Investigating the role of inhibitory and immunoregulatory pathways in the development of memory CD8 T cells following acute infection

by

Tracey D. Beauchamp

Department of Microbiology and Immunology, McGill University, Montreal

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Abstract

Generation of memory CD8 T cells provides enhanced protection against pathogen reinfection. While it is known that the selection of memory CD8 T cells from the effector pool is not a stochastic event, the mechanisms which determine memory differentiation remain incompletely understood. It has been demonstrated that enhanced accumulation of activating and inflammatory signals throughout the course of infection reduces the capacity of effector CD8 T cells to seed the memory pool. It is likely that the development of memory T cells is reliant on shielding a portion of the total T cell population from becoming fully activated, allowing them to retain the pluripotency required for differentiation from effector to memory cells; inhibitory receptors may be important for this shielding of memory T cells. Inhibitory and immunoregulatory signalling pathways, such as PD-1 and IL-10 have been implicated in the development of CD8 T cell exhaustion, a hypofunctional state, in the context of chronic infections. In this context IL-10 signalling upregulates N-glycan branching on surface glycoproteins, which reduces signalling through the T cell receptor and decreases CD8 T cell activation. PD-1 signalling directly decreases CD8 T cell activation by reducing signalling through the T cell receptor and costimulatory molecules. However, the physiological roles of PD-1 and IL-10 signalling during acute infection remain poorly defined, although it has been demonstrated that the suppressive activity of PD-1 and IL-10 signalling during acute infection is vital for preventing the development of immunopathologies. Herein we demonstrate that PD-1 signalling and N-glycan branching are elevated in memory precursor cells (MPC) compared to terminal effector cell (TEC) subsets of effector CD8 T cells. In addition, disruption of either immunoregulatory pathway does not alter the size or functionality of the memory pool formed following acute infection, indicating that these pathways do not play a functional role in the generation of memory CD8 T cells. Despite this, our data indicate that, at least initially, MPC receive stronger signals than TEC during responses to acute infections, which may contribute to memory CD8 T cell selection. Advances in this field could lead to improved T cell vaccines as well as therapies for treatment of a variety of diseases such as cancer and autoimmune diseases.

Résumé

La génération des cellules T CD8 mémoire améliore la protection contre les réinfections pathogéniques. Il est clair que la sélection des cellules T CD8 effectrice qui vont devenir mémoire n'est pas stochastique, mais les mécanismes qui déterminent la différentiation des cellules T CD8 ne sont pas entièrement connus. Il a été démontré que la signalisation par la voie du récepteur des cellules T et ainsi que par les cytokines inflammatoires durant le cours d'infection réduisent la capacité des cellules T CD8 effectrice de devenir des cellules à mémoire. Il est probable que le développement des cellules T mémoire dépend d'une protection d'une portion de la population totale de cellules T contre les signaux d'activation, permettent la rétention de la pluripotence requise pour la transformation de cellule T effectrice mémoire. Les voies de signalisation inhibitrices comme PD-1 et IL-10 sont peut-être importantes pour cette protection des cellules T CD8 à mémoire. Les deux sont impliqués dans le développement de l'épuisement des cellules T CD8 dans le contexte des infections chroniques. Dans ce contexte, la signalisation IL-10 résulte dans une accumulation des N-glycans ramifiés sur les glycoprotéines au surface cellulaire qui diminue la signalisation par le récepteur des cellules T, ce qui réduit l'activation des cellules T CD8. La signalisation PD-1 réduise l'activation des cellules T par réduire la signalisation par la voie du récepteur des cellules T et les voies costimulatoires. Cependant, le rôle physiologique des voies de signalisation de PD-1 et IL-10 pendant l'infection aiguë est encore peu défini, bien qu'il ait été démontré que l'activité suppressive de PD-1 et IL-10 sont essentiels pour prévenir l'évolution des immunopathologies. On a démontré que la signalisation PD-1 et les N-glycans ramifiés sont élevés dans les précurseurs des cellules mémoire (MPC) en comparaison aux cellules effectrice terminales (TEC). Les deux sont les sous-populations de cellules T effectrice bien étudiés avec des différences connues dans leur capacité de devenir des cellules T mémoire. De plus, la disruption de l'un ou l'autre n'a pas modifié la taille non plus la fonctionnalité de la population des cellules CD8 T à mémoire formé au cours d'infection aiguë, indiquant que ces voies de signalisation ne sont pas directement impliquées dans la formation des cellules T à mémoire. Quand même, nos résultats indiquent qu'au moins initialement, les MPC reçoivent des signaux de plus haute intensité que les TEC

pendant l'infection aiguë, ce qui peut contribuer donc à la sélection des cellules T CD8 à mémoire. Les avancements futurs dans ce domaine peuvent mener à l'amélioration des vaccins ainsi qu'au thérapies pour le traitement du cancer et les maladies autoimmunes.

Contribution of Authors

In the work that makes up this M.Sc. thesis, I designed and executed the majority of experiments, performed data analysis, interpreted results, generated figures and wrote the manuscript. Stefanie F. Valbon prepared TEC and MPC RNA for RT-qPCR analysis. Dr. Esther Tarrab from the laboratory of Dr, Alain Lamarre at the Institut Armand-Frappier prepared MHC Class I tetramers for analysis of epitope-specific endogenous CD8 T cells. Stephanie A. Condotta prepared all viral and bacterial stocks used for the experiments conducted in this thesis. Martin J. Richer designed and planned experiments performed data analysis and edited the manuscript.

List of abbreviations

actA-LM-OVA :	*Acta*$^{-/-}$ *Listeria monocytogenes* expressing the ovalbumin protein
APC :	Antigen presenting cell
ATP :	Adenosine triphosphate
Bcl-6 :	And B cell lymphoma 6 protein
BLIMP-1 :	B lymphocyte-induced maturation protein 1
BrfA :	Brefeldin A
CD4 :	Cluster of differentiation 4
CD8 :	Cluster of differentiation 8
CD25 :	Cluster of differentiation 25
CD28 :	Cluster of differentiation 28
CD44 :	Cluster of differentiation 44
CD62L :	L-selectin
CD69 :	Cluster of differentiation 69
CD127 :	Cluster of differentiation 127
CFU :	Colony forming units
CTLA-4 :	Cytotoxic T-lymphocyte-associated protein 4
CXCR3 :	Chemokine receptor, G protein-coupled receptor 9
DC :	Dendritic cell
Dpi :	Days post infection
Dll1 :	Delta-like ligand 1
EAE :	Experimental autoimmune encephalitis
EC50 :	Effective concentration 50
EOMES :	Eomesodermin
Gal3 :	Galectin 3
gMFI :	Geometric mean of fluorescence intensity
GP33 :	GP_{33-41} epitope of LCMV.
HIV :	Human immunodeficiency virus
ICS :	Intracellular cytokine staining
IFNAR :	Interferon α/β-receptor
IFN :	Interferon
IFNγ :	Interferon gamma
IL-2 :	Interleukin 2
IL-7 :	Interleukin 7
IL-10 :	Interleukin 10
IL-12 :	Interleukin 12
IL-15 :	Interleukin 15
KLRG1 :	Killer cell lectin-like receptor family G1
LAG3 :	Lymphocyte-activation gene 3

