IL-10 reduces CD8 T cell antigen sensitivity during the establishment of chronic viral infection by modifying N-glycan branching

by

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Abstract (English)

Chronic viral infections remain a global health concern. The early events that facilitate viral persistence have been linked to the activity of the immunoregulatory cytokine IL-10. However, the mechanisms by which IL-10 facilitates the establishment of chronic infection are not fully understood. Herein, I demonstrate that the antigen sensitivity of CD8 T cells is decreased during chronic infection and that this is directly mediated by IL-10. Mechanistically, we show that IL-10 induces the expression of *Mgat5*, a glycosyl-transferase that enhances N-glycan branching on surface glycoproteins. Increased N-glycan branching on CD8 T cells promotes the formation of a galectin 3-mediated membrane lattice, which restricts the interaction of key glycoproteins, ultimately increasing the antigenic threshold required for T cell activation. Our study identifies a novel regulatory loop in which IL-10 directly restricts CD8 T cell activation and function through modification of cell surface glycosylation allowing the establishment of chronic infection. This work will inform the design of therapeutics which could serve to better control chronic infections and cancer by maintaining heightened function of effector CD8 T cells.

Résumé (Français)

Les infections virales chroniques restent un important problème de santé mondial. Les premiers événements qui facilitent la persistance d'un virus ont été corrélé à l'activité de la cytokine immunorégulatrice IL-10. Par contre, les mécanismes par lesquels IL-10 facilite l'établissement d'une infection chronique ne sont pas bien compris. Je démontre ici que la sensibilité antigénique des lymphocytes T CD8 est diminuée pendant une infection chronique et que cette fonction des lymphocytes T CD8 est coordonné directement par IL-10. Nous montrons que l'IL-10 induit l'expression de Mgat5, une glycosyltransférase qui catalyse les branchements du N-glycan sur les glycoprotéines à la surface. L'augmentation des branchements du N-glycan sur les lymphocytes T CD8 contribue à la formation d'un treillis sur la membrane qui est médié par la galectine 3. Ce treillis limite l'interaction de certaines glycoprotéines ce qui cause une augmentation dans le seuil antigénique requis pour l'activation des lymphocytes T. Notre recherche identifie un nouveau système de régulation par lequel IL-10 diminue directement l'activation des cellules T CD8 par la modification de la glycosylation à la surface cellulaire, qui en fin du compte permet l'établissement d'une infection chronique. Cette recherche informera le développement de nouvelles approches thérapeutiques qui pourrait servir à mieux contrôler les infections chroniques et les cancers en augmentant la fonction des cellules T CD8 effectrices.

List of Abbreviations

AIDS:	Autoimmune deficiency syndrome
AKT:	Ak derived thymoma
APC:	Antigen presenting cell
BFA:	Brefeldin A
CD28:	Cluster of differentiation 28
CD3ε:	Cluster of differentiation 3 epsilon
CD4:	Cluster of differentiation 4
CD45:	Cluster of differentiation 45
CD8:	Cluster of differentiation 8
CD80:	Cluster of differentiation 80
CD86:	Cluster of differentiation 86
DC:	Dendritic cell
DNA:	Deoxyribonucleic acid
EAE:	Experimental autoimmune encephalomyelitis
EC50:	Effective concentration 50
ERK1/2:	Extracellular response kinase 1 and 2
FasL:	Fas ligand
FF_{Luc}:	Firefly luciferase
FRET:	Förster Resonance Energy Transfer
Gal3:	Galectin 3
GlcNAc:	N-acetyl glucosamine
HBV:	Hepatitis virus B
HCV:	Hepatitis virus C
HIV:	Human immunodeficiency virus
IFNL1:	Interferon lambda 1
IFN-γ:	Interferon gamma
IL-10:	Interleukin 10
IL10Ra:	Interleukin 10 receptor alpha
IL-12:	Interleukin 12
IL-2:	Interleukin 2
IL-22:	Interleukin 22
IL-26:	Interleukin 26
IL-28:	Interleukin 28
IL-6:	Interleukin 6
JAK:	Janus kinase
LCMV:	Lymphocytic choriomeningitis virus
Mgat5:	Mannosyl(alpha-1,6) glycoprotein (beta-1,6) N-acetyl glucosaminyltransferase
MHC:	Major histocompatibility complex
PD-1:	Programmed cell death protein 1

PFU:	Plaque forming unit
PHA-L:	Phaseaolus vulgaris leukoagglutinin
PLCγ:	Phospholipase C gamma
PMA:	Phorbol myristate acetate
RNA:	Ribonucleic acid
RT-qPCR:	Real time-quantitative polymerase chain reaction
SH2:	Src homology 2
shRNA:	short hairpin ribonucleic acid
TBP:	TATA binding protein
TCR:	T cell receptor
TGF-β:	Transforming growth factor beta
TNFα:	Tumor necrosis factor alpha
ZAP70:	Zeta associated protein of 70 kDa

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Chapter I Literature review and general introduction

CD8 T Lymphocytes

The adaptive immune response provides highly specific recognition of pathogens to facilitate coordinated responses and protect the host from various disease causing agents. This process is fueled by diversity established in the lymphocyte compartment during development as well as selective pressure during the course of infection. As CD8 T lymphocytes develop in the thymus they induce rearrangement of the T cell receptor alpha/beta loci to generate a unique TCR with a distinct binding capacity for antigen (Cox et al., 2013). These cells then undergo a rigorous selection process to remove T cell clones that arranged TCRs with high specificity for self-antigens, in a process called negative selection (Klein et al., 2014). Similarly, T cells undergo positive selection ensuring their unique TCR is able to recognize peptide antigen in the context of host Major histocompatibility complex (MHC). At this stage T cells express both co-receptors CD4 and CD8 which facilitate binding to MHC class II and MHC class I, respectively. Developing CD8 T cells will arrange TCRs which bind to MHC class I and will subsequently recruit CD8 to the contact site contributing to stable interaction and cellular signalling. This will commit the T cell to the CD8 lineage, and following a series of developmental steps, these cells will down regulate CD4 while maintaining expression of CD8 and egress from thymus entering peripheral circulation as naïve cytotoxic CD8 T cells (Germain, 2002).

During the course of an infection, danger signals and pathogen associated molecular patterns are recognized by cells of the innate immune system, which induce their activation. Of particular importance is the activation of dendritic cells (DCs), a heterogeneous group of cells arising from lymphoid and non-lymphoid progenitors that play a key role in antigen presentation to T cells (Joffre et al., 2009). DCs are professional antigen presenting cells (APC) which take-up, process, and present peptide antigen in MHC to T cells. After receiving a danger signal, such as ligation of a Toll-like receptor (TLR), DCs become activated and upregulate molecules involved in antigen presentation and T cell activation, such as CD80 and CD86 costimulatory molecules (Kapsenberg, 2003).

Naïve CD8 T cells must interact with a dedicated antigen presenting cell (APC) such as a DC to become activated and develop effector functions (Curtsinger and Mescher, 2010). DCs provide three essential signals required for T cell activation. Signal 1 being cognate antigen peptide-MHC complex, and signal 2 being costimulatory molecules such as CD80 and CD86, which bind to CD28 on the T cell resulting in enhanced activation while also providing survival signals during expansion. Signals 1 and 2 are the minimal unit required for T cell activation, in the absence of signal 2 activated T cells undergo anergy. Signal 3, the production of inflammatory cytokines, is also provided by DCs and serves to tune and shape the ensuing immune response (Figure I – 1).

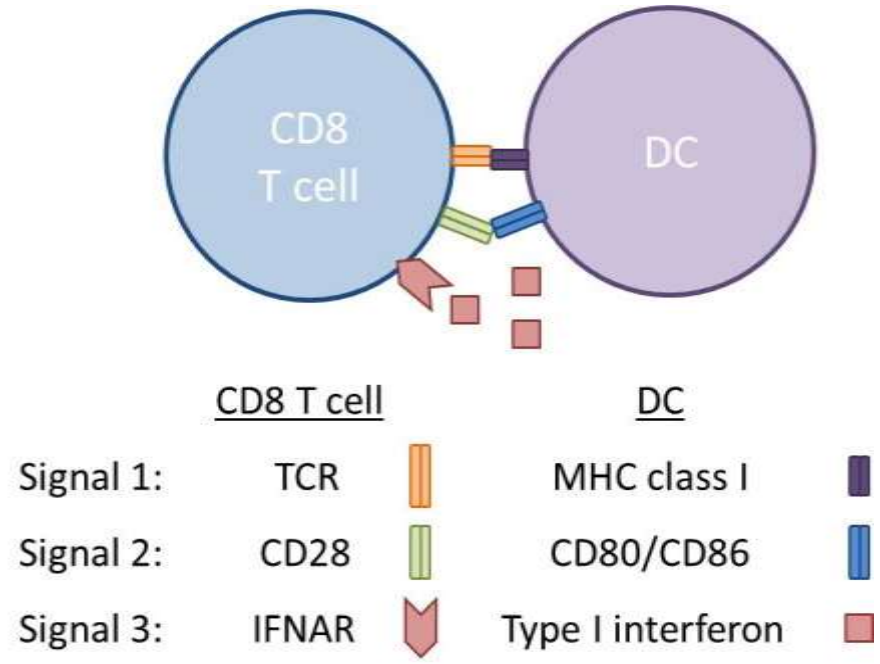

Figure I – 1. CD8 T cell activation involves 3 signals from DCs. Dendritic cells present cognate antigen on MHC class I to CD8 T cells, stimulating their antigen specific TCR (signal 1). The ligation of CD28 on the surface of T cells by CD80/CD86 on the surface of DCs provides the necessary costimulation to prevent T cell anergy (signal 2). Inflammatory cytokines produced from the DC and received by the T cells influence the ensuing response (signal 3, e.g. type I interferons depicted here).

Following interaction and subsequent activation by a DC presenting cognate antigen, antigen-specific CD8 T cells enter an expansion phase where an individual cell will undergo rapid proliferation and produce up to 10^4 daughter cells (Wong and Pamer, 2003). This clonal expansion is coupled with the acquisition of effector functions as the expanding cells induce the expression of inflammatory cytokines such as IFN-γ and TNF-α as well as several cytolytic molecules such as perforins, granzyme B and FasL. The increase in cell number coupled with the inflammatory and cytolytic capacity of expanding effector cells facilitate target cell cytolysis when CD8 T cells encounter an infected cell in the periphery displaying cognate antigen on their surface MHC class I. In this way CD8 T cells are able to control infection with intracellular pathogens (Tscharke et al., 2015).

CD8 T cell activation and expansion is carefully regulated to prevent inappropriate host damage, therefore several immunomodulatory mechanisms are in place to dampen the CD8 T cell response following expansion such as regulated contraction and concurrent expression of inhibitory receptors. Programmed contraction of the effector pool results in 90-95% of the responding cells dying via apoptosis, the remaining 5-10% of antigen specific CD8 T cells seed the memory pool. Memory CD8 T cells develop a number of functional advantages compared to their naïve counterparts, thereby providing enhanced protection against subsequent infections with that pathogen (Wong and Pamer, 2003).

Antigen sensitivity

The threshold of antigen required to elicit an effector response is commonly known as antigen sensitivity, or functional avidity. This functional attribute of T cells considers not only the direct affinity of the receptor for peptide ligand but also the avidity of surface receptor interactions, efficiency of signal transduction and mechanical interactions which all culminate in T cell activation (Vigano et al., 2012). CD8 T cells must sense and respond to low levels of antigen in peripheral tissue to mitigate host pathology; however the direct affinity of the TCR for specific peptide ligands is relatively low, antigen recognition is therefore regulated by other cellular factors. The sensitivity of a T cell for its cognate antigen changes throughout the course of an infection. Selective expansion of T cell clones bearing high affinity TCRs results in a CD8 T cell pool that is able to respond to lower antigen levels during secondary infections (Busch and

Pamer, 1999; Zehn et al., 2009). However, infection induced changes in antigen sensitivity also occur at a single cell level, whereby transgenic T cells constituting a monoclonal population increase in antigen sensitivity following infection (Slifka and Whitton, 2001). Similarly, research using monoclonal transgenic models, has demonstrated that the concentration of antigen presented during priming imprints lasting effects on the antigen sensitivity of CD8 T cells(Kroger and Alexander-Miller, 2007).

Sensitivity to antigen is modulated during thymic selection through the regulation of microRNAs and phosphatases that dampen signal transduction from the TCR (Li et al., 2007). Similarly, age related decline in the expression of specific microRNAs like miR181a lead to inhibited antigen sensitivity in antigen specific CD8 T cell responses due to increased phosphatase expression, such as DUSP6, which antagonizes ERK activation (Li et al., 2012). Additionally, organization of the plasma membrane has been shown to regulate antigen sensitivity by limiting the interaction between the co-receptor CD8 and the peptide MHC molecule (Cawthon and Alexander-Miller, 2002). As the TCR and CD8 co-receptor exist in distinct membrane microdomains, structural factors like the cortical actin cytoskeleton can regulate the redistribution of these molecules, thereby increasing the receptor dwell time needed to induce optimal signal transduction (Filipp et al., 2012) (Figure I – 2).

Antigen sensitivity is not only a hardwired or systematic phenomenon, but is also dynamically regulated by the inflammatory context. Research from our group has demonstrated that type I interferons and IL-12 produced during infection increases the antigen sensitivity of CD8 T cells, likely to aid in rapid pathogen clearance (Richer et al., 2013). We have shown that exposure to type I interferons and IL-12 causes T cell intrinsic enhancement of the TCR signalling machinery, allowing for heightened signal transduction following antigen stimulation. This provides an important regulatory link between inflammatory cytokine production and antigen sensitivity, and demonstrates that the cytokine milieu can regulate cytotoxic effector functions. Antigen sensitivity is a clear correlate of CD8 T cell mediated protection but the regulatory networks that dictate this process are poorly understood, given that extrinsic factors such as cytokine signalling can manipulate antigen sensitivity this opens areas for therapeutic development. Modulating the activation threshold of CD8 T cells could potentially alter the treatment strategies for many diseases.

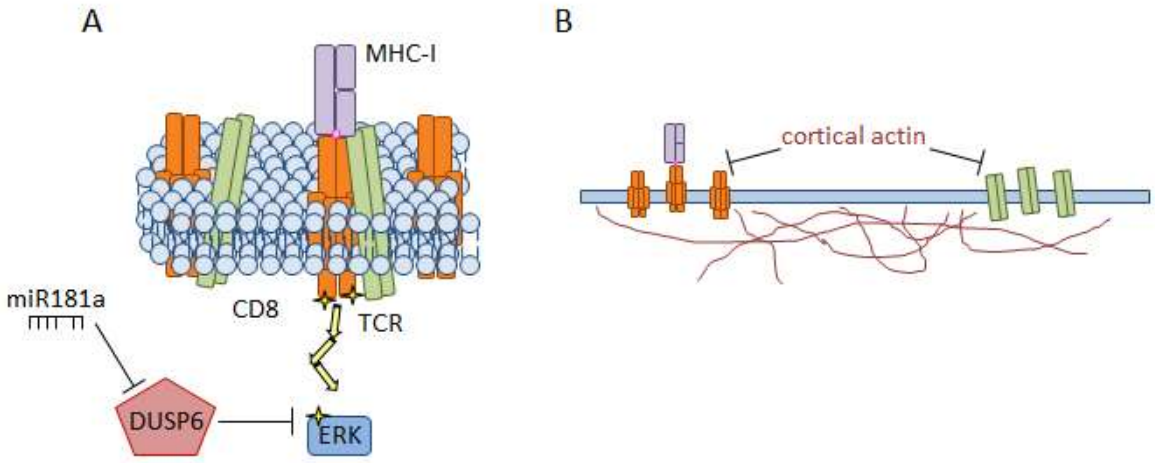

Figure I – 2. Antigen sensitivity is regulated by intrinsic factors in T cells. The expression of negative regulators such as phosphatases inhibits signal transduction downstream of TCR activation. Phosphatase expression is regulated, in part, by microRNAs (A). Structural regulators that prevent the membrane redistribution of the CD8 co-receptor with the TCR also limit antigen sensitivity by reducing the stability of the TCR:MHC-I interaction (B).

Chronic viral infections and immunity

Many infections are quickly and efficiently removed by the immune response, either through rapid elimination by the innate immune compartment or induction of highly specific adaptive immunity. However, some pathogens are able to establish persistent infections within the host. These infections typically result from impaired pathogen control at early stages during infection tipping the balance into an immunosuppressive environment to mitigate self-damage (Norris et al., 2013). This is observed in several chronic viral infections in human patients, where individuals presenting chronic human immunodeficiency virus (HIV) infection, hepatitis C virus (HCV) infection, or hepatitis B virus (HBV) infection demonstrate impaired immune function both in the innate compartment as well as reduced functionality in the antigen-specific CD8 T cell pool (Kurktschiev et al., 2014; Kwon and Kaufmann, 2010). In addition, persistent antigenic stimulation of CD8 T cells drives T cell exhaustion, a hypofunctional state where CD8 T cells fail to respond to antigen and express high levels of inhibitory receptors (Wherry, 2011).

Murine models have been instrumental in dissecting the contributions of various immune cells and subcellular mediators in the establishment of chronic viral infections. The naturally occurring mouse pathogen lymphocytic choriomeningitis virus (LCMV) is a robust model for evaluating host-viral interactions. This negative sense RNA virus is a natural mouse pathogen that elicits a dominant and extensively characterized CD8 T cell response. The immune response to the prototypical strain (LCMV Armstrong) as well as other natural variants (e.g. LCMV clone 13) has been well established (Wherry et al., 2003). In fact, despite possessing only two functional amino acid substitutions from the parental Armstrong strain, LCMV clone 13 establishes a chronic infection in mice, while LCMV Armstrong is rapidly cleared by day 5 post infection (Ahmed et al., 1984). Thus, this virus provides an ideal model to study host-pathogen interactions in the establishment of chronic infection.

