gut, lungs, kidneys, pancreas, or hematopoietic system[421]. Furthermore, genetic ablation of checkpoint receptors in laboratory animals[215,216,226,227], along with rare cases of human deficiencies[422], result in severe autoimmune disorders in multiple organs. These studies stress the importance of checkpoint receptors in maintaining immunological tolerance and raise the possibility of targeting and blocking their activity as potential treatments for autoimmunity. The goal of such therapies would be to target and increase their inhibitory activity to suppress overreactive immune responses. Attempts to use checkpoints receptors as a target for autoimmune therapies are already underway, with efforts to develop agonists agents or antibodies that enhance the inhibitory signaling of checkpoint receptors such as PD-1, BTLA, TIGIT, TIM3[423]. Identifying Siglec-5 as a novel inhibitory checkpoint receptor adds another potential target for autoimmunity therapy development. Based on our data showing the strong suppression that Siglec-5 activation can mediate, we hypothesize

that in auto reactive T cells this could be beneficial and would serve to dampen the inflammation and effector functions targeted against self-antigens. Combination therapies are superior to single agent in cancer treatments, and we can predict that targeting multiple inhibitory pathways in autoimmunity may be more efficient in mediating immunosuppression. Identifying new checkpoint receptors for the design of immune therapies is of great importance as it would allow for the careful design of therapies that combine different targets, with or without redundant signaling pathways, and would help to really unravel the true potential that this class of therapeutics have.

Section II

Development of Novel Tools to Identify TGF-β Producing Cells

Transforming growth factor β (TGF-β) is a pleiotropic cytokine involved in regulating of many cellular processes[372]. Initially secreted as a part of an inactive complex with the latency-associated protein (LAP), TGF-β signaling is initiated only upon release of active TGF-β from the inhibitory complex[424]. However, even though the biological functions of TGF-β are well established, where, when, or how latent TGF-β is activated is not well understood. Several studies suggest that active TGF-β is expressed on cell surfaces[329,339]. Although several anti-TGF-β antibodies are commercially available, detecting cell-surface bound active TGF-β remains challenging and is not well documented. The most widely used 1D11 antibody clone serves well for neutralizing TGF-β, but not for immunofluorescent detection purposes. This technical hurdle leaves a knowledge gap in the TGF-β field.

Using phage display technology, we generated a reagent that binds active but not latent TGF-β. We chose phage clone 6 (Ph6) for further analysis and

characterization because of its superior affinity for active TGF-β compared to the other clones (Figure 23A). We also confirmed that not only does Ph6 bind to TGF-β, but it also neutralizes TGF-β's physiological functions, such as phosphorylation of SMAD2/3 proteins, as well as the induction of epithelial to mesenchymal transition (Figure 23B and 23C). From our previous studies we knew that human CD14+ monocytes require cell-cell contact to induce TGF-β mediated differentiation of naïve T cells into regulatory T cells[375]. Using fluorescently labeled Ph6 we stained and found that Ph6 binds to human CD14+ monocytes, suggesting that these cells express active TGF-β on their surface. Human CD14+ monocytes have been reported to activate high levels of TGF-β as a mechanism to dampen inflammation. Mechanistically, these cells use the integrin αVβ8 to mediate the activation of TGF-β. The integrin expression and the ability to activate TGF-β is still maintained even when monocytes differentiate into macrophages. This type of macrophage plays a vital role in maintaining tolerance in the gut. During active inflammatory bowel disease, the frequency of these macrophages and the levels of integrin αVβ8 are significantly decreased, stressing their importance during inflammatory responses and the maintenance of intestinal homeostasis[425]. Studies from our lab and others show that CD14+ monocytes also express Thrombospondin 1 (TSP), another well characterized cofactor involved in the conversion of latent to active TGF-β [327,375]. Altogether, our lab and others have documented that CD14+ monocytes mediate TGF-β specific functions leading to tolerance and tissue homeostasis. But, for the first time we report that CD14+ monocytes can perform such functions by activating and directly presenting active TGF-β to the target cells in the environment. However, active TGF-β does not have a membrane anchoring region, and its presence on the cell

surface would suggest that unidentified mechanisms enable cells to retain active TGF-β on the cell surface. Our method of detection of surface-bound active TGF-β can serve as a tool to study how TGF-β is retained on the cell surface, what other molecules it associates with on the surface, and how it interacts with other cells using biochemical or immunofluorescent methods.