LCMV Arm :	The Armstrong strain of the Lymphocytic choriomeningitis virus
LCMV :	Lymphocytic choriomeningitis virus
Mgat5 :	N-glucosyltransferase mannoside acetylglucosaminyltransferase 5
MHC :	Major histocompatibility complex
MPC :	Memory precursor cell
mTORC1 :	Mammalian target of rapamycin complex 1
NFAT :	Nuclear factor of activated T cells
NP396 :	Endogenous CD8 T cells specific for the $NP_{396-404}$ epitope of LCMV.
OVA :	Ovalbumin
PBL :	Peripheral blood leukocytes
PD-1 :	Programmed cell death protein 1
PD-L1 :	Programmed death-ligand 1
PFU :	Plaque forming units
PHA-L :	Phaseolus vulgaris leukoagglutinin
RNA :	Ribonucleic acid
RA :	Rheumatoid arthritis
RP :	Red pulp of lymphoid tissues
SF :	Synovial fluid
TBP :	TATA binding protein
TCR :	T cell receptor
TCR-tg :	T cell receptor transgenic
TEC :	Terminal effector cell
TNFα :	Tumour necrosis factor alpha
WP :	White pulp of lymphoid tissues
WT :	Wild type
ZAP70 :	Zeta associated protein of 70 kDa

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Chapter I Literature review and general introduction

The CD8 T cell response to infection

CD8 T cells are critical for host protection against intracellular pathogens, such as viruses. These cells control viral replication via targeted cell lysis of host cells, which are sites of viral replication. During an infection, naïve antigen specific CD8 T cells must receive three signals in order to become activated. These three signals are 1) ligation of the T cell receptor (TCR) by cognate antigen in the context of the major histocompatibility complex (MHC) Class I, 2) co-stimulatory signalling through the CD28 co-receptor and 3) signalling by inflammatory cytokines (Alexander-Miller 2005; Condotta *et al*. 2012; Hashimoto *et al*. 2017; Huber and Farrar 2011; Kaech and Cui 2012; Kaech *et al*. 2002; Khan *et al*. 2015; Khanolkar *et al*. 2007; Kim *et al*. 2016; Richard *et al*. 2018; Valbon *et al*. 2016). Signals 1 and 2 alone are adequate to provoke activation of naïve CD8 T cells; however, optimal CD8 T cell accumulation does not occur in the absence of signal 3 (Ely *et al*. 1999; Hashimoto *et al*. 2017; Huber and Farrar 2011; Kim *et al*. 2016; Laidlaw *et al*. 2015; Richer *et al*. 2013; Rubinstein *et al*. 2008; Starbeck-Miller *et al*. 2014; Urban *et al*. 2016; Valbon *et al*. 2016).

Once activated, naïve CD8 T cells undergo a period of expansion while simultaneously differentiating into effector CD8 T cells (Alexander-Miller 2005; Condotta *et al*. 2012; Hashimoto *et al*. 2017; Huber and Farrar 2011; Kaech and Cui 2012; Kaech *et al*. 2002; Khan *et al*. 2015; Khanolkar *et al*. 2007; Kim *et al*. 2016; Richard *et al*. 2018; Valbon *et al*. 2016). Effector cells possess the capacity to produce cytolytic molecules perforin and granzyme B as well as effector cytokines interferon-γ (IFNγ) and tumour necrosis factor-α (TNFα) (Huber and Farrar 2011; Stemberger *et al*. 2007a; Stemberger *et al*. 2007b; Valbon *et al*. 2016). Following peak expansion, the effector pool undergoes a period of contraction wherein 90-95% of the effector cells die via apoptosis and the remaining 5-10% will go on to seed the long-lived memory pool (Kaech and Cui 2012; Kaech *et al*. 2003). The selection of effector cells that will seed the memory pool is not stochastic: the capacity of individual effector cells to become memory is influenced by the signals received throughout the course of the immune response. Key pro-inflammatory cytokines such as interleukin 12 (IL-12) promote the terminal differentiation of effector CD8 T cells. Though it is clear that signals from the

inflammatory milieu guide the fate of individual effector cells, how these signals are integrated globally and temporally as well as the downstream signalling pathways which ultimately regulate the fate decision of effector CD8 T cells remain to be fully elucidated (Figure I – 1) (Buchholz *et al*. 2016; Kaech and Cui 2012).

Memory CD8 T cells provide enhanced long-term host protection against pathogen re-infection and mount a faster and more protective response than their naïve counterparts. The advantage of memory CD8 T cells over naïve cells is due to comparatively enhanced proliferative capacity and response time of memory cells following activation as well as increased sensitivity to their cognate antigen (Huber and Farrar 2011; Kaech *et al*. 2003; Stemberger *et al*. 2007b; Valbon *et al*. 2016; Youngblood *et al*. 2013). Furthermore, the memory pool can be subdivided into distinct populations, some of which recirculate through the blood and lymphoid organs while constantly scanning for cognate antigen, and some which become resident in various tissues such as the skin, gut and lungs (Mani *et al*. 2019; Mueller and Mackay 2016). These tissue-resident memory cells are identified by expression of surface markers CD62L and CD103 alongside canonical CD8 T cell memory markers like CD127. Tissue-resident memory cells provide enhanced protection against localized infections and are enriched in tissues which are common entry sites for infectious pathogens, such as in the lung and gut (Mueller and Mackay 2016). The pool of memory CD8 T cells is maintained for the lifetime of the host in an antigen-independent manner. Signalling by IL-7 and IL-15 is required for the maintenance of the memory pool via homeostatic proliferation (Figure I – 1) (Kaech *et al*. 2003; Rubinstein *et al*. 2008).