This differential disease pathology between LCMV Armstrong and LCMV clone 13 has been implicated, in part, to the increased binding affinity of the clone 13 glycoprotein to its receptor alpha-dystroglycan due to a mutation in the viral glycoprotein (L260F) (Bonhomme et al., 2013). This point mutation in turn influences viral tropism as it permits LCMV clone 13 to better infect macrophages and plasmacytoid DCs that express high amounts of alpha-dystroglycan on their

surface. Furthermore, rapid viral replication within these cells due to mutation K1079Q in the L protein (viral polymerase) induces a robust wave of type I interferons which paradoxically drive chronicity in this model (Sullivan et al., 2011). Though type I interferon production is known to promote anti-viral immunity, the excessive levels of type I interferons released following infection with LCMV clone 13 drives the production of immunosuppressive cytokines such as transforming growth factor beta (TGF-β) and interleukin 10 (IL-10) from several cell types (Oldstone, 2015). Using IL-10 reporter mice, researchers have shown that many immune cell types produce IL-10 during LCMV clone 13 infection (Parish et al., 2014). IL-10 transcripts are detectable as early as day 2 following infection in DC and macrophage populations, and IL-10 reporter expression can be measured in these same cells as well as B and T cells (particularly in Th1 polarized cells). Thus, infection with LCMV clone 13 induces an immunoregulatory response that is likely aimed at protecting the host from immunopathology driven by excessive CD8 T cell activation.

IL-10: an immunomodulatory cytokine

Clinical manifestation of chronic viral infections coincides with an immunosuppressive state in patients, corresponding with reduced pathogen control. Similarly, infections such as HCV and HIV are characterized by increased expression of the immunomodulatroy cytokine IL-10 (Ng and Oldstone, 2014a). Systemic levels of IL-10 detectable in the serum of these patients are inversely correlated with IFN-γ production by antigen-specific T cells, a measure of activation and function (Couper et al., 2008). Furthermore, individuals harboring genetic polymorphisms in the IL-10 pathway demonstrate improved viral control during chronic infection, such as patients with active HIV infection that do not progress to AIDS (Kwon and Kaufmann, 2010). Thus, IL-10 plays an important role during chronic infections.

IL-10 is an established broad spectrum immunoregulatory cytokine with diverse and suppressive functions on several cell types. IL-10 has been demonstrated to reduce the expression of costimulatory molecules on the surface of APCs, ultimately leading to impaired T cell priming (Ng and Oldstone, 2014b). Additionally, IL-10 has been shown to impair glycolytic metabolism in DCs, an essential process in their activation and subsequent immune priming (Krawczyk et al.,

2010). Furthermore, although some stimulatory effects of IL-10 on CD8 T cells have been reported (Emmerich et al., 2012), IL-10 can also play a role in limiting effector responses in antigen specific CD8 T cells, such as expansion and cytokine production (Biswas et al., 2007).

IL-10 signals through the IL-10 receptor on the surface of target cells; this receptor is comprised of the CD210 and CDW210B, the alpha and beta chains, respectively. The beta chain is common to IL-10, IL-22, IL-26, IL-28 and IFN-L1; however, the alpha chain provides specificity to soluble IL-10 (Moore et al., 2001). The IL-10 receptor heterodimer is a canonical JAK-STAT receptor (Janus kinase and Signal transducer and activator of transcription, respectively), which possesses large intracellular domains for docking of signalling molecules. The IL-10 receptor associates with Jak1 and Tyk2 kinases on the intracellular leaflet of the plasma membrane; docking of soluble IL-10 induces auto-phosphorylation of these kinases, which then phosphorylate tyrosine residues on the intracellular domain of the IL-10 receptor. These provide phospho-tyrosines to recruit SH2 domain containing proteins of the STAT family. The IL-10 receptor has reported binding sites for STATS 1, 3, and 5 however the multiplicity and high affinity of STAT3 binding sites preclude the binding of other STATS, thereby favoring STAT3 recruitment. As STATs themselves possess a phosphorylatable tyrosine residue, their recruitment to the activated IL-10R results in phosphorylation by the activated kinases. Phosphorylated STATs dissociate from the receptor and form homo- and hetero-dimers with other activated STAT proteins. Dimerization results in the formation of a bipartite nuclear localization signal, allowing for nuclear import and subsequent transcriptional modulation by activated STATs.

The IL-10 receptor is transcriptionally controlled through the expression of the alpha chain. Most cells of the immune system have been documented to express the IL-10 receptor including CD8 T cells, thus contributing to the broad spectrum of IL-10's cellular targets (Moore et al., 2001). Given that IL-10 signals predominantly through STAT3, a transcription factor shared with other proinflammatory cytokines such as IL-6, the pleiotropic nature of STAT3 has been largely attributed to cell type specific and epigenetic landscapes which modulate its binding capacity and function (Durant et al., 2010). Thus the specific effects of IL-10 on responding cells is subject to a great degree of heterogeneity influenced by cell type, activation and cell cycle status.

Galectin-glycan interactions as a regulator of immune function

Post-translational modification of proteins affects their localization and function within the cell (Prabakaran et al., 2012). Rapid responses such as signal transduction are mediated through sophisticated phosphorylation cascades. However, modifications added to proteins as they form will go on to influence their subcellular compartmentalization, turn over and their interactome. The addition of carbohydrate sugars to proteins acts as an information rich mechanism of modifying proteins, as carbohydrates have exponentially greater combinatorial arrangements compared to conventional phosphorylation or ubiquitination (Moremen et al., 2012). These glycan moieties are covalently linked to proteins through either a hydroxyl or amide residue, providing the nomenclature O-linked or N-linked glycosylation, respectively.

Glycosylation serves many purposes in the adaptive immune response due to interactions with various carbohydrate binding lectins (Elola et al., 2015). Differential glycosylation patterns on activated T cells facilitate interactions with selectins on endothelial cells promoting migration and adhesion. Importantly, glycosylation patterns on the regulatory phosphatase CD45 coincide with various stages of activation in humans and have given rise to many useful diagnostic markers ($CD45RO^+$ memory CD8 T cells for example) (Earl and Baum, 2008).

O-linked glycosylation results in highly complex glycan moieties with diverse chemical properties and binding capacities. Conversely, though N-linked glycans have a great degree of heterogeneity, they share a common precursor, namely, a dolichol-linked GlcNAc sugar containing 9 branched mannose residues. This shared base is transferred onto the asparagine residues at specific sites (N-X-S/T) on nascent proteins as they are cotranslationally imported into the endoplasmic reticulum (Figure I – 3A) (Dennis et al., 2009). This sugar is then cleaved, modified and processed as the protein progresses through the endomembrane system, with many of the N-glycan modifications occurring in the golgi apparatus.

Importantly, N-glycans can result in extended and branched antennae comprised of variable lengths of repeating poly-lactosamine subunits, which may terminate in a sialic acid residue (Taniguchi and Korekane, 2011). These antennae act as binding substrates for the galectin family of soluble galactoside binding lectins which bind to poly-lactosamine with a high affinity. The number of branched antennae on an N-glycan is positively correlated with the binding

affinity of galectins (Nabi et al., 2015). Branched antennae production on N-glycans is regulated by a family of golgi-resident glycosyl-transferases which catalyse the covalent addition of GlcNAc to the mannose core. Golgi processing results in the production of mono, bi, tri and tetra-antennary glycans which are catalysed by the glycosyl-transferases Mgat1, Mgat2, Mgat4a/b, and Mgat5, respectively. The kinetics of these branching enzymes is highly dependent on enzyme availability as well as substrate abundance (Mkhikian et al., 2016). As each successive enzyme in the pathway has a reduced binding affinity for its substrate UDP-GlcNAc (a by-product of the hexosamine biosynthesis pathway) the frequency of fully branched glycans is highly dependent on the levels of Mgat5 expression (Figure I – 3B) (Grigorian et al., 2007). Tetra-antenneray glycans (products of the branching enzyme Mgat5) serve as the highest affinity binding sites for galectins, likely due to the multiplicity of the poly-lactosamine chains. The galectin family is highly diverse, consisting of several members with varying glycan valences depending on their oligomerization status and the structural context (Yang et al., 2008).

Mgat5 mediated glycosylation of surface receptors has been shown to regulate T cell activation and function at steady state (Demetriou et al., 2001). Branched glycosylation of the TCR allows for its incorporation into the galectin lattice, impeding lateral redistribution and clustering upon antigenic stimulation (Chen et al., 2007). Genetic deletion of this regulatory system results in spontaneous autoimmunity, demonstrating the role of Mgat5 in restricting inappropriate T cell activation at steady state (Grigorian and Demetriou, 2011). Furthermore, research has demonstrated that Mgat5 derived glycans and the galectin lattice similarly prevents the exclusion of the regulatory phosphatase CD45 from the contact site, impairing signal transduction (Chen et al., 2007). Additionally, the galectin lattice plays an important role in cancer immune evasion. Cytotoxic T cells isolated from tumours have reduced co-localization of the TCR with the co-receptor CD8, inhibition of galectin binding has been shown to restore this association concurrent with restoring T cell function, increasing functional and cytolytic capacity, and reducing tumour burden in mice (Demotte et al., 2008; Demotte et al., 2010).

Transmembrane proteins are highly glycosylated during export through the golgi complex while being routed to the plasma membrane, and given the ubiquitous nature of extracellular galectins, multivalent galectin-surface glycoprotein interactions results in formation of a galectin lattice which structurally regulates cellular activity (Ilarregui et al., 2005). The galectin lattice can

reduce internalization of glycoproteins causing retention of cytokine receptors, influencing their bioavailability (Grigorian et al., 2009; Lau et al., 2007). Furthermore, the galectin scaffold has been reported to oppose membrane re-organization, an essential step in signal amplification for many immune receptors (Antonopoulos et al., 2012). In this fashion, galectin-glycoprotein interactions as regulated at the level of protein glycosylation serve as potent modulators of immune cell function.

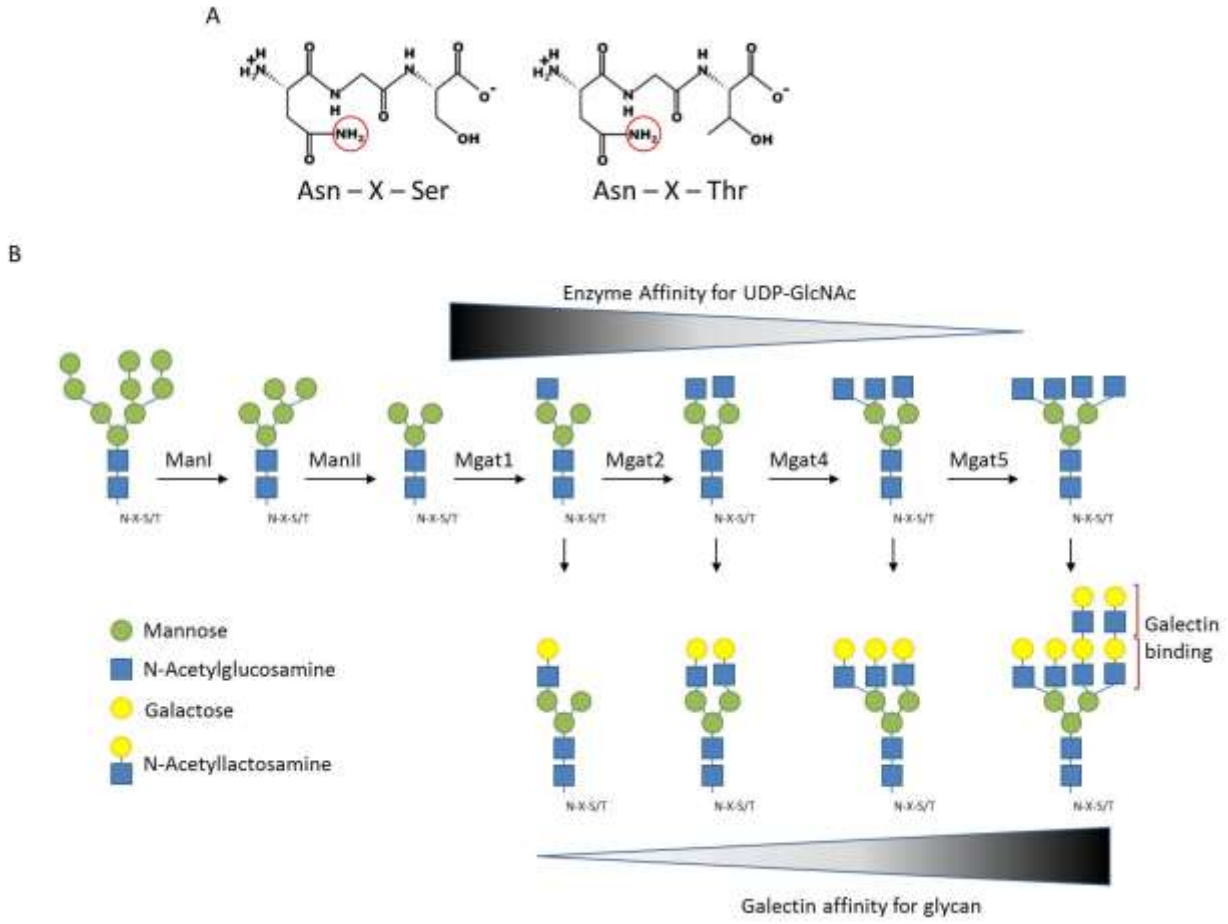

Figure I – 3. N-linked glycosylation and glycan processing. Asperigine linked glycans are covalently linked via an amide linkage at one of two consensus sites (A). Golgi resident enzymes process N-linked glycans as they progress through the endomembrane system. The Mgat family of glycosyl-transferases catalyse stepwise additions of N-acetylglucosamine to the growing glycan. These branched moieties can then be extended with poly-lactosamine repeats, providing high affinity binding sites for galectins (B). Adapted from Grigorian et al., 2007.

Hypothesis and Rationale

Antigen-specific CD8 T cells rely on high sensitivity for their cognate antigen to adequately control infection and protect the host (Vigano et al., 2012). Antigen sensitivity is modulated by the inflammatory milieu to tune CD8 T cell responses during the course of infection (Richer et al., 2013). Some viruses are able to impair immune responses and establish chronic infections, correlating with heightened and prolonged production of IL-10 by the host (Brooks et al., 2006). IL-10 plays an important role in facilitating chronicity but the mechanism of action still remains incompletely defined (Brooks et al., 2008). Several immunoregulatory circuits are in place to control T cell responses, including production of branched N-glycans and galectin mediated restriction of the TCR clustering dynamics (Demetriou et al., 2001). Therefore, *I hypothesize that IL-10 produced following infection with LCMV clone 13 impairs CD8 T cell antigen sensitivity, facilitating the development of chronic infection, by modulating global glycosylation.*

Chapter II Manuscript

IL-10 DIRECTLY INHIBITS CD8 T CELL FUNCTION BY ALTERING N-GLYCOSYLATION

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Author contributions

In the manuscript that makes up this M.Sc. thesis, I designed and executed the majority of experiments, analyzed the data, interpreted the results, generated the figures, and wrote the manuscript. Giselle M. Boukhaled prepared retrovirus and aided in experimentation and analysis. Stephanie A Condotta prepared LCMV stocks as well as execution, analysis and interpretation of viral quantification. Sabrina Mazouz isolated cells from human peripheral blood and extracted RNA. Jenna J. Guthmiller and Rahul Vijay performed experiments with *P. yoelii* and provided cDNA. Noah S. Butler, Julie Bruneau, Naglaa H. Shoukry, Connie M. Krawczyk designed experiments and provided guidance and reagents. Martin J. Richer designed and planned experiments, analyzed the data and edited the manuscript.

Introduction

Chronic viral infections, such as human immunodeficiency virus (HIV), hepatitis C virus (HCV) and hepatitis B virus (HBV), still represent a tremendous global health burden threatening a significant proportion of the population. These infections lead to progressive immune dysfunction, including the functional exhaustion and eventual deletion of the responding T lymphocytes (Wherry and Kurachi, 2015). While the consequences of chronic viral infections on the host immune system are well described, less is known of the early events that allow for the establishment of viral persistence. CD8 T cells play a critical role in the immune response to viral infections and are central to the capacity of the host to prevent the establishment of chronic infection (Barber et al., 2006). Efficient pathogen control relies on the capacity of CD8 T cells to rapidly respond and develop effector functions in the presence of low levels of antigen, also known as antigen sensitivity or functional avidity (Alexander-Miller, 2005; Vigano et al., 2012; Walker et al., 2010). The importance of antigen sensitivity is best illustrated by the findings that patients who can resolve multiple HCV infections harbor highly sensitive HCV-specific CD8 T cells compared to patients who progress to chronic infection (Abdel-Hakeem et al., 2017). Thus, antigen sensitivity of the responding CD8 T cells may be a key characteristic that determines whether the immune response is able to rapidly and efficiently control the invading pathogen.

The affinity of the T cell receptor (TCR) for peptide antigen is relatively low and, as opposed to the B cell receptor, remains fixed during the lifetime of the cell. However, the antigen sensitivity of CD8 T cells is modulated during infection at both the population (Busch and Pamer, 1999; Malherbe et al., 2005; Zehn et al., 2009) and single cell level (Richer et al., 2013; Slifka and Whitton, 2001). Individual CD8 T cell clones become more sensitive to their cognate antigen when they encounter inflammatory cytokines such as type I interferons and IL-12 (Richer et al., 2013). Exposure to inflammatory cytokines enhances the TCR signal transduction capacity of CD8 T cells, thereby reducing the threshold of antigen necessary for the induction of effector functions such as the production of cytokines and cytolysis (Richer et al., 2013). This represents an important regulatory loop that couples the presence of inflammatory cytokines to the activation of T cells, which likely limits immunopathology. However, because the antigen sensitivity of CD8 T cells is modulated by extrinsic factors, these regulatory circuits could potentially favor persistence if they are inappropriately activated during the course of infection. It is currently unknown whether the antigen sensitivity of CD8 T cells is modulated in the context of chronic viral infections or how this might be regulated.

Chronic viral infections induce an inflammatory milieu that is distinct from those established by acute viral infections (Ng and Oldstone, 2014b). These differences include changes in both the duration of the inflammatory period and cytokines produced. Notably, several chronic viral infections, including HIV and HCV, are associated with sustained induction of IL-10 in humans (Mannino et al., 2015). IL-10 is a broad spectrum immunoregulatory cytokine that can inhibit the function of a variety of immune cells and plays a critical role in dampening inflammatory responses (Corinti et al., 2001; Moore et al., 2001). Importantly, increased serum detection of IL-10 coincides with disease progression in patients with active HIV infection (Kwon and Kaufmann, 2010). Additionally, elevated IL-10 signatures at early time points following HCV challenge is associated with chronic progression of the disease (Flynn et al., 2011). Thus, the induction of IL-10 is a common feature of pathogens that can establish chronic infections.