We sought then to generate a recombinant protein expressing the fibronectin type III domain, FN3, (i.e., ligand-binding domain) of Ph6. Previously, our lab had tried to generate recombinant proteins containing just the FN3 domain of Ph6. However, those experiments were unsuccessful, and no soluble proteins were recovered (data not shown). Instead, we generated a FN3-hIgG1 Fc chimeric protein that contains the FN3 sequence of Ph6 conjugated to the Fc region of hIgG1 (6FN3-Fc). As a control we used the wild-type FN3 domain (WTFN3-Fc). The FN3 and Fc domains were spaced with the hinge region of hIgG1, which allows for flexibility and dimerization of the expressed proteins. We successfully expressed the FN3-hIgG1 Fc chimeric proteins using a eucaryotic expression system and verified their ability to bind active TGF-β via ELISA. However, the proteins were not suitable for staining purposes, as we did not detect any binding to human CD14+ monocytes, like we saw with the Ph6. We tried several different detection methods for Clone 6 FN3-hIgG1 Fc binding to CD14+ monocytes: 1) Fluorochrome conjugated anti-human IgG Fc; 2) direct conjugation of Alexa Fluorochromes to clone 6 FN3-hIgG1 Fc; 3) biotinylating of clone 6 FN3-hIgG1 Fc and found that in all cases the recombinant protein can bind active TGF-β by ELISA, but not active TGF-β on cell surfaces. One thing to remember about the phage platform is that the FN3 domain is expressed as a fusion of the minor coat protein III, which has at least

4-5 copies expressed per phage particle, in contrast to the two binding sites in our recombinant protein. Thus, it is possible that in comparison to the recombinant protein, the phage particles have higher avidity and can detect active TGF-β on CD14+ monocytes. In the future, we will try to optimize the labeling, as well as staining conditions using the recombinant proteins.

Potential for Diagnostics and Therapeutic Tool Development

By having a reliable reagent that binds and detects active TGF-β specifically, we can start asking basic science questions about where and how is latent TGF-β converted into the active form. Furthermore, we can also use this reagent as a diagnostic tool intended to identify cells and tissues that express active TGF-β and predict how those cells regulate tissue homeostasis. In addition, numerous studies have reported the role of TGF-β in promoting tumor growth. Having a tool that can help us evaluate the presence of TGF-β in different cancers can help design targeted strategies to overcome TGF-β pro-tumorigenic functions.

Besides using it as a diagnostic tool, Ph6 also has the potential for therapeutic development. It is well established that cancer progression relies on avoiding immune surveillance and developing an immunosuppressive environment that hinders the anti-tumor immune response. One aspect of TGF- β mediated cancer immune evasion is the restriction of differentiation and activation of anti-tumor T cells. Single-agent inhibition of TGF-β in cancer treatment has yielded inconsistent results with limited clinical significance, and combination therapies may be a better approach to harness TGF-β immunosuppression. One such approach to enhance the anti-tumor response is engineering tumor specific T cells that are also insensitive to TGF-β suppression. Based

on our results showing that Ph6 neutralizes TGF-β physiological functions, we can hypothesize that engineering T cells that express clone 6 FN3 as a decoy receptor, along with a tumor-specific TCR, will enhance the anti-tumor effect of the engineered T cells in the presence of TGF-β. The development of cells resistant to TGF-β would be neccessary for its clinical significance as we can design combination immune therapies that can improve the survival of cancer patients. Furthermore, it will also be important from a fundamental science standpoint because it can allow us to study how T cells behave in the absence of TGF-β.

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VITA

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