Enhancing memory CD8 T cell generation during responses to infection or vaccination has been a primary focus of CD8 T cell biology for many years. It is well documented that a representative 5-10% of the total effector pool will go on to seed the memory pool, therefore it may be anticipated that a correlation exists between the number of effector cells present at the peak of effector expansion and the number of cells which survive to memory timepoints. However, DC immunization of mice with or without with an adjuvant resulted in equivalent sized memory pools despite a 100-fold increase in effector cells present at the peak of infection in the adjuvant treated group (Kim *et al*. 2016). Thus, increasing the magnitude of CD8 T cell effector expansion is

insufficient to increase the size of the memory pool generated following contraction, due to the fact that signals that enhance effector CD8 T cell expansion also enhance expression of pro-apoptotic proteins which in turn promote the death of effector cells during contraction, such as Bim (Kim *et al*. 2016), and therefore increasing the magnitude of effector expansion also increases the magnitude of effector contraction. Experiments such as this have demonstrated the effects of antigenic and inflammatory signal manipulations on the formation of effector and memory CD8 T cells and have provided insights into the mechanisms that regulate CD8 T cell memory generation (Kaech and Cui 2012; Kaech *et al*. 2003; Kim *et al*. 2016; Starbeck-Miller *et al*. 2014).

Most current vaccines elicit antibody-mediated protection against pathogen infection. Many of these vaccines provide highly effective host protection and have even been instrumental in eliminating serious threats to human health, such as smallpox. However, protection provided by humoral vaccines against intracellular or rapidly mutating pathogens may be insufficient or short-lived. In particular, the protection afforded by humoral vaccination against the influenza virus is short lived. Antibodies are restricted to targeting accessible external viral coat proteins. As such, antibody-mediated protection is restricted to homologous viral strains, and mutations of the serological coat may result in epitope escape, allowing viral infections to evade detection and persist in spite of a robust and protective humoral immune response (Amanna and Slifka 2011; Billeskov *et al*. 2018; Garman *et al*. 2014; Greenspan 2014; Korber *et al*. 2009; Thomas *et al*. 2006). In contrast, a T cell vaccine against influenza may offer improved protection due to the ability of T cells to respond to more highly conserved internal epitopes, such as nucleoproteins. Additionally, T cell vaccines may also offer broader protection against heterologous viral strains, since serologically distinct viral strains may share conserved proteins that are targeted by T cells (Amanna and Slifka 2011; Billeskov *et al*. 2018; Korber *et al*. 2009; Thomas *et al*. 2006). Improved understanding of CD8 T cell memory generation is critical to advance T-cell-mediated vaccination strategies, which could improve protection and treatment options against a variety of intracellular pathogens, such as influenza and human immunodeficiency virus (HIV) (Amanna and Slifka 2011; Billeskov *et al*. 2018; Greenspan 2014; Kaech *et al*. 2002; Korber *et al*. 2009). Furthermore, improved understanding of CD8 T cell activation and function could

lead to the development of improved therapies for tissue transplants and autoimmune diseases, where CD8 T cell activity target and damage transplanted or healthy host tissues, and cancers, where the ability of T cells to infiltrate and respond to tumour antigens has been demonstrated to be critical for disease regression (Cho *et al*. 2012; Goodman *et al*. 2017; Harper *et al*. 2015; Tsai and Hsu 2017).

Regulation of CD8 T cell activation

CD8 T cells are highly cytotoxic and therefore are capable of damaging host tissues. Thus, the activation and function of CD8 T cells must be strictly regulated to avoid causing damage to healthy host tissues. Signals received from the inflammatory milieu have been demonstrated to greatly impact CD8 T cell function and differentiation, as such, manipulations of these signals may offer opportunities to improve therapeutic options for chronic infections, transplants, cancers, and autoimmune diseases (Amanna and Slifka 2011; Cho *et al*. 2012; Dyck and Mills 2017; Greenspan 2014; Harper *et al*. 2015; Kaech *et al*. 2002; Korber *et al*. 2009; Provine *et al*. 2016; Sarkander *et al*. 2016).

It is known that the inflammatory milieu regulates many aspects of the immune response, including cell recruitment, activation and differentiation. This milieu includes both pro- and anti-inflammatory signals and its composition is pathogen specific. Furthermore, the signals present undergo dynamic changes throughout the course of the immune response (Joshi *et al*. 2007; Pipkin *et al*. 2010; Richer *et al*. 2013; Rubinstein *et al*. 2008; Stelekati *et al*. 2014; Valbon *et al*. 2016). Both cytokine and chemokine signalling molecules are secreted by immune cells during responses to infection. While cytokines regulate the function and differentiation of responding immune cells, chemokines primarily attract immune cells to the site of infection (Hashimoto *et al*. 2017; Joshi *et al*. 2007; Massena *et al*. 2010; Richer *et al*. 2013). Pro- and anti-inflammatory signals work in opposition of one another, and balance between the two is critical for host health. Disruption of this balance is associated with pathologies, such as the establishment of persistent infections or immunopathologies (Demetriou *et al*. 2001; Grigorian and Demetriou 2011; O'Shea and Plenge 2012; Zamani *et al*. 2016; Zhang *et al*. 2019). Thus, the inflammatory milieu influences the overall immune response, and

specifically influences the capacity of effector CD8 T cells to generate memory (Joshi *et al*. 2007; Laidlaw *et al*. 2015).

Signalling by type I interferons during responses to acute infections, as well as signalling by IL-12, IL-2 and IL-33 are pro-inflammatory and have been demonstrated to enhance the activation, function and proliferation of CD8 T cells (Cox *et al*. 2013; Hashimoto *et al*. 2017). Furthermore, pro-inflammatory signalling by both type I IFNs and IL-12 have been demonstrated to directly enhance the antigen sensitivity of effector CD8 T cells (Richer *et al*. 2013). In contrast, inhibitory signals such as IL-10 and immune checkpoint blockade receptors such as PD-1 and LAG3 negatively regulate the activation and function of responding immune cells, these inhibitory signals play a critical role in restraining immune responses and additionally play a critical role in preventing immune-mediated damage to host tissues and ultimately restrict the development of immunopathologies (David *et al*. 2019; Jakobshagen *et al*. 2015; Keir *et al*. 2008; Sinha *et al*. 2015; Sinha *et al*. 2014; Zamani *et al*. 2016). As such, the impacts of inflammatory signalling on CD8 T cells during responses to acute infections are complex, and the overall integration of both stimulatory and inhibitory signalling pathways govern the outcome of individual immune responses.