The role of IL-10 during the establishment of viral persistence is clearly supported in mouse models of chronic viral infection. Challenging mice with lymphocytic choriomeningitis virus strain clone 13 (LCMV clone 13) establishes chronic infection and induces heightened and sustained production of IL-10, compared to acute infection with the closely related LCMV

Armstrong strain. The induction of IL-10 plays an important role in the establishment of persistence by LCMV clone 13 as genetic deficiency of IL-10 or antibody mediated blockade of the IL-10 receptor alpha chain results in enhanced viral control (Brooks et al., 2006; Ejrnaes et al., 2006). The mechanisms linking the production of IL-10 to the establishment of chronic infection remain poorly understood. What is currently known is that the mechanism through which IL-10 favors viral persistence is distinct from the T cell exhaustion pathways mediated by the expression of co-inhibitory receptors such as PD-1 (Brooks et al., 2008).

Since we have previously shown that cytokines impact the antigen sensitivity of CD8 T cells (Richer et al., 2013), we tested the hypothesis that IL-10 induced during chronic infection leads to a reduction in the antigen sensitivity of CD8 T cells and increases the threshold of antigen necessary for CD8 T cell activation. This important regulatory loop therefore enables the pathogen to outpace the immune response and establish persistence.

Methods

Mice and Pathogens

C57BL/6 and IL10KO mice were originally purchased from Charles River Laboratories and The Jackson Laboratory, respectively and bred in house. Mice with TCR-tg P14 cells were previously described (Pircher et al., 1987) and were provided by Dr. A. Lamarre (INRS-Institut Armand-Frappier). Gal3KO mice were provided by Dr. D. Sheppard (McGill University). Infected mice where housed in biocontainment level 2 and all animal procedures were carried out in accordance with the Canadian Council on Animal Care and were approved by the McGill University Animal Care Committee. LCMV Armstrong and LCMV clone 13 were kindly provided by J. Harty (University of Iowa) and Dr. T. Watts (University of Toronto) from a strain originally propagated by Dr. M. Oldstone (The Scripps Research Institute), respectively. LCMV was propagated as described (Slifka and Whitton, 2001). Mice were infected with 2×10^5 plaque forming units (PFU) of LCMV Armstrong by intraperitoneal (ip) injection, or 2×10^6 PFU LCMV clone 13 intravenous (iv) injection, respectively (Richer et al., 2013; Wherry et al., 2003). Viral titers were determined by plaque assay conducted on Vero cells as described previously (Ahmed et al., 1984).

Plasmodium infection

Plasmodium yoelii yoelii clone 17XNL expressing GP_{33-41} of LCMV was routinely passaged through mosquitoes. 1×10^4 P14 TCR-tg T cells were transferred into congenially mismatched recipients at day -1. 24 hours later mice received 1×10^6 parasitized red blood cells iv serial transfer to initiate infections. At day 7 post-infection mice were sacrificed and P14s were sorted by flow cytometry. RNA was extracted (Trizol) and cDNA synthesized. RT-qPCR analysis of gene expression was conducted as described below.

Adoptive Transfer

$2 – 100 \times 10^3$ Naïve TCR tg CD8 T cells were transferred by iv injection into congenically mismatched recipients at the Thy1 locus. 1 day following transfer mice were infected with the appropriate strain of LCMV. At day 8 post-infection mice were sacrificed and splenic CD8 T cells were analysed.

Ex vivo cytokine production

1 – 3 x 10^6 splenocytes were incubated *ex vivo* with titrated concentrations of GP_{33-41} (KAVYNFATM) peptide in the presence of BFA (eBioscience). Cells were stimulated for 6 hours at 37°C, 5% CO2 and then stained for the production of IFNγ by intracellular cytokine staining (Richer et al., 2013).

TCR signaling and Immunoblot

1 - 10 x 10^4 Naïve P14 cells were injected iv into naïve congenically mismatched recipients, 1 day later mice were infected with LCMV Armstrong or LCMV clone 13, as indicated. On day 8 post-infection, spleens were harvested and transgenic cells were isolated by Thy1.1 or Thy1.2-PE positive-selection, accordingly. In brief, cells were stained with the appropriate PE conjugated antibody and purified using anti-PE-magnetic separation according to standard AutoMACS protocols (Miltenyi Biotec). 1 x 10^7 cells were stimulated by CD3ε crosslinking or PMA stimulation at 37°C, as described previously (Richer et al., 2013). 15 – 25 μg of protein was resolved by SDS-PAGE, transferred to PVDF membranes and probed with the indicated antibodies. Antibody binding was detected using goat-anti-rabbit conjugated to horseradish peroxidase (Jackson ImmunoResearch) and Amersham Prime ECL (GE Lifesciences).

FRET

TCR-CD8 FRET was measured using flow cytometry, as described previously (Perica et al., 2012). Briefly, 1 – 5 x 10^6 cells were incubated for 30 minutes with PE-conjugated anti-Vα2 (eBiosciences; a component of the transgenic TCR) as a fluorescence donor and APC-conjugated anti-CD8α (Biolegend) as a fluorescence acceptor. Samples were stained with either antibody (E_{PE}, or E_{APC}), both (E_{Both}) or neither (E_{none}). Cells were concurrently labeled with CD3ε-biotin and stimulated by crosslinking with streptavidin. FRET emission was assessed by flow cytometry without compensation, observing emission in the APC wavelength without direct laser excitation. FRET efficiency was calculated in FRET units as described previously (Perica et al., 2012).

FRET unit = $(E3_{Both} – E3_{none}) – [(E3_{APC} - E3_{none}) x (E2_{both}/E2_{APC})] – [(E3_{PE} - E3_{none}) x (E1_{Both}/E1_{PE})]$

E1: emission in the donor channel upon excitation of the donor
E2: emission in the acceptor channel upon excitation of the acceptor
E3: emission in the acceptor channel upon excitation of the donor

Lactose treatment

For experiments measuring antigen sensitivity, $1 – 3 \times 10^6$ splenocytes were incubated with titrated concentrations of peptide in the presence of BFA as described above. Prior to the 6 hour incubation cells were supplemented with 50 mM D-Lactose (Fisher L5-500). For signaling experiments and FRET analysis purified or total cells were supplemented with 50 mM D-Lactose and incubated for 30 min in 37°C water bath to disrupt galectin binding (Demetriou et al., 2001). Cells were then processed as described above.

Branched N-glycan surface expression analysis

Cells were treated with 50 mM D-Lactose, as described above, to remove bound galectins, which may impair staining through steric hindrance. Cells were stained with fluorescently labeled antibodies for 20 minutes, then washed and fixed. Cells were incubated with 50 μg/mL biotinylated *Phaseolus vulgaris* Leukoagglutinin (PHA-L; EY laboratories) for 1+ hour at room temperature, washed and incubated with PE-conjugated streptavidin (Biolegend) and analyzed by flow cytometry.

PHA-L pull-down

P14 cells were enriched from total splenocytes by standard AutoMACS protocol as described above. Isolated cells were treated with 50 mM D-Lactose for 30 minutes, at 37^0C, to remove any surface bound galectin and expose branched N-glycans. Cells were then pelleted by centrifugation, lysed in NP-40 lysis buffer, and quantified using a Bradford assay. 10% of whole cell lysate was reserved and loaded as the input fraction. 250 μg of total protein was incubated with 150 μg of PHA-L conjugated agarose beads (Vector Laboratories) at room temperature overnight. Beads were pelleted by centrifugation and washed several times with lysis buffer. Proteins were eluted using Laemmli sample buffer (Sigma Aldrich), and detected by SDS-PAGE followed by immunoblotting with anti-TCRα/β (clone H59-597, Cedarlane).

Gene expression analysis

For RT-qPCR analysis, P14s were isolated by PE-selection as described above. RNA was then extracted (Trizol reagent) and 1 μg of total RNA was used to generate cDNA with iScript reverse transcriptase (Bio-Rad). RT-PCR analysis was then conducted using SensiFAST SYBR (Bioline) and the following primer sets (Integrated DNA Technologies; 5' – 3'): mouse *il10ra* forward: GCCAAGCCCTTCCTATGTGT; mouse *il10ra* reverse: TCCGTACTGTTTGAGGGCCA; mouse *mgat5* forward: CAAGGCTGCTGGAGAGTGAA; mouse *mgat5* reverse: GGGCTTCCATTCTGATGACCT; mouse *TBP* forward: TGGAATTGTACCGCAGCTTCA: mouse *TBP* reverse: ACT GCAGCAAATCGCTTGGG; human *MGAT5* forward: CCATGTTCCCTCATACCCCAG; human *MGAT5* reverse: TCCCGTCCACTGAGGATACC; human *TBP* forward: GTGACCCAGCATCACTGTTTC; human *TBP* reverse: GTAAGGTGGCAGGCTGTTGT; human *IL10RA* forward: GCGCTCCTGAGGTATGGAAT; human *IL10RA* reverse: CCCGAGGATGAAGCCATTGT. Transcript expression was normalized to TATA binding protein as an internal control and depicted as a relative fold change using the ΔΔCt method, compared to the mean of the control group (Livak and Schmittgen, 2001).

CD8 T cell transduction

Naïve P14 cells were isolated from spleens by negative CD8 selection (STEMCELL technologies). 2 x 10^6 cells per well were stimulated with plate bound CD3ε and CD28 in the presence of 20 units/mL of recombinant murine IL-2 (eBioscience). 18 hours later, activated cells were either transduced using retrovirus produced in HEK293Ts transfected with LMPd-based retroviral vectors provided by M. Pipkin (The Scripps Research Institute, Florida) encoding the shRNA of interest (Chen et al., 2014; Paddison et al., 2004) or mock-transduced. Cells were allowed to recover for 6 hours prior to adoptive transfer into recipient mice that were infected with LCMV 6 hours prior.

Ex vivo IL-10 treatment

Naïve CD8 T cells were isolated from spleens by negative CD8 selection (STEMCELL Technologies). 2 x 10^6 cells/mL were activated with plate bound CD3ε/CD28 in the presence of 20 units/mL of IL-2. Cells were treated with or without 200 units/mL of recombinant murine IL-10 (eBioscience) and with or without 0.5 nmol/mL the STAT3 inhibitor WP1066 (Sigma). After 2 days cells were transferred to plates without CD3ε/CD28 and maintained up to day 8 at 37°C with 5% CO_2.

MGAT5 expression from patient samples

Study subjects were enrolled among people who inject drugs (PWIDs) participating in the Montreal Acute Hepatitis C Cohort Study (HEPCO)(Grebely et al., 2013). This study was approved by the Institutional Ethics Committee of CRCHUM (Protocol SL05.014). All samples were anonymized. Chronic HCV infection was identified in participants who tested positive for HCV RNA for more than 6 months post initial infection as previously described (Badr et al., 2008; Grebely et al., 2013). CD8 T cells were isolated from cryopreserved peripheral blood mononuclear cells (PBMCS) from five chronically infected or HCV naïve participants by MACS separation (Miltenyi Biotech). RNA was isolated using RNeasy Plus kit (Qiagen). cDNA synthesis and RT-qPCR were performed as described above.

Statistical Analyses

Data were analyzed with GraphPad Prism software. The Specific tests used to determine statistical significance are indicated in each figure legend. P values of less than 0.05 were considered statistically significant.

Results

IL-10 directly reduces the antigen sensitivity of antigen specific CD8 T cells

We previously showed that cytokines regulate the antigen sensitivity of CD8 T cells (Richer et al., 2013). Since chronic viral infections establish a distinct inflammatory milieu characterized by the induction of IL-10 (Brooks et al., 2006; Ejrnaes et al., 2006; Parish et al., 2014), we asked whether a virus that establishes chronic infections can negatively regulate antigen sensitivity of CD8 T cells to counter immune defenses. To address this question, we transferred congenically marked P14 TCR transgenic T cells into wildtype (WT) or IL-10-deficient hosts (IL10KO). CD8 T cells from this transgenic mouse line express a TCR specific to GP_{33-41} (an immunodominant epitope of LCMV), allowing us to measure the influence of the inflammatory milieu on a population sharing identical TCRs and therefore identical affinity for their cognate antigen. Following adoptive transfer, mice were infected with either LCMV Armstrong or LCMV clone 13 to induce either acute or chronic infection, respectively. At day 8 post-infection, total splenocytes were incubated with titrated concentrations of GP_{33-41} peptide and the antigen sensitivity of responding P14s was quantified by the percentage of cells producing interferon gamma (IFN-γ) as measured by intracellular cytokine staining (Richer et al., 2013).

Infection with LCMV clone 13 significantly impaired the antigen sensitivity of effector P14s compared to effector P14s from mice infected with LCMV Armstrong (Figures II - 1A and B). Compared to P14s from LCMV clone 13 infected mice, the antigen sensitivity of P14s from LCMV Armstrong infected mice was 4.4-fold higher based on the peptide concentration required to induce 50% of the maximum IFN-γ production (effective concentration 50 [EC_{50}]) (Figure II - 1B). Strikingly, in the absence of IL-10 the antigen sensitivity of P14s from LCMV clone 13 infected mice was completely restored (Figures II- 1A and B). Conversely, IL-10 deficiency had no effect on antigen sensitivity of effector P14s during LCMV Armstrong infection (Figures II - 1C and D). Thus, infection with LCMV clone 13 reduces the antigen sensitivity of CD8 T cells in an IL-10-dependent manner.

During established chronic infections persistent TCR stimulation leads to T cell exhaustion, a hypo-functional state where T cells fail to respond to antigen and express a high level of inhibitory receptors (Yi et al., 2010). At day 8 post-infection P14 cells from LCMV clone 13 infection maintain heightened PD-1 surface expression irrespective of the presence of

IL-10 (Figures II - 1E and F). Thus, the antigen sensitivity of CD8 T cells can be restored in the absence of IL-10 independently of changes in the expression of PD-1, further supporting that IL-10 and PD-1 can induce T cell dysfunction through distinct mechanisms (Brooks et al., 2008).

Though CD8 T cells express the IL-10 receptor, the direct effects of IL-10 on CD8 T cells remain poorly defined. While our experiments so far established a role for IL-10 in restricting the antigen sensitivity of CD8 T cells, they did not allow us to determine whether this occurred through direct or indirect mechanisms. Thus, we asked whether inhibition of IL-10 signaling specifically on CD8 T cells was sufficient to restore antigen sensitivity during LCMV clone 13 infection. To investigate this, we employed retroviral transduction to express shRNAs targeting the *Interleukin-10 receptor subunit alpha* (*Il10ra*) or the irrelevant gene *firefly luciferase* (sh*Il10ra* and shFF_{luc}, respectively). Consistent with our previous results, knockdown of the *Il10ra* gene (but not transduction with the irrelevant shRNA targeting FF_{luc}) was sufficient to restore antigen sensitivity during LCMV clone 13 infection (Figures II - 1G and H). These data establish that IL-10 regulates antigen sensitivity in a CD8 T cell intrinsic manner.

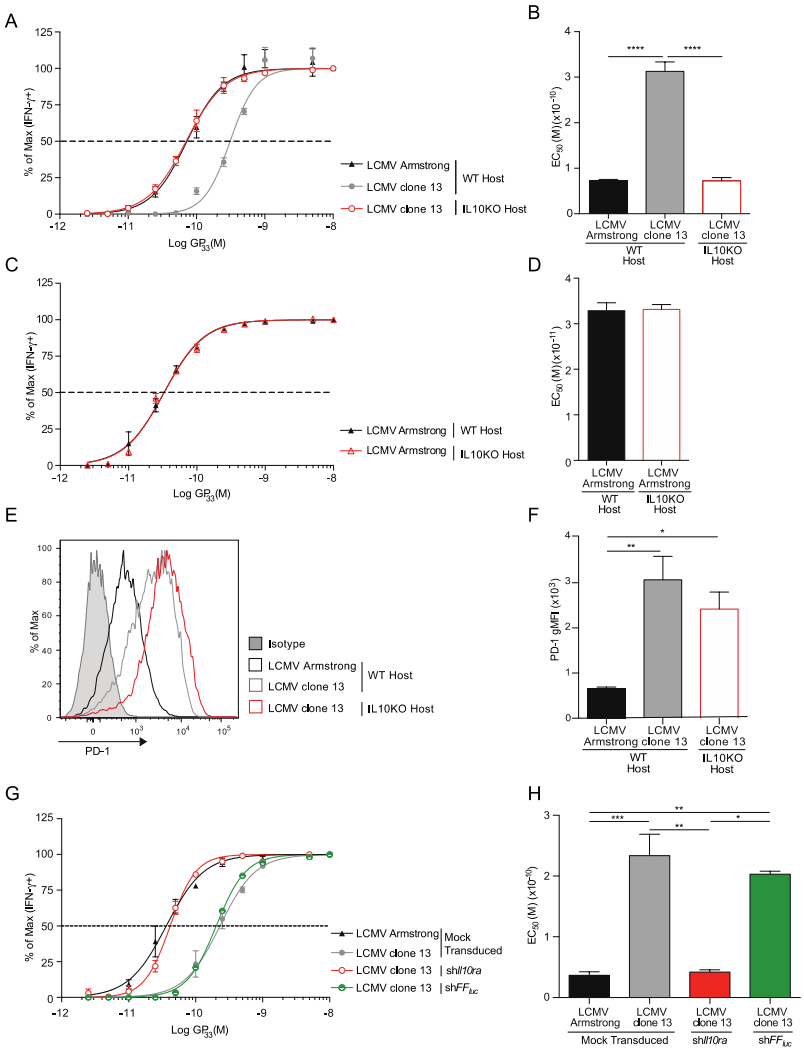

Figure II - 1. LCMV clone 13 infection reduces the antigen sensitivity of effector CD8 T cells in an IL-10-dependent manner. A) The percentage of IFN-γ^+ effector P14 cells at day 8 post-infection with LCMV Armstrong or LCMV clone 13 in the indicated host was determined following *ex vivo* stimulation with titrated concentrations of $GP_{33\text{-}41}$ peptide. Data (Mean ± SEM) are normalized to the proportion of IFN-γ^+ cells at saturating peptide concentration (10 nM). B) Summary (mean ± SEM) of EC_{50} for stimulation of IFN-γ^+ production by P14 cells. C) Antigen sensitivity as in Figure 1A at day 8 post-infection with LCMV Armstrong in the indicated host. D) Summary (mean ± SEM) of EC_{50} for stimulation of IFN-γ^+ production by P14 cells. E) Representative histograms of expression of PD-1 on P14 cells at day 8 post-infection with indicated strains of LCMV. Shaded histogram represents antibody isotype control. F) Summary (mean ± SEM) of geometric mean fluorescence intensity (gMFI) of PD-1 expression on P14 cells. G) Antigen sensitivity as in Figure 1A for P14 cells mock transduced or expressing shRNAs targeting *Firefly luciferase* (FF_{luc}) or the alpha chain of the IL-10 receptor (*Il10ra*). H) Summary (mean ± SEM) of EC_{50} for stimulation of IFN-γ^+ production by P14 cells.. Data in A-H represent 3 mice per group and are representative of at least 2 independent experiments. Data in B, D, F and H were analysed by one-way ANOVA with Tukey's post-hoc analysis of multiple comparisons. *p<0.05, **p<0.01, ****p<0.0001.