The role of TCR signalling in regulating CD8 T cell activation and function

In addition to signals received from the inflammatory milieu, CD8 T cells must be able to detect cognate antigen in order to become activated and respond to infection. During development and maturation, thymocytes undergo processes of positive and negative selection, these selection mechanisms ensure that mature CD8 T cells are capable of receiving critical survival signalling through low affinity interactions with self-peptide in the context of MHC while ensuring that cells are not so responsive as to instigate development of immunopathologies (Slifka and Whitton 2001; Zehn *et al*. 2009). Thus, the circulating pool of naïve CD8 T cells contains a wide range of TCR affinities (Viganò *et al*. 2012; Walker *et al*. 2010; Zehn *et al*. 2009). Similar to pro-inflammatory signalling, TCR signal strength induces CD8 T cell proliferation and effector differentiation. As such, antigenic, co-stimulatory and inflammatory signals are intricately interconnected and difficult to separate from one another during *in vivo*

responses to infections, and thus it is probable that effector cell fate is determined not only by overall accumulation of activating and inflammatory signals, but also by the relative contribution of inhibitory and immunoregulatory signalling, as these signals may act to shield effector cells, thereby reducing their activation to favour memory development, even in the presence of a robustly activating milieu (Figure I – 1).The recruitment of high-affinity CD8 T cells during immune responses has been demonstrated to improve control of viral replication, due to the enhanced ability of high-affinity CD8 T cells to respond to low concentrations of cognate antigen (Viganò *et al*. 2012; Walker *et al*. 2010; Zehn *et al*. 2009). However, it has also been documented that CD8 T cells with low affinity for their cognate antigen are present and activated alongside high-affinity clones, and low affinity clones have been demonstrated to emigrate from lymphoid organs and reach the peak of expansion quicker than their high affinity counterparts (Viganò *et al*. 2012; Zehn *et al*. 2009). Though recruitment of high-affinity T cells is critical for efficient control of viral infections, low affinity clones may offer the host a competitive advantage against rapidly mutating pathogens, such as the influenza virus, by responding to more highly conserved epitopes which have reduced capacity to undergo epitope escape mutation (Amanna and Slifka 2011; Billeskov *et al*. 2018; Greenspan 2014; Zehn *et al*. 2009).

Though the affinity of individual TCR to its cognate antigen is a static property, the overall sensitivity, or avidity of CD8 T cells to signalling by cognate antigen is plastic and factors such as co-localization with the CD8 receptor and the spatial clustering of TCRs on the plasma membrane have been demonstrated to modulate the antigen sensitivity of responding CD8 T cells (Artyomov *et al*. 2010; Richer *et al*. 2013). Furthermore, both pro- and anti-inflammatory signals have been demonstrated *in vivo* to alter the antigen sensitivity of responding CD8 T cells (Richer *et al*. 2013; Smith *et al*. 2018; Viganò *et al*. 2012). CD8 T cells of both high and low affinity have been demonstrated to have the capacity to generate memory cells, and as such it is unclear what roles TCR signal strength and inflammatory signalling play in the selection of effector cells that go on to seed the long-lived memory pool (Zehn *et al*. 2009). TCR signalling likely integrates with signals received from the inflammatory milieu, and

downstream integration of activating and inhibitory signals likely combine to determine effector CD8 T cell fate (Figure I – 1).

CD8 T cell memory differentiation

The selection of effector cells that go on to seed the long-lived memory pool is not stochastic and thus not all effector cells have equivalent memory potential. It has been well documented that only 5-10% of the effector pool will survive contraction and attempts to enhance the fraction of the effector pool surviving contraction have shown limited success (Hand *et al*. 2007; Kim *et al*. 2016). Effector cells with enhanced memory potential can be distinguished early during the CD8 T cell response. In fact, the effector pool can be divided into two groups with known differences in memory potential. These populations are known as the terminal effector cells (TEC) and memory precursor cells (MPC) and are differentiated from one another using the expression of the cell surface markers Killer Cell Lectin-like Receptor G1 (KLRG1) and the alpha chain of the IL-7 receptor (CD127) (Figure I – 1) (Hand *et al*. 2007).

MPC ($KLRG1^{LO}CD127^{HI}$) have been robustly demonstrated to have enhanced capacity compared to TEC ($KLRG1^{HI}CD127^{LO}$) to go on to seed the long-lived memory pool (Hand *et al*. 2007; Kaech and Cui 2012; Kaech *et al*. 2003; Laidlaw *et al*. 2015). It is well known that expression of CD127 on MPC is critical for the long-term survival of these cells, as cells which survive contraction but do not express CD127 are not maintained long-term. (Hand *et al*. 2007). Though IL-7 signalling is critical for memory cell maintenance, expression of CD127 alone is not sufficient to enhance the size of the memory pool and effector CD8 T cells with constitutive expression of CD127 contract similarly to wild type cells (Hand *et al*. 2007). This effect was demonstrated to be due to limited supply of IL-7, and as such, injection of additional recombinant IL-7 present during CD8 T cell contraction resulted in a corresponding increase in the number of CD8 T cells constitutively expressing the CD127 receptor which survived contraction (Hand *et al*. 2007). Clearly, the ability of effector cells to receive IL-7 signalling is critical for memory transition, however the mechanisms which select effector cells to express CD127 throughout response to infection are incompletely understood (Figure I – 1).

Though both TEC and MPC have been shown to contribute to CD8 T cell effector responses during infections, these populations have been demonstrated to localize independently within lymphoid tissues (Jung *et al*. 2010). MPC have been demonstrated to reside primarily within the white pulp (WP) of lymphoid organs during and following resolution of immune responses while TEC have been demonstrated to localize to the red pulp (RP) (Jung *et al*. 2010). Stromal cells in the WP, such as fibroblastic reticular cells are known producers of IL-7, and it is possible that WP localization of MPC enhances the IL-7 survival signals they receive, furthermore, IL-7 availability may be a factor limiting the potential size of memory pools generated following acute infection (Jung *et al*. 2010) (Figure I -1).

In general, studies investigating mechanisms underlying differentiation of TEC and MPC, such as expression of CD127, or enhancement of proliferation and cytotoxic function have found that signals which enhance effector activation and proliferation simultaneously decrease the memory potential of responding CD8 T cells (Kaech and Cui 2012; Khanolkar *et al*. 2007; Kim *et al*. 2016; Urban *et al*. 2016; Youngblood *et al*. 2013). For example, early elimination of antigen following CD8 T cell activation via treatment with antibiotics results in reduced clonal expansion of responding CD8 T cells but enhances the size of the MPC compartment of the effector pool (Badovinac *et al*. 2005; D'Souza and Hedrick 2006; Joshi *et al*. 2007). Similarly, increasing either antigenic and pro-inflammatory signals such as type I IFNs, IL-12 and IL-2 without altering presence of antigen has been demonstrated to increase the relative ratio of TEC to MPC, while also increasing overall expansion of effector CD8 T cells (Badovinac *et al*. 2004; Cui *et al*. 2009). Furthermore, deficiency of either IFNγ or IL-12 during responses to infection results in a greatly enhanced frequency of MPC (Badovinac *et al*. 2004; Cui *et al*. 2009). The timing and magnitude of important signals such as IL-2 which enhance proliferation of effector CD8 T cells and thus increases the size of the effector pool have also been shown to decrease the relative size of the MPC compartment (Khan *et al*. 2015; Starbeck-Miller *et al*. 2014). Thus, enhancing antigenic and pro-inflammatory signals during CD8 T cell responses increases effector activation, proliferation and function, but decreases the overall memory potential of the effector pool (Figure I -1).

Transcriptional regulation of CD8 T cell differentiation

Although we do not yet fully understand the mechanisms underlying memory generation, the integration of TCR and inflammatory signalling clearly plays an important role in the fate decision of effector CD8 T cells. As such, antigenic and inflammatory signals have been demonstrated to collaboratively influence the balance between pairs of reciprocally acting transcription factors in CD8 T cells (Kaech and Cui 2012). The relative level of expression of each transcription factor compared to its partner has been demonstrated to influence the fate of effector cells and is known to undergo dynamic changes throughout the course of CD8 T cell responses (Kaech and Cui 2012). Two of the major reciprocally regulating transcription factor pairs which have been identified as regulators of CD8 T cell activation and differentiation are EOMES and T-bet, and BLIMP-1 and Bcl-6 (Figure I -1) (Kaech and Cui 2012).

Eomesodermin (EOMES) and T-bet are T-box transcription factors with partially redundant functions in the context of CD8 T cells. Both EOMES and T-bet have been demonstrated to be required for the canonical function of effector CD8 T cells, and loss of both results in loss of cytotoxic T cell identity and the development of immunopathologies (Intlekofer *et al*. 2008; Kaech and Cui 2012; Pipkin *et al*. 2010; Takemoto *et al*. 2006). Expression of T-bet is induced by TCR signalling and amplified by pro-inflammatory IL-12 signalling while EOMES expression occurs subsequently to T-bet and is amplified by IL-2 signalling (Joshi *et al*. 2007; Kaech and Cui 2012; Pipkin *et al*. 2010; Takemoto *et al*. 2006). T-bet is required for TEC formation, and overexpression of T-b*et al*one is sufficient to induce the formation of TEC (Joshi *et al*. 2007; Kaech and Cui 2012; Takemoto *et al*. 2006). Though memory CD8 T cells are generated normally in the absence of EOMES, these cells have irregular expression of memory associated surface markers such as CD62L and CXCR3 and have reduced sensitivity to IL-15 signalling (Joshi *et al*. 2007; Kaech and Cui 2012; Pipkin *et al*. 2010; Takemoto *et al*. 2006). Thus, memory CD8 T cells deficient in EOMES have impaired homeostatic proliferation and reduced long-term persistence (Joshi *et al*. 2007; Kaech and Cui 2012; Pipkin *et al*. 2010; Takemoto *et al*. 2006). The ratio of T-bet to EOMES changes throughout the course of the CD8 T cell response, with higher relative T-bet expression in effector cells and higher relative EOMES expression in memory cells,

although both T-bet and EOMES have been demonstrated to cooperate to sustain the long-lived memory pool (Kaech and Cui 2012). Thus, manipulation of the signals regulating T-bet and EOMES expression ratios may be one mechanism by which memory CD8 T generation could be influenced (Figure I – 1).

B lymphocyte-induced maturation protein 1 (BLIMP-1) and B cell lymphoma 6 protein (Bcl-6) are reciprocally acting transcriptional repressors which have been labelled as genetic switches in B and T lymphocytes (Crotty *et al*. 2010; Kaech and Cui 2012). BLIMP-1 is robustly expressed in effector CD8 T cells and has been demonstrated to be critical for the production of effector cytokines and cytolytic molecules such as IFNγ and granzyme B (Fu *et al*. 2017; Kaech and Cui 2012; Kallies *et al*. 2009; Rutishauser *et al*. 2009; Shin *et al*. 2009). BLIMP-1 has also been demonstrated to actively repress expression of certain memory-associated molecules (Crotty *et al*. 2010; Kallies *et al*. 2009; Rutishauser *et al*. 2009). BLIMP-1 expression decreases as CD8 T cells progress from effector to memory phenotypes. Although expression of BLIMP-1 is virtually undetectable in mature memory CD8 T cells. Bcl-6 expression is antagonistic to BLIMP-1 and is expressed at the highest level in memory CD8 T cells (Crotty *et al*. 2010; Ichii *et al*. 2004). Bcl-6 expression is required for memory CD8 T cell formation and Bcl-6 overexpression is sufficient to increase the size of the MPC compartment of effector CD8 T cells (Cui *et al*. 2011; Ichii *et al*. 2004; Kaech and Cui 2012). BLIMP-1 and Bcl-6 reciprocally repress the expression of one another, and thus manipulation of the BLIMP-1 and Bcl-6 expression ratio is another mechanism by which memory CD8 T cell generation could be influenced (Figure I – 1).

Therefore, based on these data, integration of antigenic and inflammatory signals modulates the expression of transcription factors including EOMES, T-bet, BLIMP-1 and Bcl-6, which ultimately regulates the transcriptional programs which regulate the fate decision of individual effector CD8 T cells. Furthermore these transcription factor pairs do not necessarily act independently of one another, and in fact T-bet and Bcl-6 have been shown to directly interact with one another (Kaech and Cui 2012; Oestreich *et al*. 2011; Oestreich *et al*. 2012). Ultimately, the balance of activating and immunoregulatory signals received decides the fate of individual effector CD8 T cells. How integration of antigenic and inflammatory signals regulates TEC and MPC diversification of the

effector pool remains to be fully elucidated. This problem has been a driving question is CD8 T cell functional biology since the discovery of MPC and TEC subpopulations and there exist many models that attempt to explain the complex process of CD8 T cell diversification.