IL-10 impairs the TCR signal transduction capacity of CD8 T cells

We have previously shown that inflammatory cytokines can impact antigen sensitivity by increasing the efficiency of signal transduction downstream of the TCR (Richer et al., 2013). Therefore, we asked whether IL-10 antagonizes TCR signal transduction leading to the increased antigen threshold required for activation. TCR ligation induces rapid phosphorylation of the kinase Zap-70 and activation of PLCγ, leading to the eventual phosphorylation of AKT and ERK1/2, distal kinases activated downstream of the signaling cascade (Smith-Garvin et al., 2009). To measure these signaling events at day 8 post-infection we stimulated enriched P14s by CD3ε-crosslinking to induce activation in the absence of antigen presenting cells (APC). P14s isolated from LCMV clone 13 infected mice demonstrated a clear defect in the activation of these signaling pathways despite no changes in overall protein expression (Figure II - 2A). P14s isolated from IL-10 deficient mice infected with LCMV clone 13 exhibited completely restored TCR signaling capacity (Figure II - 2A). Importantly, P14s from LCMV clone 13 infected mice showed no defect in ERK1/2 activation following stimulation with the diacyl-glycerol (DAG) analog phorbol myristate acetate (PMA; Figure II - 2B), which bypasses the proximal signaling machinery. This establishes that P14s from LCMV clone 13 infected mice maintain the capacity for robust MAP kinase activation when the TCR proximal signaling machinery is bypassed. This defect in signal transduction is not due to a change in the expression of surface components of the TCR or costimualtory molecules, as we observed that infection with LCMV clone 13 did not alter the surface expression of the alpha (Vα2) and beta (Vβ8.1) chains of the TCR, CD3ε, co-receptor CD8 or the costimulatory molecule CD28 (Figure II - 3). Together, this demonstrates that infection with LCMV clone 13 induces an IL-10-dependent TCR proximal signaling defect that likely results in decreased antigen sensitivity.

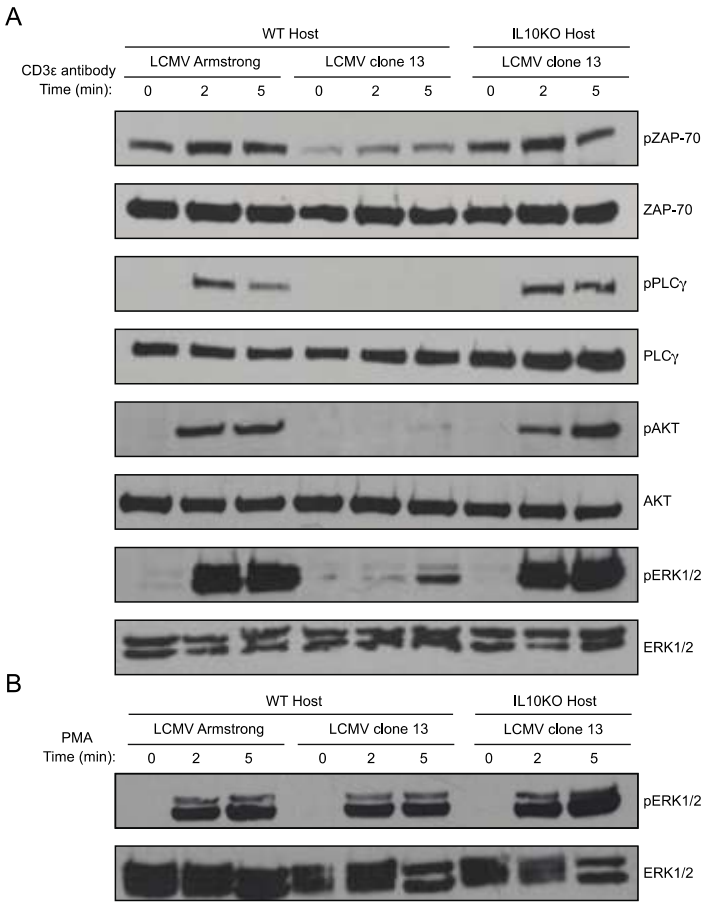

Figure II - 2. IL-10 impairs TCR proximal signal transduction. A) Immunoblot analysis of cell lysates from P14 CD8 T cells isolated at day 8 post-infection with indicated strains of LCMV from the indicated mouse strain. Cells were stimulated by CD3-crosslinking for the indicated time and equivalent amounts of total protein loaded into each lane. Samples were pooled from n = 3 mice. Total and phospho-proteins were probed, as indicated. B) Same as A, but using PMA stimulation. Data are representative of at least 2 independent experiments.

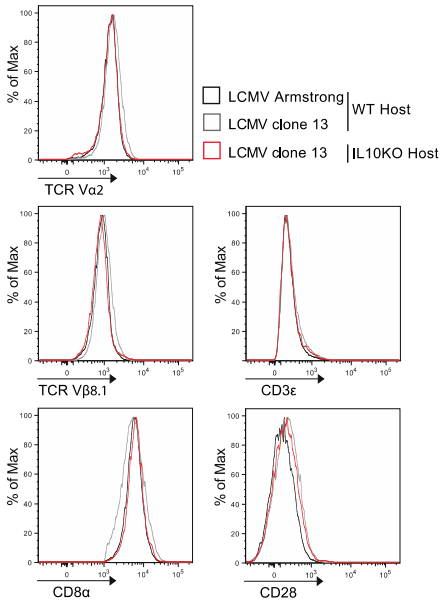

Figure II – 3. IL-10 does not influence the surface expression of TCR signalling machinery. Representative histograms of indicated receptor expression on P14 cells isolated from LCMV Armstrong or LCMV clone 13 infection in WT hosts or LCMV clone 13 infection in IL10KO hosts. Data are representatives from 3 mice per group and are representative of 3 experiments.

IL-10 impairs TCR:CD8 co-localization during LCMV clone 13 infection

T cell activation requires the clustering of several membrane proteins to induce effective and robust signal transduction. The TCR and co-receptor CD8 exist in distinct lipid microdomains within the T cell plasma membrane (Demotte et al., 2008). Upon interacting with antigen, large scale re-organization of the plasma membrane allow for both TCR and CD8 to interact with peptide:MHC class I on the APC leading to signal transduction. Importantly, the interaction between TCR and the CD8 co-receptor increases the affinity for peptide:MHC complex leading to increased antigen sensitivity (Borger et al., 2014; Cawthon and Alexander-Miller, 2002). In addition, memory T cells form nano- and micro-scale clusters of receptors on their surface and this has been suggested to facilitate rapid responses to low levels of antigen upon re-challenge (Kumar et al., 2011).

Because the capacity of the TCR to interact with the CD8 co-receptor and to form micro-clusters is integral to dictating antigen sensitivity, we hypothesized that reduced TCR and CD8 co-receptor association is linked to a decrease in CD8 T cell antigen sensitivity. To test whether TCR:CD8 co-localization is impaired during LCMV clone 13 infection we used a well-defined flow cytometry adapted Förster resonance energy transfer (FRET) approach (Demotte et al., 2008). Briefly, the alpha chains of the TCR and CD8 were labelled with donor and acceptor fluorophores, respectively (Figure II - 4A). Co-localization is determined by measuring the emission from the acceptor fluorophore without its direct excitation (only the donor is excited) following cross-linking of CD3ε. As the emission spectrum of the donor fluorophore overlap with the excitation of the acceptor fluorophore, FRET occurs if both proteins are within 10 nm of each other (Figure II- 4A). On day 8 post-infection with LCMV Armstrong, we observed robust co-localization of TCR and CD8 on P14s and this was significantly reduced on P14s isolated from LCMV clone 13 infected mice (Figure II - 4B and Figure II - 5A). TCR and CD8 co-localization was rescued in P14s isolated from LCMV clone 13 infected IL10KO mice (Figure II - 4B and Figure II - 5A), correlating with their restored TCR signaling capacity and antigen sensitivity (Figures II - 1 and 2). These data demonstrate that infection with LCMV clone 13 leads to IL-10-induced impairment in the capacity of CD8 T cells to co-localize the TCR and CD8 co-receptors, potentially leading to reduced T cell responsiveness to antigen.

LCMV clone 13 infection increases N-glycan branching on T cells in an IL-10 dependent manner

The expression of *Mgat5*, a golgi resident glycosyl-transferase, has been shown to restrict TCR clustering by increasing the branching of N-glycans on the surface of T cells at steady-state (Demetriou et al., 2001). Mgat5-modified N-glycans can be further extended resulting in high affinity binding sites for the galectin family of soluble β-galactoside binding lectins (Figure II - 4C) (Ilarregui et al., 2005). Galectin binding of surface glycoproteins forms a lattice that restricts protein diffusion due to multivalent interactions with multiple glycoproteins (Elola et al., 2015; Ilarregui et al., 2005). However, whether this is a mechanism governing CD8 T cell activation and whether this can be modulated by IL-10 remains unknown. Thus, we asked whether *Mgat5* expression is linked to IL-10-mediated regulation of CD8 T cell antigen sensitivity. At day 8 post-infection we observed an 11-fold increase in *Mgat5* transcript expression in CD8 T cells responding LCMV clone 13 infection relative to naïve T cells (Figure II - 4D). Similarly, we observed an IL-10-dependent increase in *Il10ra* transcript expression by P14s during LCMV clone 13 infection (Figure II -5B). Increased *Mgat5* expression was not observed in CD8 T cells responding to LCMV Armstrong infection or by CD8 T cells responding to LCMV clone 13 infection in IL10KO mice, establishing that IL-10 expression during chronic infection regulates *Mgat5* expression (Figure II - 4D). To determine whether increased *Mgat5* expression resulted in enhanced N-glycan branching on CD8 T cells, CD8 T cells were stained with PHA-L, a lectin isolated from *Phaseolus vulgaris* that binds to Mgat5-modified branched N-glycans (Figure II - 4C) (Demetriou et al., 2001). We observed increased binding of PHA-L on CD8 T cells from mice infected with LCMV clone 13 infection and this increase was dependent on IL-10 (Figures II - 4E and F). Mgat5-mediated glycosylation is known to facilitate binding of galectin 3 (Gal3) to the TCR thereby limiting its redistribution (Demetriou et al., 2001). Thus, we asked if Mgat5-modified glycans were detectable on the TCR specifically. At day 8 post-infection we precipitated Mgat5-modified glycoproteins from P14 whole cell lysates using PHA-L-conjugated agarose beads. PHA-L beads only precipitated TCR from T cells from WT mice infected with LCMV clone 13 and not from LCMV Armstrong infected WT mice or LCMV clone 13 infected IL10KO mice (Figure II - 4G). Thus Mgat5-mediated glycosylation of the TCR is enhanced during LCMV clone 13 infection in an IL-10-dependent manner.

Chronic plasmodium and HCV infections regulate expression of Mgat5

In order to address whether other pathogens that establish chronic infection can also regulate the expression of *Mgat5* in CD8 T cells during the early stages of infection, we used a murine model of chronic parasitic infection. We adoptively transferred congenically marked P14s and subsequently infected mice with *Plasmodium yoelii* (a parasite that induces sustained IL-10 production and stimulates T cell exhaustion) (Butler et al., 2012; Kobayashi et al., 1996) expressing the model antigen GP_{33-41} of LCMV. At day 7 post-infection we isolated the P14s and observed increased expression of *Mgat5* compared to naïve P14s (Figure 3H). In addition, *Plasmodium yoelii* infection resulted in increased expression of *Il10ra* (Figure II - 5C) on CD8 T cells. Thus, these data suggest that the induction of *Mgat5* may represent a conserved mechanism through which pathogens limit T cell responses in order to establish persistent or prolonged infections.

We next asked whether this mechanism of regulation is also observed during chronic viral infections in humans. To address this, we examined the expression of *MGAT5* and *IL10RA* in CD8 T cells isolated from peripheral blood of subject with chronic infection HCV (a chronic viral infection that has also been associated with the induction of IL-10 (Frebel et al., 2010)). We observed that CD8 T cells from subjects chronically infected with HCV expressed higher levels of *MGAT5* and *IL10RA* compared to CD8 T cells from HCV naïve individuals from the same cohort (Figure II - 4I and Figure II - 5D). Therefore, our data establish that chronic infections in humans also result in increased *MGAT5* expression by CD8 T cells.

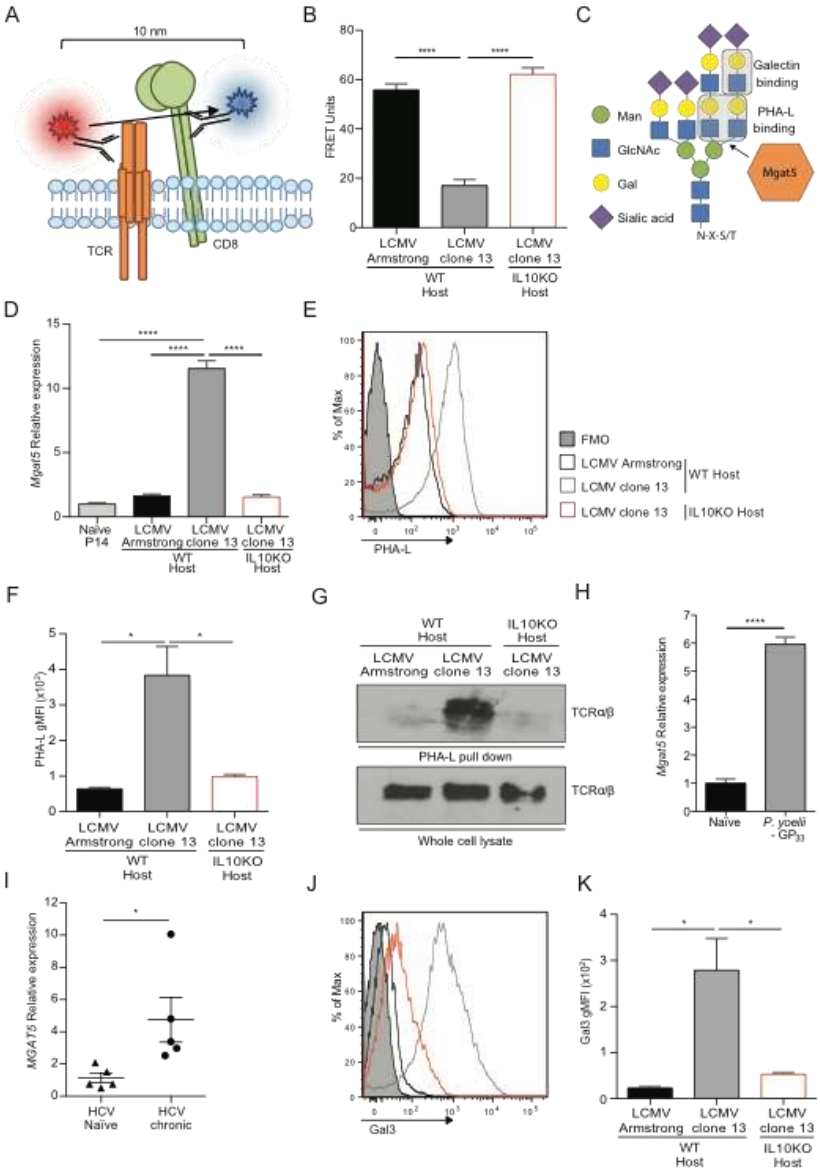

Figure II - 4. IL-10 restricts the co-localization of TCR with CD8 co-receptor and changes the N-glycosylation of CD8 T cells. A) Schematic of FRET approach. B) Summary (mean ± SEM) of FRET Units for P14 cells at day 8 post-infection with indicated strains of LCMV in the indicated mouse strain. C) Schematic of Mgat5-modified N-glycan and respective galectin and PHA-L binding sites. D) qRT-PCR analysis of *Mgat5* expression in day 8 effector P14 cells from indicated conditions, relative to naïve P14s (mean ± SEM for n = 3 mice). Representative of 2 independent experiments. E) Representative histogram of PHA-L binding on effector P14 cells at day 8 post infection. Gray shaded histogram represents fluorescence minus one (FMO) control F) Summary (mean ± SEM) of gMFI of PHA-L binding. G) Immunoblot of α/β TCR following

pull-down with PHA-L conjugated beads. H) qRT-PCR analysis of *Mgat5* expression in day 7 effector P14 CD8 T cells following infection with *P. yoellii* – GP_{33} relative to naïve P14s (mean ± SEM for n = 3 mice per group). I) qRT-PCR analysis of *MGAT5* expression in total CD8 T cells isolated from human patients with chronic HCV infection, relative to a naïve cohort (mean ± SEM for n = 5 patients per group). J) Representative histogram of Gal3 expression on effector P14 cells at day 8 post-infection with indicated strain of LCMV. Shaded histogram represents FMO control. Refer to panel E for color legend. K) Summary (mean ± SEM) of gMFI of Gal3 expression on P14 cells. Data in B, D, F and K were analysed by one-way ANOVA with Tukey's post-hoc analysis of multiple comparisons. Data in H and I were analyzed by two-tailed unpaired T-test. *p<0.05, ****p<0.0001.