Models of CD8 T cell diversification

The most simplistic model of CD8 T cell diversification is the separate-precursor model, which states that effector CD8 T cells that go on to become either TEC or MPC originate from separate naïve precursors which are pre-programmed for these fates during thymic development (Kaech and Cui 2012). By necessity, within the confines of this model naïve cells are not equipotent, and once activated will proliferate to generate either only TEC or only MPC descendants. Single-cell adoptive transfers and cellular barcoding techniques have been used to demonstrate that both TEC and MPC effector CD8 T cells can be generated from the same naïve progenitor (Diao and Pipkin 2019; Gerlach *et al*. 2010; Kaech and Cui 2012), and therefore the separate-precursor model does not accurately describe CD8 T cell diversification processes. Recent evidence has demonstrated that non-cognate interactions with DCs expressing α_V-integrins may precondition naïve CD8 T cells to favour the formation of epithelial tissue-resident CD8 T cells following activation (Mani *et al*. 2019). However, this preconditioning is reversible and does not limit pre-conditioned cells to a strictly effector or memory fate, but rather is critical for the localization and retention of tissue-resident memory CD8 T cells in the skin following resolution of the CD8 T cell effector response (Mani *et al*. 2019).

Other models of CD8 T cell diversification focus on initial strength of TCR signal strength as the factor which determines CD8 T cell fate. The signal-strength model states that CD8 T cell diversification originates from differences in the strength of signal received by naïve CD8 T cells undergoing activation, where stronger initial signals enhance clonal proliferation of responding cells. While this allows for the selection of competent CD8 T cell clones to preferentially seed the memory pool, it has been demonstrated that excessive strength of signal results in terminal differentiation and even deletion of CD8 T cells (Kaech and Cui 2012).

The asymmetric cell fate model is complementary to the signal strength model, and states that progenitors from a single naïve precursor can give rise to both memory and effector populations; however, in this model the fate decision rests on whether the cell receives the immunological synapse and other important signalling molecules, such as the high-affinity chain of IL-2 receptor during cell division following CD8 T cell activation (Arsenio *et al*. 2014; Buchholz *et al*. 2013; Buchholz *et al*. 2016; Kaech and Cui 2012). In this model, the daughter cell which receives the synapse and key signalling molecules is in closer contact to the activating APC and so receives not only the immunological synapse, but also stronger inflammatory signals compared to its sister cell. It has been demonstrated using single-cell gene expression analysis and computer modeling of early effector differentiation that the eventual lineage commitment of individual effector cells can be identified as early as the first cell division (Arsenio *et al*. 2014; Arsenio *et al*. 2015). Furthermore, although the overall effector population displays a highly reproducible level of heterogeneity, the differentiation paths of individual progenitors are highly diverse and heavily influenced by the signals encountered during activation and expansion (Buchholz *et al*. 2013; Buchholz *et al*. 2016). Though it is possible that initial strength of TCR stimulation and asymmetries between daughter cells arising from the first division of activated effector cells may play a role in establishing progenitor fate, it is unlikely that this is the full mechanism, and it is highly probable that signals received throughout the immune response contribute to the eventual fate of individual effector cells (Buchholz *et al*. 2016).

The best-supported model of CD8 T cell diversification is the decreasing-potential model. This model assumes that the outcome of each individual effector cell is related directly to the complete history of signals accumulated throughout the course of infection. This model states that T cells which accumulate more activating signals from both antigen and inflammatory cytokines proliferate more and become more terminally differentiated than cells which do not. This model does not eliminate the possible contributions of asymmetric cell division or contribution of differences in signal strength in determining the fate of individual cells. However, on a population level, the decreasing-potential model suggests that characteristics which are attributed to memory cells, such as proliferative potential and expression of CD127 are in direct contrast to

characteristics attributed to effector function such as cytolytic capacity. Supporting evidence for this model includes increased memory formation following abrogation of antigen-exposure or inflammation through treatment with drugs or in controlled dendritic cell immunization models of T cell activation (Figure I – 1) (Buchholz *et al*. 2016; Kaech and Cui 2012).

Implications of the decreasing potential model and memory development

For effector CD8 T cells to diversify, TEC and MPC-fated effector cells must accumulate different signals throughout the course of the response. Although it is probable that TEC and MPC are exposed to different signalling and inflammatory milieux, for example through differential tissue localization or through recruitment of naïve CD8 T cells at later timepoints during an ongoing immune response, spatial and temporal segregation of TEC and MPC effector subpopulations is unlikely to be the full explanation of TEC and MPC diversification (Jung *et al*. 2010; Snell *et al*. 2018). This explanation does not explain how both TEC and MPC which arise from a single progenitor would differentiate separately, assuming both daughter cells would be exposed to an identical milieu before and following cell division. It is also possible that inhibitory and immunoregulatory signalling pathways provide a mechanism whereby CD8 T cells are, at least partially, shielded from the activating effects of the signals encountered, allowing for TEC and MPC to further diversify while co-existing within an identical milieu (Figure I – 1).

If inhibitory signalling pathways are playing a role in limiting the activation of T cells in the context of acute infection, then a balance between activation and immunoregulation of these cells must exist. CD8 T cells enter a hypofunctional state known as exhaustion following exposure to prolonged antigen signalling during responses to cancers and chronic infections (Bucks *et al*. 2009). Exhaustion is a unique physiological state of T cell hypofunctionality with a distinct epigenetic signature (Blackburn and Wherry 2007; Kamphorst *et al*. 2017; Utzschneider *et al*. 2013; Wherry 2011; Wherry and Kurachi 2015; Zehn and Wherry 2015). Exhausted CD8 T cells progressively lose expression of effector cytokines IFNγ, TNFα, as well as cytolytic molecules perforin and granzyme B. Additionally, their proliferative capacity declines

and they display increased expression of inhibitory receptors such as PD-1, CTLA-4 and LAG3 (Wherry 2011; Wherry and Kurachi 2015; Yi *et al*. 2010). If chronic infection persists, exhausted T cells will undergo clonal deletion via apoptosis (Blackburn and Wherry 2007; Wherry 2011).

Immune checkpoint blockade targets the inhibitory receptors which are overexpressed by exhausted CD8 T cells such as PD-1, LAG3 and CTLA-4. These therapies have been demonstrated to dramatically reinvigorate exhausted T cells, thus improving viral control (Barber *et al*. 2006; Dyck and Mills 2017; Tsai and Hsu 2017). Overexpression of inhibitory receptors in this context promote the development of an equilibrium where effector cells are able to be maintained long term rather than undergoing clonal deletion, while still exercising important, although diminished control of viral replication (Dyck and Mills 2017). In addition to their role in mediating CD8 T cell dysfunction in the context of chronic infections, inhibitory and immunoregulatory signalling pathways also play an important role during responses to acute infections. In fact, blockade of signalling by PD-1 during acute responses has been demonstrated to result in the development of severe spontaneous immunopathologies (Ahn *et al*. 2018; Barber *et al*. 2006; Nishimura *et al*. 2001; Zamani *et al*. 2016). During chronic infections these critical regulatory pathways are inappropriately activated and result in dysregulation of the host's immune system, conferring a survival advantage to the pathogen.

Therefore, it is expected that many pathways implicated in the development of exhaustion are also expressed in the context of acute infection, where they have been shown to play an important role in limiting the activation of responding CD8 T cells. In particular, we are interested in the roles of programmed cell death protein 1 (PD-1) and interleukin 10 (IL-10) in limiting T cell activation to favour memory CD8 T cell development in the context of acute infection. Both pathways have been primarily studied during responses to chronic infections. However, in the context of acute infections, mice lacking these key immunoregulatory signalling pathways are at risk of developing spontaneous immunopathologies, which may be fatal (Ahn *et al*. 2018). Therefore, both PD-1 and IL-10 signalling pathways play a critical role in regulating CD8

T cell activation in the context of acute infection, making them ideal candidates for this project.

The role of PD-1 signalling during acute infections

PD-1 is a regulatory co-receptor expressed by CD8 T cells. PD-1 is a member of the CD28 co-receptor family and signalling through the PD-1 receptor results in direct dephosphorylation of CD28 (Hui *et al*. 2017; Kamphorst *et al*. 2017; Krueger and Rudd 2017). Interruption of CD28 signalling decreases downstream survival and proliferative signals and results in an overall decrease in CD8 T cell activation (Hui *et al*. 2017; Kamphorst *et al*. 2017; Krueger and Rudd 2017). PD-1 signalling during responses to chronic infections has been demonstrated to play a role in the induction and maintenance of CD8 T cell exhaustion (Blackburn and Wherry 2007; Wherry 2011; Wherry and Kurachi 2015). Blockade of PD-1 or its ligand PD-L1 have been shown in clinical trials to have a potent ability to re-invigorate exhausted T cells, greatly enhancing CD8 T cell-mediated control of tumours or viral replication (Dyck and Mills 2017; Goodman *et al*. 2017; Kamphorst *et al*. 2017; Kunimasa *et al*. 2018; Utzschneider *et al*. 2013). As such, PD-1 signalling potently reduces CD8 T cell activation in the context of chronic infections.

PD-1 signaling has also been demonstrated to regulate T cell activation in the context of acute infections, in fact, mice lacking PD-1 signaling have been shown to develop of spontaneous immunopathologies (Ahn *et al*. 2018; David *et al*. 2019). Additionally, cells lacking PD-1 signaling during T cell activation have been reported to have higher rates of proliferation, produce more cytotoxic molecules and become more strongly activated than those which receive PD-1 signaling (Ahn *et al*. 2018). Therefore PD-1 signalling reduces CD8 T cell activation and function in the context of acute infection as well. As such, PD-1 signalling may play a role in shielding a fraction of the CD8 T cell pool to favour memory development.

PD-1 expression has been shown to be rapidly induced in CD8 T cells during responses to acute infections, and in fact, PD-1 expression has been shown to mirror the expression patterns of canonical markers of T cell activation such as CD25, CD44 and CD69 (Ahn *et al*. 2018). Many inhibitory receptors commonly implicated in T cell

exhaustion, such as LAG3 and CTLA-4 have been demonstrated to undergo similar patterns of expression to PD-1 in the context of CD8 T cell activation during acute infection (Ahn *et al*. 2018; David *et al*. 2019). This rapid expression of PD-1 following CD8 T cell activation has been demonstrated to be a direct downstream effect of TCR signalling, and thus stronger TCR activation may enhance shielding of higher affinity CD8 T cells from further activating signals (Ahn *et al*. 2018). Therefore, CD8 T cells which receive stronger TCR signals may express more PD-1, thereby enhancing shielding from activating and inflammatory signals and cells with enhanced shielding may be preferentially selected to seed the memory pool.

The role of interleukin 10 and Mgat5 in responses to acute infections

IL-10 is an immunoregulatory cytokine that is highly expressed during chronic infections, and IL-10 signalling during chronic infections has been demonstrated to influence the ability of a persistent pathogen to establish a chronic infection by modifying immune responses (Brooks *et al*. 2006). IL-10 is also expressed at a basal level during immune responses to many acute infections (Brooks *et al*. 2008; Brooks *et al*. 2006; Smith *et al*. 2018). IL-10 expression is significantly increased during chronic infections and increased IL-10 signalling has been demonstrated to directly decrease the antigen-sensitivity of CD8 T cells in an Mgat5 and Gal3-mediated manner (Smith *et al*. 2018). This decrease in antigen-sensitivity is a result of increased global N-glycan branching, which when bound by galectin-3 (Gal3) creates a lattice that changes the way surface receptors interact with one another. The increase in N-glycan branching observed on CD8 T cells during responses to chronic infections is controlled directly by the enzyme N-glucosyltransferase mannoside acetylglucosaminyltransferase 5 (Mgat5) (Smith *et al*. 2018).

Mgat5 belongs to the Mgat family of glycosyltransferases, which reside in the Golgi apparatus. Mgat5 has been shown to actively regulate CD8 T cell activation, and CD8 T cells deficient in Mgat5 have been demonstrated to be hyperresponsive to TCR stimulation (Demetriou *et al*. 2001; Grigorian and Demetriou 2011). In fact, Mgat5 deficiency has been shown to play a role in the development of delayed-type hypersensitivity, spontaneous autoimmunity in the kidney, and experimental

autoimmune encephalitis (EAE- a murine model of multiple sclerosis) (Demetriou *et al*. 2001; Grigorian and Demetriou 2011). The Mgat enzymes (Mgat1, Mgat2, Mgat4, Mgat5) act sequentially to build complex branching N-glycans on transmembrane glycoproteins destined for the plasma membrane (Boscher *et al*. 2011). Specifically, Mgat5 is responsible for the addition of a quaternary β1→6 branch on these complex glycosidic structures (Boscher *et al*. 2011). Elongations from the Mgat5-generated branch point are binding sites for a class of ubiquitously expressed carbohydrate-binding protein known as galectins, and specifically for Gal3.