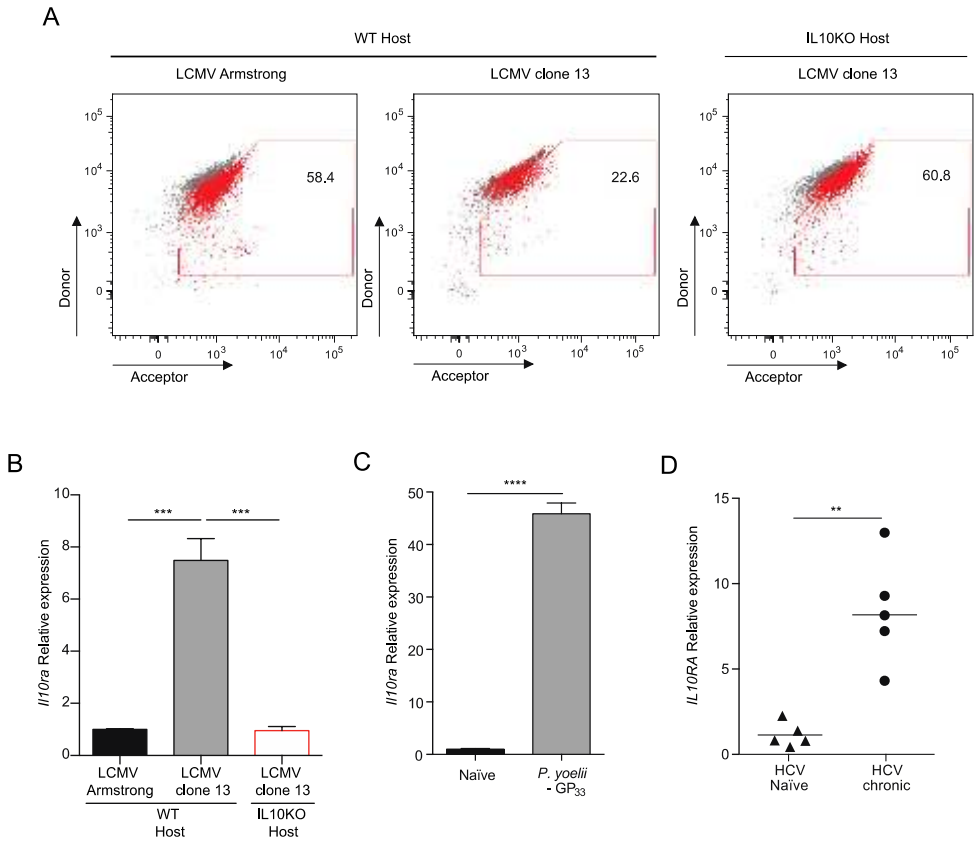

Figure II - 5. IL-10 reduces colocalization of CD8:TCR and induces expression of Il10ra. A) Representative plots of FRET between the TCR Vα2 (donor) and CD8α co-receptor (acceptor) on P14 cells following stimulation by CD3-crosslinking. Gray indicates the donor only control, red represents donor + acceptor stained FRET sample. B and C) qRT-PCR expression of *Il10ra* in P14 cells isolated from indicated infections. D) qRT-PCR expression of *IL10RA* in CD8 T cells isolated from patients chronically infected with HCV compared to a naïve cohort. Data in A, B, and C are representatives of 3 mice per group and represent 2 experiments. Data in D are representative of a cohort of 5 infected and 5 naïve patients. Data in B were analysed by one-way ANOVA with Tukey's post-hoc analysis of multiple comparisons. Data in C and D were analyzed by two-tailed unpaired T-test. **p<0.01, ***p<0.001, ****p<0.0001

Galectin inhibition restores CD8 T cell antigen sensitivity

While we observed an increase in *Mgat5* expression and N-glycan branching, this would not be sufficient to restrict surface receptor interaction unless the N-glycans are engaged to form a restrictive network. The galectin protein family has several diverse members that are ubiquitously expressed in both the intracellular and extracellular environment. Previous studies have demonstrated that in T cells, Gal3 (the only member of this family able to form pentamers), is the predominant galectin involved in restricting TCR clustering (Demetriou et al., 2001). To investigate if Gal3 binding could explain the impaired TCR:CD8 co-receptor association during LCMV clone 13 infection, we measured extracellular Gal3 expression on CD8 T cells by flow cytometry. Consistent with our previous results, we observed increased in Gal3 binding on the surface of CD8 T cells during LCMV clone 13 infection that corresponds with enhanced PHA-L binding and is lost in the absence of IL-10 (Figure II - 4E, J and K). As staining was performed directly *ex vivo* (without addition of exogenous Gal3), these data establish that CD8 T cells bind more Gal3 *in vivo* during the establishment of chronic infection and that this is regulated by IL-10.

To identify a functional role of galectin binding in reducing CD8 T cell antigen sensitivity and TCR signaling, we asked whether inhibition of galectin binding using the competitive inhibitor D-Lactose is sufficient to restore CD8 T cell function. Splenocytes from LCMV clone 13 infection were incubated with or without D-Lactose and antigen sensitivity following was measured. Treatment with D-Lactose was sufficient to completely remove bound Gal3 from the surface of P14s (Figure II - 6A and B) without reducing the surface expression of the co-inhibitory receptor PD-1 (Figure II - 6C and D). Strikingly, when comparing the function of P14s isolated from the same LCMV clone 13 infected donor mice, we observed that the addition of D-Lactose during stimulation with peptide completely restored CD8 T cell antigen sensitivity (Figures II - 7A and B). Similarly, galectin inhibition was sufficient to restore TCR:CD8 membrane co-localization (Figures II - 7C and Figure II - 6E) and to rescue the TCR signaling capacity of CD8 T cells (Figure 4D). These data demonstrate that inhibiting galectin binding is able to rescue IL-10-mediated CD8 T cell suppression. Importantly, treatment with D-Lactose had no effect on P14s isolated from LCMV Armstrong infected mice (Figures II - 6F and G). Together, these data indicate that the inhibitory effects of IL-10 on antigen sensitivity and signal transduction are mediated by the binding of galectins on the surface of T cells.

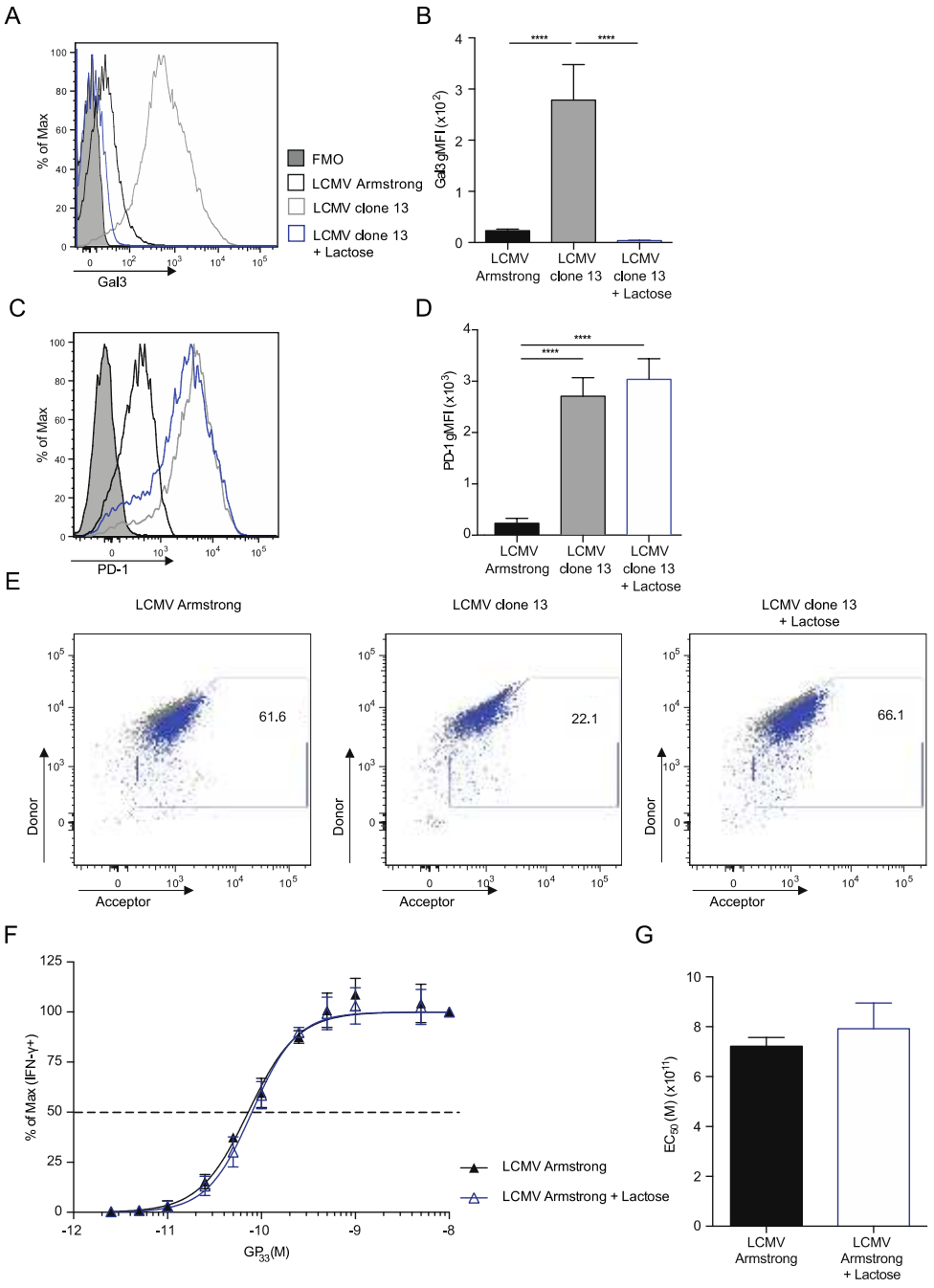

Figure II - 6. Lactose treatment inhibits Gal3 binding and restores function during LCMV clone 13 infection. A) Representative histograms of galectin 3 (Gal3) expression on P14s at day 8 post infection. Cells were isolated from LCMV Armstrong or clone 13 infected mice and were subsequently treated with or without 50 mM D-Lactose. Shaded histogram represents FMO control. B) Summary (mean ± SEM) of gMFI of Gal3 expression on P14 cells. C) Representative histograms of PD-1 expression on P14 cells isolated from LCMV Armstrong or LCMV clone 13 infected mice with or without treatment with D-Lactose. Shaded histogram represents isotype control. Refer to panel A for color legend. D) Summary (mean ± SEM) of gMFI of PD-1

expression on P14 cells. E) Representative plots of FRET. F) Antigen sensitivity in P14s isolated from LCMV Armstrong infection with or without treatment with D-Lactose as in Figure 1A. G) Summary (mean ± SEM) EC_{50} for IFN-γ^+ P14s. Data represent 3 mice per group and are representative of at least 2 experiments. Data in B were analysed by one-way ANOVA with Tukey's post-hoc analysis of multiple comparisons. Data in G were analyzed by two-tailed unpaired T-test. ****$p<0.0001$

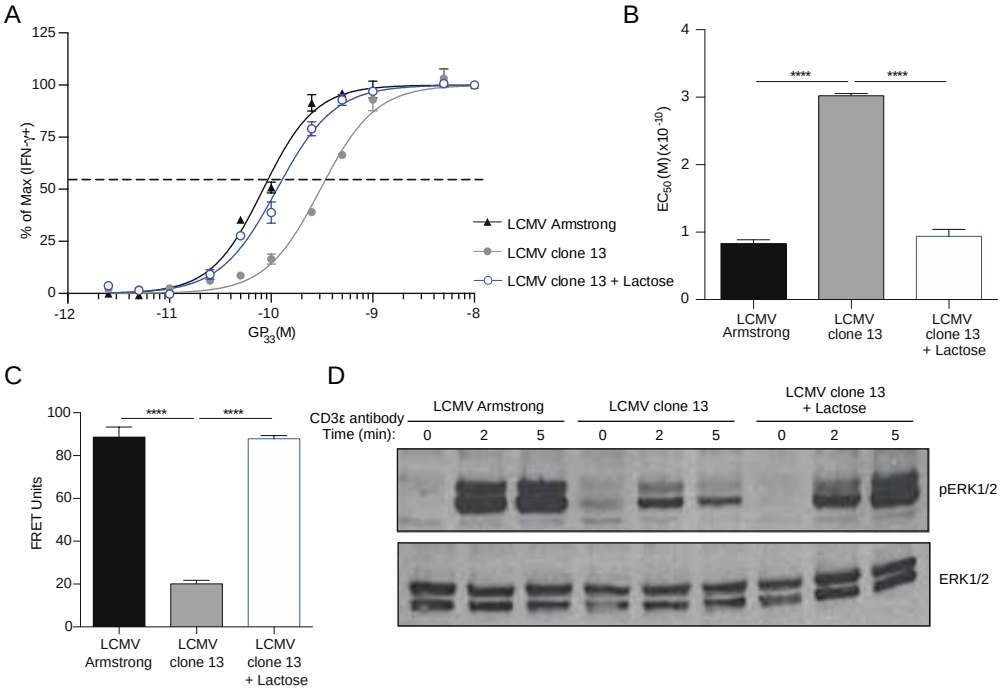

Figure II - 7. Galectin inhibition rescues T cell function during LCMV clone 13 infection. A) Antigen sensitivity as in Figure 1A of P14s from day 8 post-infection with the indicated strain of LCMV in WT hosts. Cells were stimulated in the presence or absence of 50 mM D-Lactose. B) Summary (mean ± SEM) EC_{50} values of IFN-γ^+ P14s. C) Summary FRET units (mean ± SEM) following a 30 minute treatment with or without 50 mM D-Lactose. D) Immunoblotting for phosphorylated and total ERK1/2 as in Figure 2A following treatment with or without 50 mM D-Lactose. Data in A, B and C represent 3 mice per group and are representative of at least 3 experiments. Data in D represent total protein of 3 pooled mice and is representative of at least 2 experiments. Data in B and C were analysed by one-way ANOVA with Tukey's post-hoc analysis of multiple comparisons. ****p<0.0001.

Gal3 deficiency restores antigen sensitivity and viral control despite increased N-glycan branching

Our data show that broad galectin inhibition with the competitive inhibitor D-Lactose is sufficient to rescue antigen sensitivity and TCR signaling capacity (Figure II - 7). Because Gal3 is believed to be the dominant lectin involved in restricting TCR diffusion, we asked whether Gal3 plays a role in IL-10-mediated CD8 T cell dysfunction during LCMV clone 13 infection. To address this, we adoptively transferred congenically marked WT P14 cells into Gal3 deficient mice (Gal3KO) and subsequently infected them with LCMV clone 13. At day 8 post-infection we observed that the absence of Gal3 resulted in the complete restoration of antigen sensitivity of P14s with an EC_{50} comparable to that of P14s from LCMV Armstrong infected mice (Figures II - 8A and B). The absence of Gal3 did not impact PD-1 expression, which remained high during LCMV clone 13 infection, further supporting that the effects of IL-10 on T cell function are independent of PD-1 expression (Figures II - 9A and B). In addition, reduced Gal3 binding on the surface of CD8 T cells restored TCR:CD8 co-localization (Figures II - 8C and Figure II - 9C-E) despite elevated PHA-L binding that was equivalent on P14s recovered from both WT and Gal3KO mice infected with LCMV clone 13 (Figures II - 8D and E). Thus, this demonstrates that LCMV clone 13 infection can still induce increased Mgat5-modified N-glycan branching in GAL3KO mice (as indicated by elevated PHA-L binding), but that binding of host derived-Gal3 is necessary to restrict T cell function. Together, these data demonstrate that Gal3 binding to glycoproteins on the surface of T cells plays a central role in mediating the IL-10 induced reduction in CD8 T cell antigen sensitivity.

Importantly, the Gal3KO mouse model allowed us to determine whether restoring CD8 T cell antigen sensitivity leads to an improvement in the capacity of the host to control viral infection. Genetic deletion of Gal3 dramatically improved viral control with some mice clearing LCMV clone 13 infection by day 8 and others exhibiting approximately 2 log reductions in viral titers in both the kidneys and the liver (Figures II - 8F and G). Enhanced virus control in Gal3KO mice was not simply due to an increase in CD8 T cell numbers, as T cell expansion was reduced relative to P14s responding to LCMV Armstrong infection (Figure II -9F). Thus, despite increased N-glycan branching on T cells responding to LCMV clone 13 in the presence of IL-10, Gal3 deficiency is sufficient to restore CD8 T cell antigen sensitivity and enhance the capacity of the host to control viral infection.

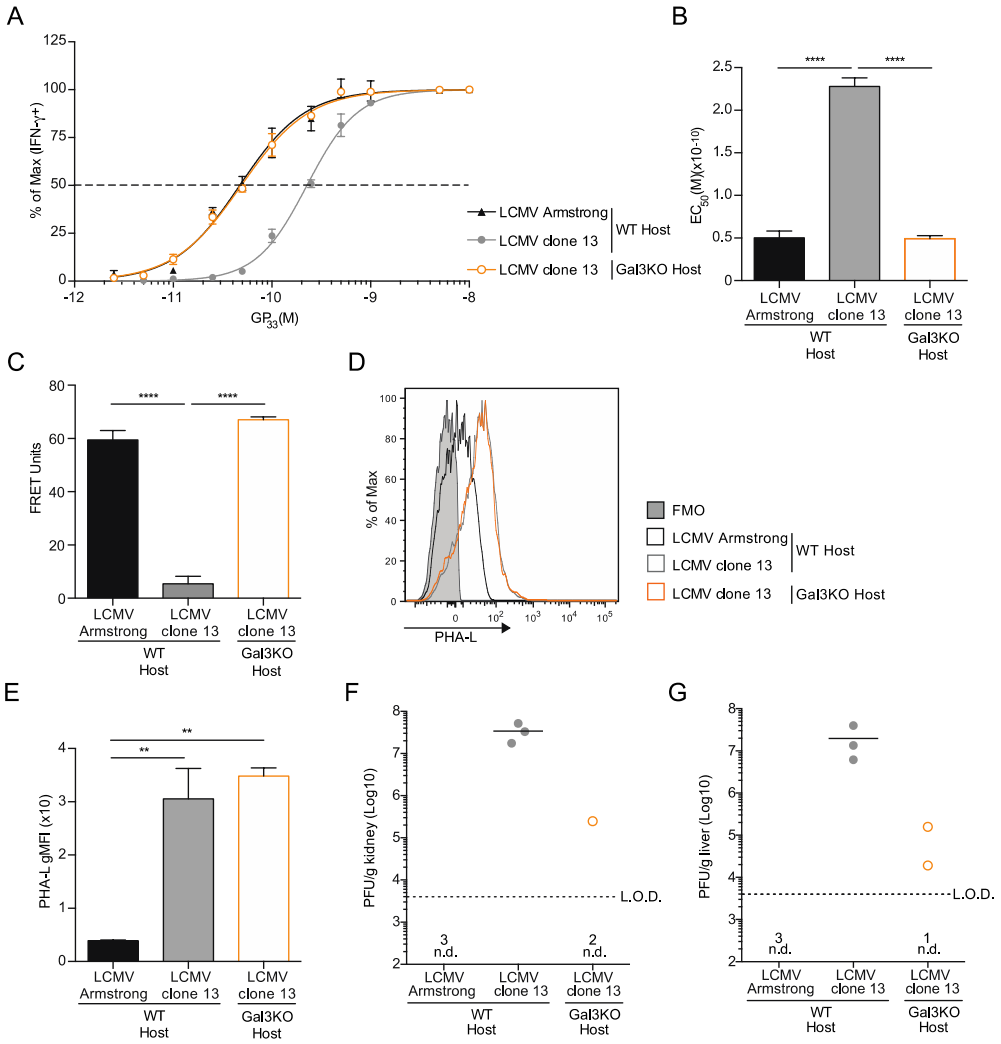

Figure II - 8. Gal3 regulates antigen sensitivity and limits T cell function during LCMV clone 13 infection. A) Antigen sensitivity as in Figure 1A for P14 cells at day 8 post-infection with the indicated LCMV strain in the indicated host. B) Summary (mean ± SEM) EC_{50} of IFN-γ^+ P14s. C) Summary (mean ± SEM) FRET Units. D) Representative histograms of PHA-L binding on P14s. Shaded histogram represents FMO control. E) Summary (mean ± SEM) gMFI of PHA-L binding. F) and G) Plaque Forming Units (PFU) per gram of indicated organ at day 8 post-infection with indicated strains of LCMV in the indicated host. Data represent 3 mice per group and are representative of 2 experiments. Data in B, C, and E were analysed by one-way ANOVA with Tukey's post-hoc analysis of multiple comparisons. **p<0.01, ****p<0.0001.

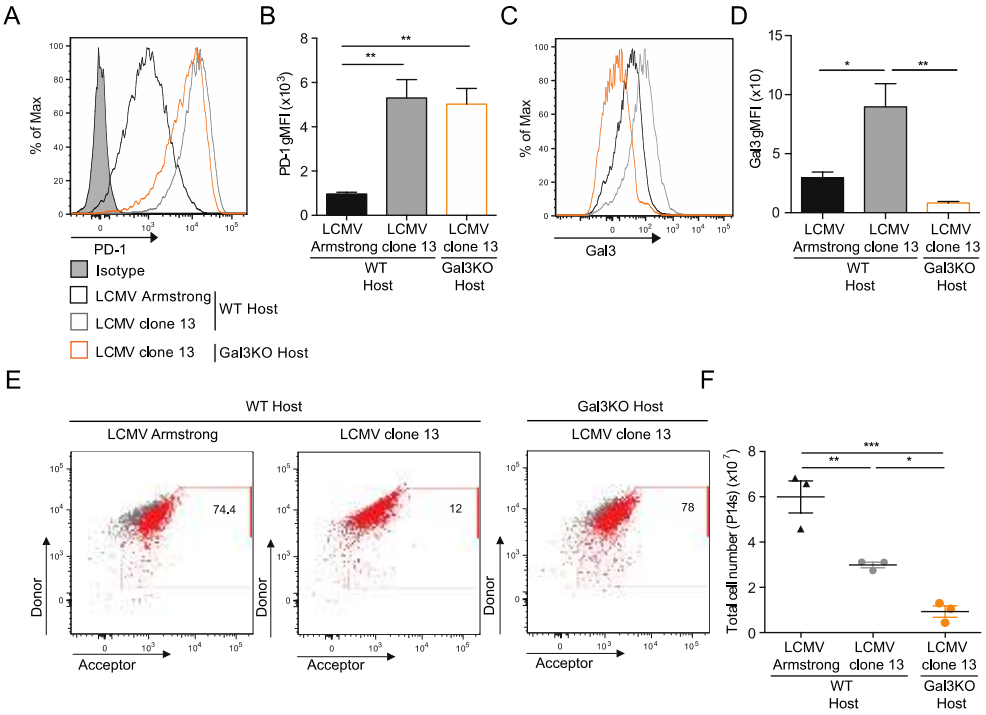

Figure II - 9. Gal3 deficiency does not alter PD-1 expression or recover cell accumulation. A) Representative histograms of PD-1 expression on P14 cells. Shaded histogram represents antibody isotype control B) Summary (mean ± SEM) gMFI of PD-1 expression. C) Representative histograms of Gal3 expression as on P14 cells. Refer to panel A for color legend. D) Summary (mean ± SEM) gMFI of Gal3 expression. E) Representative FRET plots. F) Total cell numbers of P14 cells isolated from the spleen at day 8 post infection, as indicated. Data represent 3 mice per group and are representative of 2 independent experiments. Data in B, D and F were analysed by one-way ANOVA with Tukey's post-hoc analysis of multiple comparisons. *p<0.05, **p<0.01, ***p<0.001

Our previous experiments allowed us to establish a central role for IL-10 in facilitating Gal3 mediated repression of T cell function and host protection. To investigate whether Mgat5 directly mediates these effects, we transduced P14 cells with retroviral constructs expressing shRNAs targeting *Mgat5* or the irrelevant gene *Firefly luciferase* (sh*Mgat5* and shFF_{luc}, respectively). At day 8 post-infection with LCMV clone 13, *Mgat5* knockdown in P14 cells was sufficient to restore antigen sensitivity independently of any changes in the surface expression of PD-1 (Figure II - 10A-D). *Mgat5* knockdown also resulted in decreased Gal3 and PHA-L binding on CD8 T cells showing that the reduction of *Mgat5* expression decreased N-glycan branching (Figure II – 10E-H). Importantly, retroviral transduction alone is not sufficient to restore antigen sensitivity as P14 cells expressing the control shRNA showed similar EC_{50}s and similar surface expression of PHA-L and Gal3 compared to mock transduced cells (Figure II - 10). Collectively, our data demonstrate that the IL-10 mediated increase in *Mgat5* expression is important to modify the glycosylation pattern on CD8 T cells and is associated with T cell dysfunction during LCMV clone 13 infection.

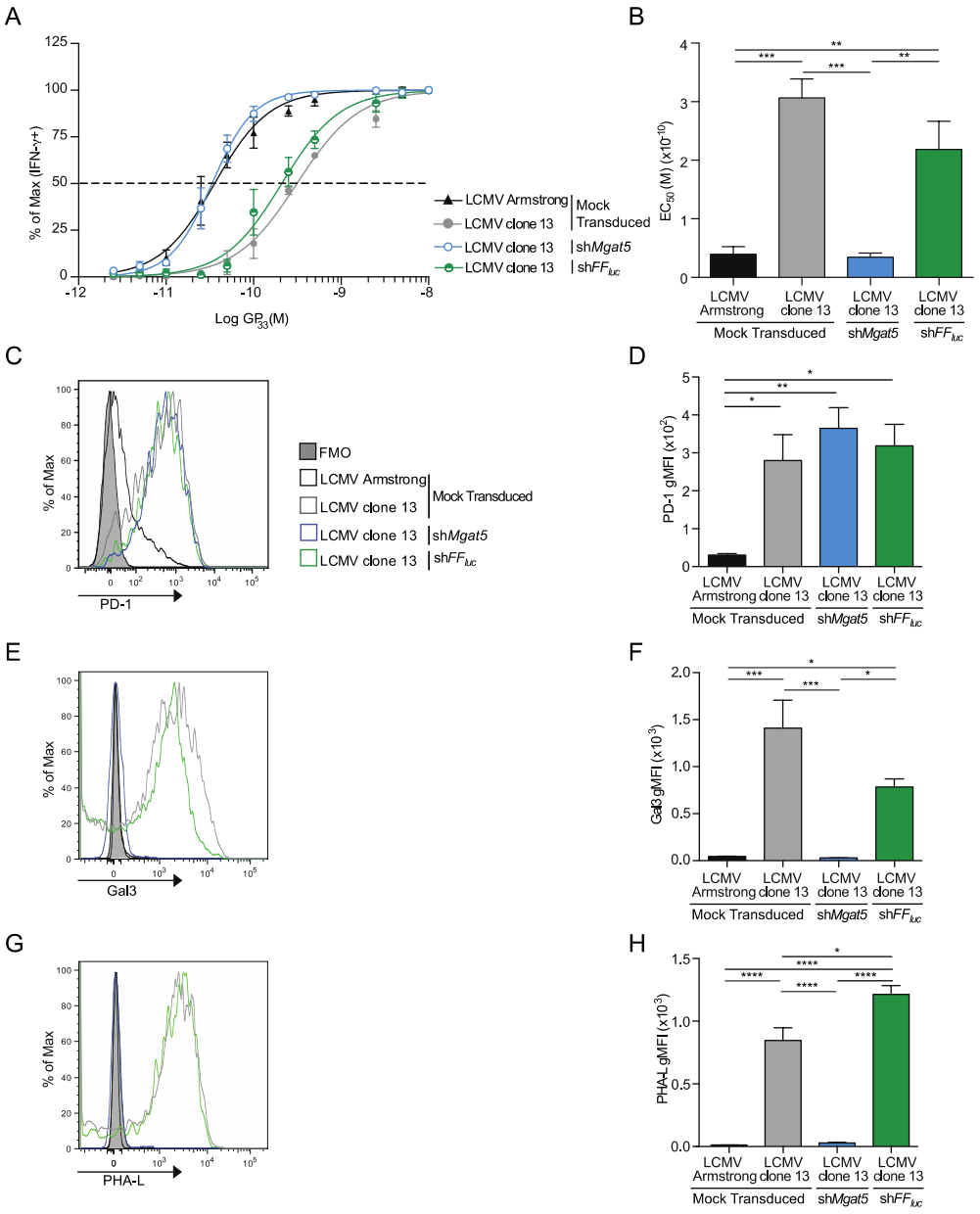

Figure II - 10. *Mgat5* knockdown rescues CD8 T cell antigen sensitivity during LCMV clone 13 infection. A) Antigen sensitivity as in Figure 1G for P14 cells expressing an shRNA targeting *Mgat5*, FF_{luc} or mock transduced. B) Summary (mean ± SEM) of EC_{50} for IFN-γ^+ P14s. C) Representative histograms of PD-1 expression, shaded histogram represents isotype control. D) Summary (mean ± SEM) gMFI of PD-1 expression on P14 cells. E) Representative histograms of Gal3 expression, shaded histogram represents FMO control. Refer to panel C for color legend. F) Summary (mean ± SEM) gMFI of Gal3 expression on P14 cells. G) Representative histograms of PHA-L binding on P14s, shaded histogram represents FMO control. Refer to panel C for color legend. H) Summary (mean ± SEM) gMFI of PHA-L expression on P14 cells. Data represent 3 mice per group and are representative of 2 experiments. Data in B, D and F were analysed by one-way ANOVA with Tukey's post-hoc analysis of multiple comparisons. *p<0.05, **p<0.01, ****p<0.0001.

IL-10 directly regulates Mgat5 expression and enhances N-glycan branching

To address whether IL-10 directly regulates the expression of *Mgat5* and altered glycosylation, we established an *ex vivo* system where we activated WT enriched polyclonal endogenous CD8 T cells *ex vivo* using plate bound CD3ε and CD28 antibodies in the presence or absence of IL-10. To further investigate whether IL-10 acts on CD8 T cells through canonical STAT3 dependent signaling pathways, we additionally employed a previously described STAT3 inhibitor (WP1066) (Iwamaru et al., 2007). We observed upregulation of *Mgat5* expression following activation in the presence of IL-10, which was impaired by STAT3 inhibition (Figure II - 11A). Furthermore, upregulation of *Mgat5* was associated with increased binding of PHA-L that was lost upon inhibition of STAT3 (Figures II - 11B and C). Because galectins are ubiquitously expressed, substrate (branched N-glycans) availability likely acts as the rate-limiting step to galectin binding. Thus, we asked whether recombinant Gal3 supplied exogenously could recapitulate our *in vivo* observations. Indeed, exogenous recombinant Gal3 only bound to cells treated with IL-10 in the absence of STAT3 inhibitor (Figures II - 11D and E), supporting our model that Mgat5-mediated glycan modifications act as the limiting step to galectin-mediated repression and that this directly induced by IL-10. Together, these data demonstrate that IL-10 acts directly on CD8 T cells in a STAT3 dependent manner to induce *Mgat5* expression, which sensitizes T cells to Gal3-mediated inhibition.

Our *ex vivo* experiments clearly demonstrate a regulatory pathway through which IL-10 can inhibit CD8 T cells by inducing *Mgat5* expression. Importantly, knockdown of *Il10ra* in P14s also resulted in reduced binding of Gal3 and reduced Mgat5-modified N-glycans (as measured by PHA-L) compared to mock-transduced cells (Figure II - 11F-I) and control shRNA transduced cells following LCMV clone 13 infection. Thus, these data firmly establish that, IL-10 acts directly on CD8 T cells to regulate N-glycan branching, leading to increased Gal3 binding and reduced antigen sensitivity contributing to T cell dysfunction during the establishment of chronic viral infection.

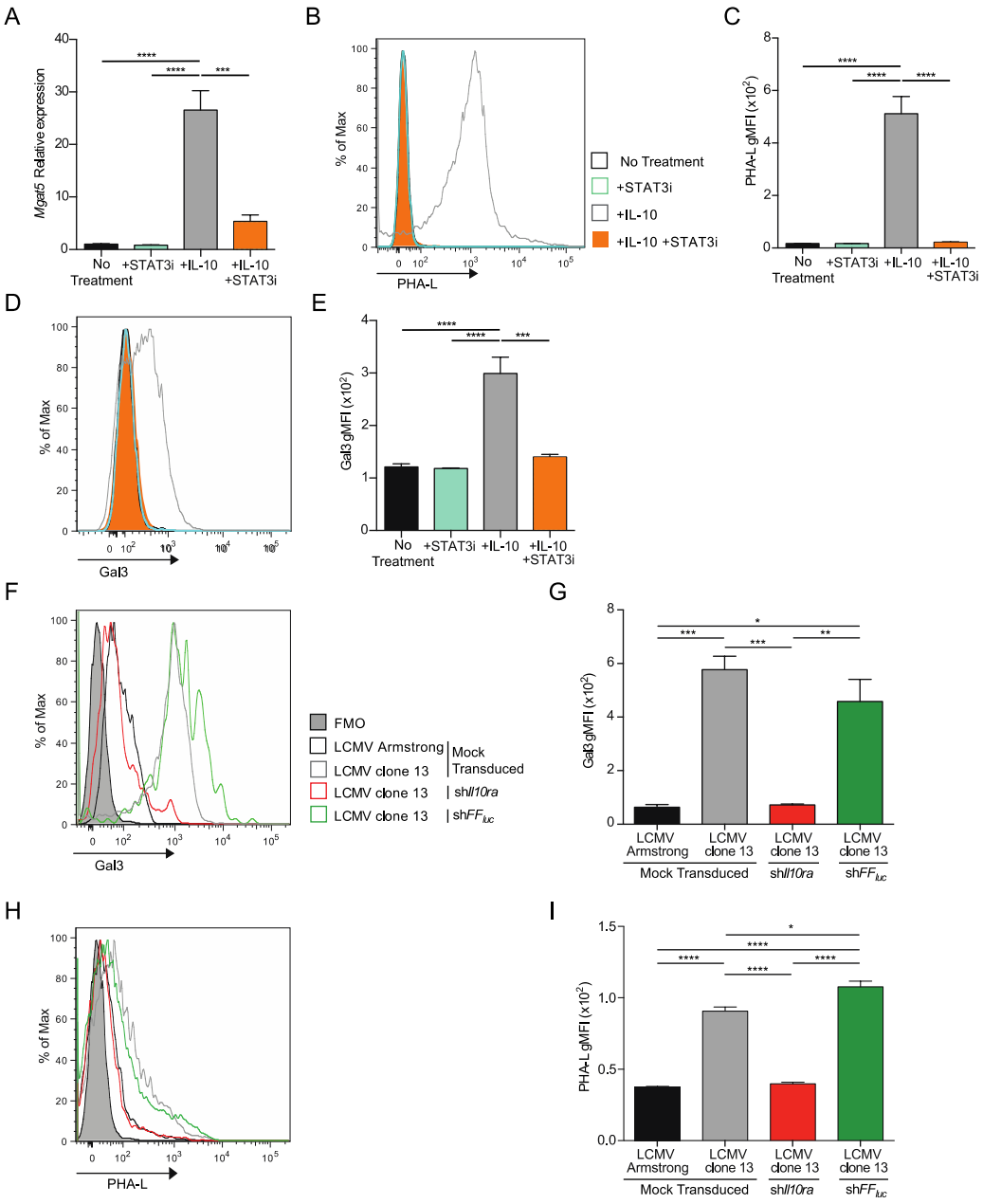

Figure II - 11. IL-10 directly regulates *Mgat5* expression and reduces antigen sensitivity in a T cell intrinsic manner. A) qRT-PCR analysis of *Mgat5* expression in CD8 T cells activated *ex vivo* in the presence or absence of recombinant IL-10 with and without STAT3 inhibitor WP1066 (STAT3i) for 8 days. B) Representative histograms of PHA-L binding to CD8 T cells following treatment with IL-10 or WP1066 as indicated. C) Summary (mean ± SEM) gMFI of PHA-L on CD8 T cells. D) Representative histograms of Gal3 expression on CD8 T cells following 30 minute incubation with 8 μg/mL of recombinant Gal3. Refer to panel B for color legend. E) Summary (mean ± SEM) gMFI of Gal3 expression on CD8 T cells. F) Representative

histograms of Gal3 expression on P14s transduced or mock-transduced as indicated, shaded histogram represents FMO control. G) Summary (mean ± SEM) gMFI of Gal3 expression on P14s. H) Representative histograms of PHA-L binding on P14s transduced or mock-transduced as indicated, shaded histogram represents FMO control. Refer to panel F for color legend. I) Summary (mean ± SEM) gMFI of PHA-L binding. Data represent 3 mice per group and are representative of 2 experiments. Data in A, C, E, G, and I were analysed by one-way ANOVA with Tukey's post-hoc analysis of multiple comparisons. **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

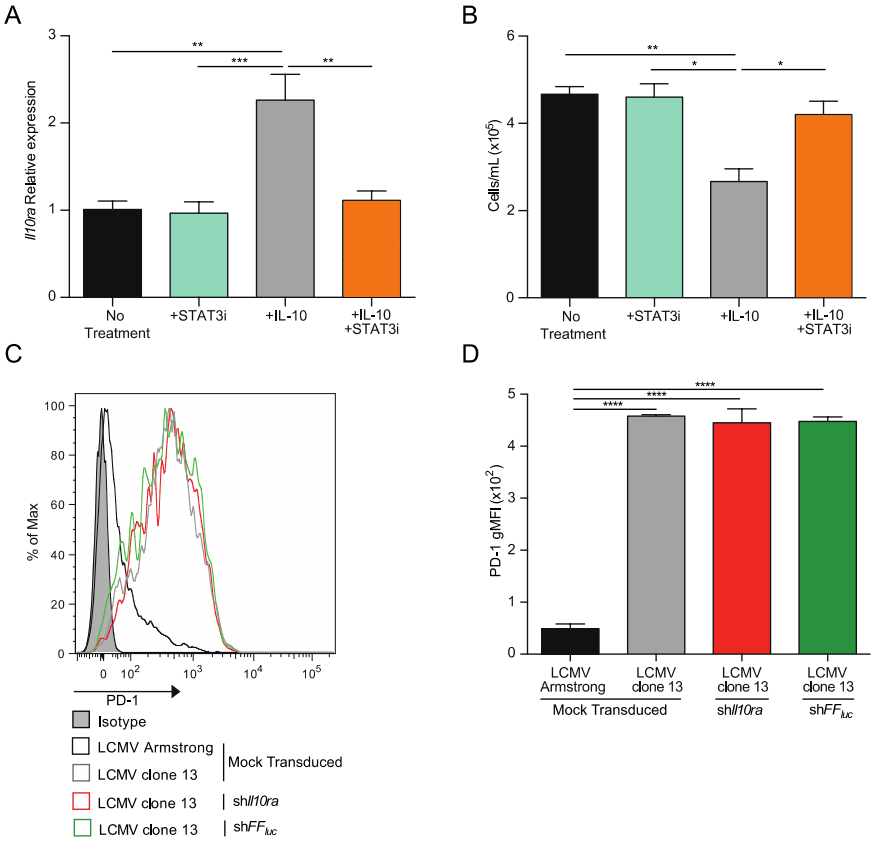

Figure II - 12. IL-10 acts on CD8 T cells and retroviral transduction does not impair PD-1 expression. A) qRT-PCR expression of *Il10ra* in CD8 T cells following expansion *ex vivo* in the presence or absence of IL-10 with or without STAT3i. B) Cell accumulation of CD8 T cells following expansion *ex vivo* in the presence or absence of IL-10 with or without STAT3i. C) Representative histograms of PD-1 expression on P14 cells isolated from LCMV infected mice transduced or mock-transduced as indicated. Shaded histogram represents isotype control. D) Summary (mean ± SEM) gMFI of PD-1 expression on P14s. Data in C and D are representative of 3 mice per group. All data are representative of at least 2 experiments. Data in A, B and D were analysed by one-way ANOVA with Tukey's post-hoc analysis of multiple comparisons. *p<0.05, **p<0.01, ****p<0.0001

Discussion

While the impact of chronic infections on host immune function, particularly the progressive development of T cell exhaustion, has been well described (Wherry and Kurachi, 2015), the early events that allow pathogens to establish persistence remain much less defined. Herein, we demonstrate that pathogens that establish chronic infections trigger reductions in CD8 T cell antigen sensitivity and that this requires CD8 T cell intrinsic IL-10 signaling. Mechanistically, we establish that IL-10 signaling, via STAT3, increases the expression of the glycosyl-transferase *Mgat5* leading to enhanced branched N-glycans on glycoproteins (including the TCR) on the surface of CD8 T cells. Mgat5-modified N-glycans, in turn, serve as ligands for the binding of Gal3 and the formation of a restrictive lattice that limits the capacity of TCR and the CD8 co-receptor to interact, resulting in a defective signal transduction cascade. This reduction in CD8 T cell antigen sensitivity increases the antigenic threshold required for T cell activation, likely allowing the rapidly replicating pathogen to outpace the immune system and establish persistence. Together, our data establish a novel mechanism of CD8 T cell regulation by anti-inflammatory cytokines that act through post-translational modification networks to modulate TCR sensitivity and CD8 T cell function. In addition, our data reveal that this regulatory loop is inappropriately activated during certain infections and favors the establishment of persistence.

IL-10 is an important immunomodulatory cytokines that can impact a variety of cell types. Interestingly, while the inhibitory role of IL-10 on APCs and CD4 T cells is becoming well defined, much less was known about its impact on CD8 T cells (Ip et al., 2017; Krawczyk et al., 2010). In fact, previous research has demonstrated that IL-10 can have a stimulatory role for CD8 T cells, aiding in tumour regression and acting as a substitute for signal 3 in the absence of IL-2 (Emmerich et al., 2012; Santin et al., 2000). Conversely, here we demonstrate a specific inhibitory effect of IL-10 directly on CD8 T cells. Similar paradoxical effects of cytokines, such as type I interferon, have been documented during chronic viral infections (Ng and Oldstone, 2014a; Wilson and Brooks, 2011). Thus, specific timing and inflammatory context likely play an important role in the transcriptional network and effects induced by IL-10 on CD8 T cells.

N-glycosylation of the TCR is an established regulator of T cell antigen sensitivity (also referred to as functional avidity), as mutational approaches reducing N-glycosylation can improve the sensitivity of T cells for their antigen (Kuball et al., 2009). Mgat5 plays a central

role in regulating the activation of T lymphocytes by modulating the branching of N-glycans. Previous studies have demonstrated that Mgat5-deficient naïve T cells spontaneously cluster their TCR and have a reduced antigenic threshold for activation at steady-state (Demetriou et al., 2001). In addition, Mgat5-deficient mice develop spontaneous autoimmunity and are more susceptible to the induction of experimental encephalomyelitis (EAE), clearly highlighting the importance of this enzyme as a gatekeeper of T cell activation (Demetriou et al., 2001; Grigorian and Demetriou, 2011). Here, we demonstrate that IL-10 signaling directly to CD8 T cells enhances the expression of *Mgat5* to negatively regulate CD8 T cell function. This novel regulatory loop appears conserved across a number of infections, and in humans, as we observed that CD8 T cells from mice infected with *Plasmodium yoelii* (a parasite that establishes chronic infection) and from patients chronically infected with HCV express elevated levels of *Mgat5* transcripts. Importantly, our data establish modulation of N-glycan branching as a critical rate-limiting step of the Gal3-mediated suppression of T cell function, as shown by the inability of CD8 T cells to bind excess Gal3 in the absence of IL-10 signaling. One question that remains is how IL-10 regulates the expression of *Mgat5*. While our data demonstrate that the increased expression of *Mgat5* is dependent on the canonical STAT3 signaling pathway, there are no STAT3 consensus binding sites within the *Mgat5* promoter region. One possibility is that STAT3 may complex with ETS-1 to potentiate *Mgat5* transcription as STAT3-ETS-1-SP1 transcriptional complexes have been described and ETS-1 is a documented regulator of *Mgat5* (Bian et al., 2011; Kang et al., 1996). Alternatively, STAT3 signaling may lead to the induction of other transcription factors leading to enhanced *Mgat5* expression. In support of this, STAT3 was shown to direct the expression and epigenetic modifications of a number of transcription factors in T cells (Durant et al., 2010). Determining how IL-10 regulates *Mgat5* expression will be the subject of future investigations.

The regulation of *Mgat5* by IL-10 over the course of infection may represent an immunoregulatory loop that plays an important role even during a protective immune response. This may serve to prevent some CD8 T cells from becoming terminally differentiated thereby maintaining their capacity to develop into memory CD8 T cells. In support of this, IL-10 plays an important role in the development of memory CD8 T cells following clearance of infection (Cui et al., 2011). While some of the role of IL-10 in that context is to insulate the T cells from the effects of other inflammatory cytokines, it is tempting to speculate that it may also have a

direct effect on CD8 T cells by regulating *Mgat5* expression and N-glycan branching. In the context of chronic infections, the sustained and heightened production of IL-10 may simply amplify this regulatory loop leading to the inability of T cells to respond efficiently, thereby favoring the establishment of pathogen persistence.

Membrane restriction by multivalent galectin interactions is a regulatory mechanism exploited by some cancers, which secrete soluble galectins that contribute to immune-evasion (Dalotto-Moreno et al., 2013). Elegant studies using murine models of tumor progression have shown systemic repression of anti-tumour immunity by galectin producing tumour implants (Dalotto-Moreno et al., 2013). Importantly, local administration of galectin inhibitors can restore immune-detection and reduce tumour burden in these models (Demotte et al., 2010). Our data suggest that substrate (branched N-glycans) availability, rather than galectin abundance is the rate-limiting step in reducing CD8 T cell antigen sensitivity during chronic viral infections and that this step is controlled by IL-10 signaling to CD8 T cells. Of note, tumour derived IL-10 plays an important role in immune-evasion in some cancers (Sun et al., 2015). Therefore, pharmacological inhibition of IL-10 or *Mgat5* regulatory circuits may also provide a more targeted approach to reinvigorating anti-tumour or anti-viral immunity. As these represent regulatory pathways that are distinct from the exhaustion pathways mediated by checkpoint blockade inhibitors, this suggests that these new therapies could be used in conjunction with checkpoint blockade to ameliorate patient outcomes.

In conclusion, our data provide novel mechanistic details about the impact of IL-10 on CD8 T cells. We establish that IL-10 induced by infection with LCMV clone 13 directly regulates the expression of *Mgat5*, leading to heightened Mgat5-modified N-glycan branching on CD8 T cells that enhances binding of Gal3. Elevated Gal3 binding restricts TCR:CD8 co-receptor association and reduces TCR signaling capacity ultimately decreasing CD8 T cell antigen sensitivity. Thus, our data describe a new regulatory pathway that leads to dysfunction of T cells during persistent infections and identifies pathways that may provide novel therapeutic targets to potentially enhance the T cell response to chronic viral infections and cancer.

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Chapter III Conclusions and future directions

Antigen sensitivity of CD8 T cells is a key correlate of anti-viral immunity (Alexander-Miller, 2005). This process is regulated by several mechanisms, including infection induced inflammation contributing to pathogen control (Richer et al., 2013). Viral infections that progress to chronic disease induce a distinct inflammatory milieu upon contraction that has been characterised by the immune-modulatory cytokine IL-10 (Mannino et al., 2015). IL-10 plays an important role in the development of chronicity as supported by rigorous studies using murine models as well as naturally occurring polymorphisms in human patients (Wilson and Brooks, 2011). The direct contribution of IL-10 and its effects on the responding anti-viral CD8 T cell pool remained a significant gap in our understanding of chronic viral infections.

Herein I demonstrate that IL-10 acts directly on CD8 T cells to limit cellular activation through the induction of branched N-glycosylation, thereby exploiting the ubiquitous galectin lattice. I show that IL-10 limits signal transduction from the TCR, coincident with increased global branch N-glycosylation and Gal3 binding. Furthermore, this regulatory mechanism can be overcome in the absence or inhibition of Gal3 ultimately resulting in improved colocalization of surface receptors and increased viral control.

Additionally, genetic knockdown of the gene *Mgat5*, the glycosyltransferase that catalyses N-glycan branching can rescue impaired antigen sensitivity mediated by IL-10, despite maintaining elevated expression of the inhibitory receptor PD-1. I have also shown that these regulatory networks are activated in human CD8 T cells as well, and an IL-10/Mgat5 network may contribute to chronic viral infections in human patients. These data represent the first mechanistic demonstration of the effects of IL-10 on CD8 T cell responses and how this interaction might be subverted to establish persistence during infection. I also provide novel insights into the functions of IL-10 in other cell types by linking IL-10 signal transduction to global changes in post-translational modification networks.

This work suggests that inhibition of Mgat5 or manipulation of the galectin lattice may act as a therapeutic agent to mitigate chronic disease. Previous research has demonstrated that galectin inhibition can reinvigorate tumour infiltrating lymphocytes and provides improved tumor control in murine models (Demotte et al., 2010). It will be important to address whether

these findings can be engineered into targetable drugs in combination with checkpoint blockade, or if genetic manipulation of *Mgat5* in CAR-T cells would provide improved immunity. These data alter our collective understanding of the early events of chronic infection, demonstrating that inflammatory context drives T cell intrinsic glycomic-changes promoting structural based inhibition. Future therapeutic design should consider the role of IL-10 and other inflammatory mediators in restraining CD8 T cell activation at these early time points, prior to the onset of T cell exhaustion.

Furthermore, there is likely an evolutionary basis for the conservation of this regulatory mechanism. Ongoing work will address the role of an IL-10/Mgat5 network in normal CD8 T cell physiological processes. As mentioned above, IL-10 plays an important role in memory generation (Laidlaw et al., 2015); it is conceivable that a mechanism evolved to promote memory generation has been coopted by pathogens to establish persistent infections. Additionally, some cytokine receptors are highly glycosylated and can remain trapped in the galectin lattice (Araujo et al., 2017). Receptors such as the IL-2 receptor have been shown to play an important role in CD4 T cell polarization, skewing the differentiation pathway toward the development of Treg cells in the presence of branched N-glycans (Araujo et al., 2017). As the expression of cytokine receptors plays a key role in CD8 T cell functions, future research should address the role of IL-10/Mgat5 mediated surface retention of cytokine receptors in CD8 T cell biology, such as the maintenance of memory CD8 T cells. Additionally, aging has been associated with deteriorated immune responses and impaired antigen sensitivity in antigen-specific CD8 T cell responses, in part to age dependent reduction in miR181a expression (Li et al., 2012). Future work will aim to address the role of this described IL-10/Mgat5 immunomodulatory network in the CD8 T cell response in aged individuals. By dissecting the mechanisms and contributions of IL-10 to CD8 T cell dysfunction, we will be better equipped to treat IL-10 mediated CD8 T cell suppression both in aging and chronic disease.

References

Abdel-Hakeem, M.S., Boisvert, M., Bruneau, J., Soudeyns, H., and Shoukry, N.H. (2017). Selective expansion of high functional avidity memory CD8 T cell clonotypes during hepatitis C virus reinfection and clearance. PLoS Pathog *13*, e1006191.

Ahmed, R., Salmi, A., Butler, L.D., Chiller, J.M., and Oldstone, M.B. (1984). Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. J Exp Med *160*, 521-540.

Alexander-Miller, M.A. (2005). High-avidity CD8(+) T cells - Optimal soldiers in the war against viruses and tumors. Immunologic Research *31*, 13-24.

Antonopoulos, A., Demotte, N., Stroobant, V., Haslam, S.M., van der Bruggen, P., and Dell, A. (2012). Loss of effector function of human cytolytic T lymphocytes is accompanied by major alterations in N- and O-glycosylation. J Biol Chem *287*, 11240-11251.

Araujo, L., Khim, P., Mkhikian, H., Mortales, C.L., and Demetriou, M. (2017). Glycolysis and glutaminolysis cooperatively control T cell function by limiting metabolite supply to N-glycosylation. Elife *6*.

Badr, G., Bedard, N., Abdel-Hakeem, M.S., Trautmann, L., Willems, B., Villeneuve, J.P., Haddad, E.K., Sekaly, R.P., Bruneau, J., and Shoukry, N.H. (2008). Early Interferon Therapy for Hepatitis C Virus Infection Rescues Polyfunctional, Long-Lived CD8(+) Memory T Cells. Journal of Virology *82*, 10017-10031.

Barber, D.L., Wherry, E.J., Masopust, D., Zhu, B.G., Allison, J.P., Sharpe, A.H., Freeman, G.J., and Ahmed, R. (2006). Restoring function in exhausted CD8 T cells during chronic viral infection. Nature *439*, 682-687.

Bian, Y., Zhou, W., Zhao, Y., Li, X., Geng, W., Hao, R., Yang, Q., and Huang, W. (2011). High-dose siRNAs upregulate mouse Eri-1 at both transcription and posttranscription levels. PLoS One *6*, e26466.

Biswas, P.S., Pedicord, V., Ploss, A., Menet, E., Leiner, I., and Pamer, E.G. (2007). Pathogen-Specific CD8 T Cell Responses Are Directly Inhibited by IL-10. The Journal of Immunology *179*, 4520-4528.

Bonhomme, C.J., Knopp, K.A., Bederka, L.H., Angelini, M.M., and Buchmeier, M.J. (2013). LCMV Glycosylation Modulates Viral Fitness and Cell Tropism. Plos One *8*.

Borger, J.G., Zamoyska, R., and Gakamsky, D.M. (2014). Proximity of TCR and its CD8 coreceptor controls sensitivity of T cells. Immunol Lett *157*, 16-22.

Brooks, D.G., Ha, S.J., Elsaesser, H., Sharpe, A.H., Freeman, G.J., and Oldstone, M.B. (2008). IL-10 and PD-L1 operate through distinct pathways to suppress T-cell activity during persistent viral infection. Proc Natl Acad Sci U S A *105*, 20428-20433.

Brooks, D.G., Trifilo, M.J., Edelmann, K.H., Teyton, L., McGavern, D.B., and Oldstone, M.B. (2006). Interleukin-10 determines viral clearance or persistence in vivo. Nat Med *12*, 1301-1309.

Busch, D.H., and Pamer, E.G. (1999). T cell affinity maturation by selective expansion during infection. Journal of Experimental Medicine *189*, 701-709.

Butler, N.S., Moebius, J., Pewe, L.L., Traore, B., Doumbo, O.K., Tygrett, L.T., Waldschmidt, T.J., Crompton, P.D., and Harty, J.T. (2012). Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage Plasmodium infection. Nature Immunology *13*, 188-195.

Cawthon, A.G., and Alexander-Miller, M.A. (2002). Optimal Colocalization of TCR and CD8 as a Novel Mechanism for the Control of Functional Avidity. The Journal of Immunology *169*, 3492-3498.

Chen, I.J., Chen, H.L., and Demetriou, M. (2007). Lateral compartmentalization of T cell receptor versus CD45 by galectin-N-glycan binding and microfilaments coordinate basal and activation signaling. J Biol Chem *282*, 35361-35372.

Chen, R.Q., Belanger, S., Frederick, M.A., Li, B., Johnston, R.J., Xiao, N.M., Liu, Y.C., Sharma, S., Peters, B., Rao, A., *et al.* (2014). In Vivo RNA Interference Screens Identify Regulators of Antiviral CD4(+) and CD8(+) T Cell Differentiation. Immunity *41*, 325-338.

Corinti, S., Albanesi, C., la Sala, A., Pastore, S., and Girolomoni, G. (2001). Regulatory Activity of Autocrine IL-10 on Dendritic Cell Functions. The Journal of Immunology *166*, 4312-4318.

Couper, K.N., Blount, D.G., and Riley, E.M. (2008). IL-10: The master regulator of immunity to infection. Journal of Immunology *180*, 5771-5777.

Cox, M.A., Kahan, S.M., and Zajac, A.J. (2013). Anti-viral CD8 T cells and the cytokines that they love. Virology *435*, 157-169.

Cui, W., Liu, Y., Weinstein, J.S., Craft, J., and Kaech, S.M. (2011). An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells. Immunity *35*, 792-805.

Curtsinger, J.M., and Mescher, M.F. (2010). Inflammatory cytokines as a third signal for T cell activation. Current Opinion in Immunology *22*, 333-340.

Dalotto-Moreno, T., Croci, D.O., Cerliani, J.P., Martinez-Allo, V.C., Dergan-Dylon, S., Mendez-Huergo, S.P., Stupirski, J.C., Mazal, D., Osinaga, E., Toscano, M.A., *et al.* (2013). Targeting galectin-1 overcomes breast cancer-associated immunosuppression and prevents metastatic disease. Cancer Res *73*, 1107-1117.

Demetriou, M., Granovsky, M., Quaggin, S., and Dennis, J.W. (2001). Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. Nature *409*, 733-739.

Demotte, N., Stroobant, V., Courtoy, P.J., Van Der Smissen, P., Colau, D., Luescher, I.F., Hivroz, C., Nicaise, J., Squifflet, J.L., Mourad, M., *et al.* (2008). Restoring the association of the T cell receptor with CD8 reverses anergy in human tumor-infiltrating lymphocytes. Immunity *28*, 414-424.

Demotte, N., Wieers, G., Van Der Smissen, P., Moser, M., Schmidt, C., Thielemans, K., Squifflet, J.L., Weynand, B., Carrasco, J., Lurquin, C., *et al.* (2010). A galectin-3 ligand corrects the impaired function of human CD4 and CD8 tumor-infiltrating lymphocytes and favors tumor rejection in mice. Cancer Res *70*, 7476-7488.

Dennis, J.W., Lau, K.S., Demetriou, M., and Nabi, I.R. (2009). Adaptive regulation at the cell surface by N-glycosylation. Traffic *10*, 1569-1578.

Durant, L., Watford, W.T., Ramos, H.L., Laurence, A., Vahedi, G., Wei, L., Takahashi, H., Sun, H.W., Kanno, Y., Powrie, F., *et al.* (2010). Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. Immunity *32*, 605-615.

Earl, L.A., and Baum, L.G. (2008). CD45 glycosylation controls T-cell life and death. Immunol Cell Biol *86*, 608-615.

Ejrnaes, M., Filippi, C.M., Martinic, M.M., Ling, E.M., Togher, L.M., Crotty, S., and von Herrath, M.G. (2006). Resolution of a chronic viral infection after interleukin-10 receptor blockade. J Exp Med *203*, 2461-2472.

Elola, M.T., Blidner, A.G., Ferragut, F., Bracalente, C., and Rabinovich, G.A. (2015). Assembly, organization and regulation of cell-surface receptors by lectin-glycan complexes. Biochem J *469*, 1-16.

Emmerich, J., Mumm, J.B., Chan, I.H., LaFace, D., Truong, H., McClanahan, T., Gorman, D.M., and Oft, M. (2012). IL-10 Directly Activates and Expands Tumor-Resident CD8(+) T Cells without De Novo Infiltration from Secondary Lymphoid Organs. Cancer Research *72*, 3570-3581.

Filipp, D., Ballek, O., and Manning, J. (2012). Lck, Membrane Microdomains, and TCR Triggering Machinery: Defining the New Rules of Engagement. Front Immunol *3*, 155.

Flynn, J.K., Dore, G.J., Hellard, M., Yeung, B., Rawlinson, W.D., White, P.A., Kaldor, J.M., Lloyd, A.R., Ffrench, R.A., and Grp, A.S. (2011). Early IL-10 predominant responses are associated with progression to chronic hepatitis C virus infection in injecting drug users. Journal of Viral Hepatitis *18*, 549-561.

Frebel, H., Richter, K., and Oxenius, A. (2010). How chronic viral infections impact on antigen-specific T-cell responses. Eur J Immunol *40*, 654-663.

Germain, R.N. (2002). T-cell development and the CD4-CD8 lineage decision. Nature Reviews Immunology *2*, 309-322.

Grebely, J., Morris, M.D., Rice, T.M., Bruneau, J., Cox, A.L., Kim, A.Y., McGovern, B.H., Shoukry, N.H., Lauer, G., Maher, L., *et al.* (2013). Cohort Profile: The International Collaboration of Incident HIV and Hepatitis C in Injecting Cohorts (InC3) Study. International Journal of Epidemiology *42*, 1649-1659.

Grigorian, A., and Demetriou, M. (2011). Mgat5 deficiency in T cells and experimental autoimmune encephalomyelitis. ISRN Neurol *2011*, 374314.

Grigorian, A., Lee, S.U., Tian, W., Chen, I.J., Gao, G., Mendelsohn, R., Dennis, J.W., and Demetriou, M. (2007). Control of T Cell-mediated autoimmunity by metabolite flux to N-glycan biosynthesis. J Biol Chem *282*, 20027-20035.

Grigorian, A., Torossian, S., and Demetriou, M. (2009). T-cell growth, cell surface organization, and the galectin-glycoprotein lattice. Immunol Rev *230*, 232-246.

Ilarregui, J.M., Bianco, G.A., Toscano, M.A., and Rabinovich, G.A. (2005). The coming of age of galectins as immunomodulatory agents: impact of these carbohydrate binding proteins in T cell physiology and chronic inflammatory disorders. Ann Rheum Dis *64 Suppl 4*, iv96-103.

Ip, W.E., Hoshi, N., Shouval, D.S., Snapper, S., and Medzhitov, R. (2017). Anti-inflammatory effect of IL-10 mediated by metabolic reprogramming of macrophages. Science *356*, 513-519.

Iwamaru, A., Szymanski, S., Iwado, E., Aoki, H., Yokoyama, T., Fokt, I., Hess, K., Conrad, C., Madden, T., Sawaya, R., *et al.* (2007). A novel inhibitor of the STAT3 pathway induces apoptosis in malignant glioma cells both in vitro and in vivo. Oncogene *26*, 2435-2444.

Joffre, O., Nolte, M.A., Sporri, R., and Sousa, C.R.E. (2009). Inflammatory signals in dendritic cell activation and the induction of adaptive immunity. Immunological Reviews *227*, 234-247.

Kang, R., Saito, H., Ihara, Y., Miyoshi, E., Koyama, N., Sheng, Y., and Taniguchi, N. (1996). Transcriptional regulation of the N-acetylglucosaminyltransferase V gene in human bile duct

carcinoma cells (HuCC-T1) is mediated by Ets-1. Journal of Biological Chemistry *271*, 26706-26712.

Kapsenberg, M.L. (2003). Dendritic-cell control of pathogen-driven T-cell polarization. Nature Reviews Immunology *3*, 984-993.

Klein, L., Kyewski, B., Allen, P.M., and Hogquist, K.A. (2014). Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). Nature Reviews Immunology *14*, 377-391.

Kobayashi, F., Morii, T., Matsui, T., Fujino, T., Watanabe, Y., Weidanz, W.P., and Tsuji, M. (1996). Production of interleukin 10 during malaria caused by lethal and nonlethal variants of Plasmodium yoelii yoelii. Parasitology Research *82*, 385-391.

Krawczyk, C.M., Holowka, T., Sun, J., Blagih, J., Amiel, E., DeBerardinis, R.J., Cross, J.R., Jung, E., Thompson, C.B., Jones, R.G., *et al.* (2010). Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. Blood *115*, 4742-4749.

Kroger, C.J., and Alexander-Miller, M.A. (2007). Dose-dependent modulation of CD8 and functional avidity as a result of peptide encounter. Immunology *122*, 167-178.

Kuball, J., Hauptrock, B., Malina, V., Antunes, E., Voss, R.H., Wolfl, M., Strong, R., Theobald, M., and Greenberg, P.D. (2009). Increasing functional avidity of TCR-redirected T cells by removing defined N-glycosylation sites in the TCR constant domain. J Exp Med *206*, 463-475.

Kumar, R., Ferez, M., Swamy, M., Arechaga, I., Rejas, M.T., Valpuesta, J.M., Schamel, W.W., Alarcon, B., and van Santen, H.M. (2011). Increased sensitivity of antigen-experienced T cells through the enrichment of oligomeric T cell receptor complexes. Immunity *35*, 375-387.

Kurktschiev, P.D., Raziorrouh, B., Schraut, W., Backmund, M., Wachtler, M., Wendtner, C.M., Bengsch, B., Thimme, R., Denk, G., Zachoval, R., *et al.* (2014). Dysfunctional CD8+ T cells in hepatitis B and C are characterized by a lack of antigen-specific T-bet induction. J Exp Med *211*, 2047-2059.

Kwon, D.S., and Kaufmann, D.E. (2010). Protective and detrimental roles of IL-10 in HIV pathogenesis. European Cytokine Network *21*, 208-214.

Laidlaw, B.J., Cui, W., Amezquita, R.A., Gray, S.M., Guan, T., Lu, Y., Kobayashi, Y., Flavell, R.A., Kleinstein, S.H., Craft, J., *et al.* (2015). Production of IL-10 by CD4(+) regulatory T cells during the resolution of infection promotes the maturation of memory CD8(+) T cells. Nat Immunol *16*, 871-879.

Lau, K.S., Partridge, E.A., Grigorian, A., Silvescu, C.I., Reinhold, V.N., Demetriou, M., and Dennis, J.W. (2007). Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation. Cell *129*, 123-134.

Li, G., Yu, M., Lee, W.W., Tsang, M., Krishnan, E., Weyand, C.M., and Goronzy, J.J. (2012). Decline in miR-181a expression with age impairs T cell receptor sensitivity by increasing DUSP6 activity. Nat Med *18*, 1518-1524.

Li, Q.J., Chau, J., Ebert, P.J., Sylvester, G., Min, H., Liu, G., Braich, R., Manoharan, M., Soutschek, J., Skare, P., *et al.* (2007). miR-181a is an intrinsic modulator of T cell sensitivity and selection. Cell *129*, 147-161.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402-408.

Malherbe, L., Hausl, C., Teyton, L., and McHeyzer-Williams, M. (2005). Clonal selection of helper T cells is determined by an affinity threshold with no further skewing of TCR binding properties. Faseb Journal *19*, A384-A384.

Mannino, M.H., Zhu, Z., Xiao, H., Bai, Q., Wakefield, M.R., and Fang, Y. (2015). The paradoxical role of IL-10 in immunity and cancer. Cancer Lett *367*, 103-107.

Mkhikian, H., Mortales, C.L., Zhou, R.W., Khachikyan, K., Wu, G., Haslam, S.M., Kavarian, P., Dell, A., and Demetriou, M. (2016). Golgi self-correction generates bioequivalent glycans to preserve cellular homeostasis. Elife *5*.

Moore, K.W., Malefyt, R.D., Coffman, R.L., and O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol *19*, 683-765.

Moremen, K.W., Tiemeyer, M., and Nairn, A.V. (2012). Vertebrate protein glycosylation: diversity, synthesis and function. Nature Reviews Molecular Cell Biology *13*, 448-462.

Nabi, I.R., Shankar, J., and Dennis, J.W. (2015). The galectin lattice at a glance. J Cell Sci *128*, 2213-2219.

Ng, C.T., and Oldstone, M.B. (2014a). IL-10: achieving balance during persistent viral infection. Curr Top Microbiol Immunol *380*, 129-144.

Ng, C.T., and Oldstone, M.B.A. (2014b). IL-10: Achieving Balance During Persistent Viral Infection. In Interleukin-10 in Health and Disease, S. Fillatreau, and A. Ogarra, eds. (Berlin: Springer-Verlag Berlin), pp. 129-144.

Norris, B.A., Uebelhoer, L.S., Nakaya, H.I., Price, A.A., Grakoui, A., and Pulendran, B. (2013). Chronic but not acute virus infection induces sustained expansion of myeloid suppressor cell numbers that inhibit viral-specific T cell immunity. Immunity *38*, 309-321.

Oldstone, M.B. (2015). A Jekyll and Hyde Profile: Type 1 Interferon Signaling Plays a Prominent Role in the Initiation and Maintenance of a Persistent Virus Infection. J Infect Dis *212 Suppl 1*, S31-36.

Paddison, P.J., Cleary, M., Silva, J.M., Chang, K., Sheth, N., Sachidanandam, R., and Hannon, G.J. (2004). Cloning of short hairpin RNAs for gene knockdown in mammalian cells. Nature Methods *1*, 163-167.

Parish, I.A., Marshall, H.D., Staron, M.M., Lang, P.A., Brustle, A., Chen, J.H., Cui, W., Tsui, Y.C., Perry, C., Laidlaw, B.J., *et al.* (2014). Chronic viral infection promotes sustained Th1-derived immunoregulatory IL-10 via BLIMP-1. J Clin Invest *124*, 3455-3468.

Perica, K., Bieler, J.G., Edidin, M., and Schneck, J. (2012). Modulation of MHC binding by lateral association of TCR and coreceptor. Biophys J *103*, 1890-1898.

Pircher, H., Michalopoulos, E.E., Iwamoto, A., Ohashi, P.S., Baenziger, J., Hengartner, H., Zinkernagel, R.M., and Mak, T.W. (1987). Molecular analysis of the antigen receptor of virus-specific cytotoxic T cells and identification of a new V alpha family. Eur J Immunol *17*, 1843-1846.

Prabakaran, S., Lippens, G., Steen, H., and Gunawardena, J. (2012). Post-translational modification: nature's escape from genetic imprisonment and the basis for dynamic information encoding. Wiley Interdisciplinary Reviews-Systems Biology and Medicine *4*, 565-583.

Richer, M.J., Nolz, J.C., and Harty, J.T. (2013). Pathogen-specific inflammatory milieux tune the antigen sensitivity of CD8(+) T cells by enhancing T cell receptor signaling. Immunity *38*, 140-152.

Santin, A.D., Hermonat, P.L., Ravaggi, A., Bellone, S., Pecorelli, S., Roman, J.J., Parham, G.P., and Cannon, M.J. (2000). Interleukin-10 increases Th1 cytokine production and cytotoxic potential in human papillomavirus-specific CD8(+) cytotoxic T lymphocytes. Journal of Virology *74*, 4729-4737.

Slifka, M.K., and Whitton, J.L. (2001). Functional avidity maturation of CD8(+) T cells without selection of higher affinity TCR. Nat Immunol *2*, 711-717.

Smith-Garvin, J.E., Koretzky, G.A., and Jordan, M.S. (2009). T Cell Activation. In Annu Rev Immunol (Palo Alto: Annual Reviews), pp. 591-619.

Sullivan, B.M., Emonet, S.F., Welch, M.J., Lee, A.M., Campbell, K.P., de la Torre, J.C., and Oldstone, M.B. (2011). Point mutation in the glycoprotein of lymphocytic choriomeningitis virus is necessary for receptor binding, dendritic cell infection, and long-term persistence. Proc Natl Acad Sci U S A *108*, 2969-2974.

Sun, Z., Fourcade, J., Pagliano, O., Chauvin, J.M., Sander, C., Kirkwood, J.M., and Zarour, H.M. (2015). IL10 and PD-1 Cooperate to Limit the Activity of Tumor-Specific CD8+ T Cells. Cancer Res *75*, 1635-1644.

Taniguchi, N., and Korekane, H. (2011). Branched N-glycans and their implications for cell adhesion, signaling and clinical applications for cancer biomarkers and in therapeutics. BMB Reports *44*, 772-781.

Tscharke, D.C., Croft, N.P., Doherty, P.C., and La Gruta, N.L. (2015). Sizing up the key determinants of the CD8(+) T cell response. Nature Reviews Immunology *15*, 705-716.

Vigano, S., Utzschneider, D.T., Perreau, M., Pantaleo, G., Zehn, D., and Harari, A. (2012). Functional avidity: a measure to predict the efficacy of effector T cells? Clin Dev Immunol *2012*, 153863.

Walker, L.J., Sewell, A.K., and Klenerman, P. (2010). T cell sensitivity and the outcome of viral infection. Clin Exp Immunol *159*, 245-255.

Wherry, E.J. (2011). T cell exhaustion. Nature Immunology *131*, 492-499.

Wherry, E.J., Blattman, J.N., Murali-Krishna, K., van der Most, R., and Ahmed, R. (2003). Viral Persistence Alters CD8 T-Cell Immunodominance and Tissue Distribution and Results in Distinct Stages of Functional Impairment. Journal of Virology *77*, 4911-4927.

Wherry, E.J., and Kurachi, M. (2015). Molecular and cellular insights into T cell exhaustion. Nat Rev Immunol *15*, 486-499.

Wilson, E.B., and Brooks, D.G. (2011). The role of IL-10 in regulating immunity to persistent viral infections. Curr Top Microbiol Immunol *350*, 39-65.

Wong, P., and Pamer, E.G. (2003). Cd8 T cell responses to infectious pathogens. Annu Rev Immunol *21*, 29-70.

Yang, R.Y., Rabinovich, G.A., and Liu, F.T. (2008). Galectins: structure, function and therapeutic potential. Expert Rev Mol Med *10*, e17.

Yi, J.S., Cox, M.A., and Zajac, A.J. (2010). T-cell exhaustion: characteristics, causes and conversion. Immunology *129*, 474-481.

Zehn, D., Lee, S.Y., and Bevan, M.J. (2009). Complete but curtailed T-cell response to very low-affinity antigen. Nature *458*, 211-214.