Gal3 is the only chimera-type galectin found in vertebrates and forms unique homo-pentameric quaternary structures with other Gal3 monomers (Fortuna-Costa *et al*. 2014). Binding of these multivalent pentamers to branched N-glycans on integrated and peripheral membrane-associated glycoproteins results in the generation of a restrictive lattice on the plasma membrane of the cell (Demetriou *et al*. 2001; Grigorian and Demetriou 2011; Li *et al*. 2013; Smith *et al*. 2018). This lattice inhibits the diffusion of molecules through the membrane, and additionally has been demonstrated to alter the turnover kinetics membrane-associated proteins (Lau *et al*. 2007). During chronic infections, this lattice reduces global co-localization of the TCR and CD8 molecules, resulting in an increased threshold of activation and a decrease in antigen sensitivity (Smith *et al*. 2018). CD8 T cells with reduced antigen sensitivity have a decreased capacity to detect infected host cells, which results in establishment of persistent infections (Smith *et al*. 2018). The activation of this pathway has not been studied in the context of acute infections; however, IL-10 signalling during CD8 T cell contraction has been shown to be critical for memory CD8 T cell development (Laidlaw *et al*. 2015). Thus, we hypothesize that the immunoregulatory signaling pathways mediated by PD-1 and IL-10 reduce T cell activation to favour memory development in the context of acute infection, and we will examine these two pathways independently of one another.

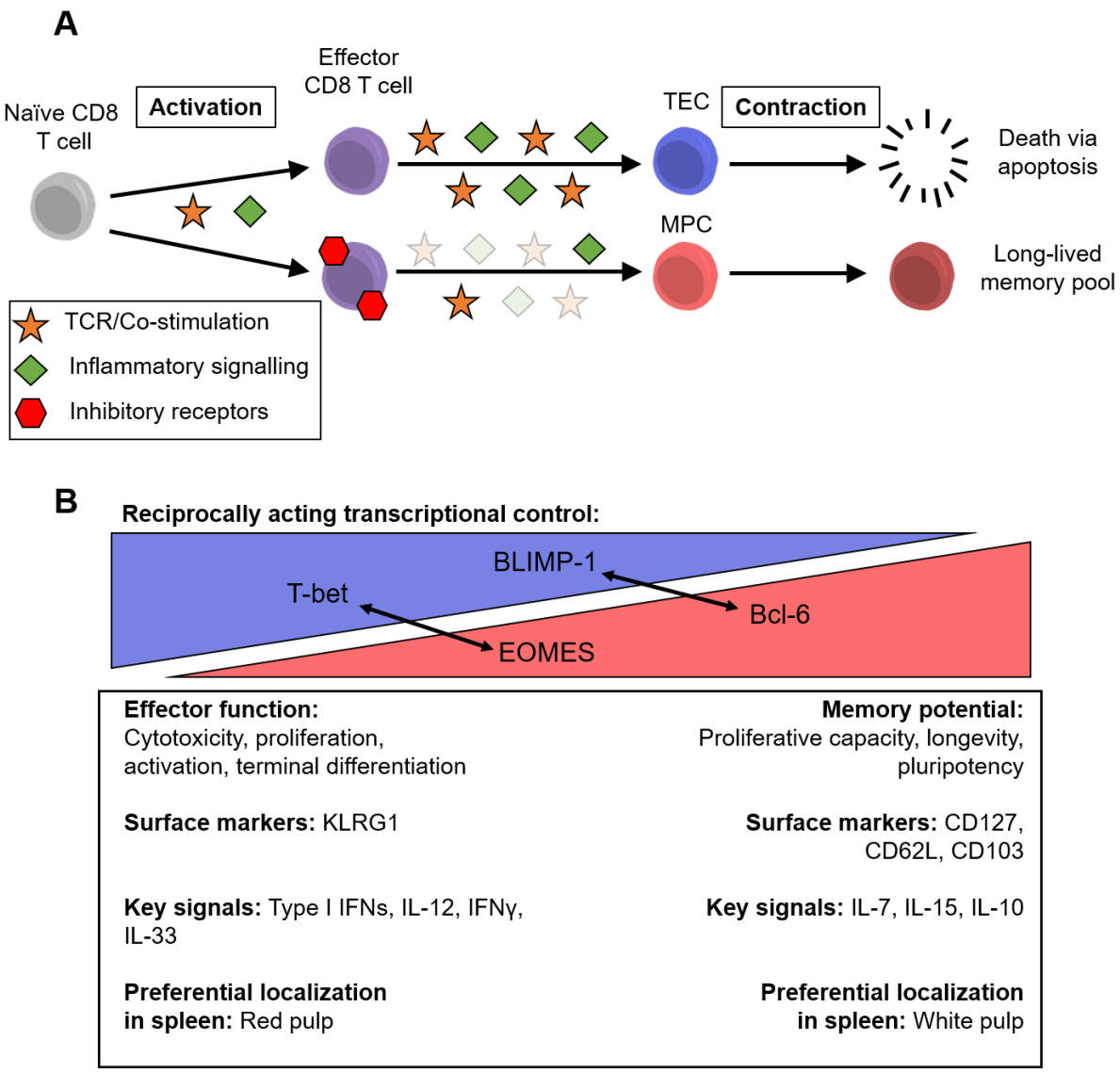

Figure I - 1. Graphical summary of CD8 T cell effector diversification and memory differentiation following acute infection. A) The decreasing potential model of CD8 T cell diversification, showing that enhanced accumulation of activating and inflammatory signals leads to a more terminally differentiated phenotype. B) TEC and MPC diversification is transcriptionally regulated by reciprocally-acting pairs of transcription factors. TEC and MPC differ in their expression of key surface markers, and signals received from the inflammatory milieu influence the differentiation of effector CD8 T cells. TEC and MPC also preferentially localize to different areas within the splenic tissue.

Hypothesis and Rationale

Memory CD8 T cells provide enhanced protection against pathogen reinfection (Kaech and Cui 2012). Selection of effector cells which will go on to seed the memory pool is not a stochastic process, however the mechanisms which underly selection of the memory pool have yet to be fully elucidated. The overall accumulation of activating and inflammatory signals throughout the course of CD8 T cell responses to acute infections has been demonstrated to enhance effector functions of CD8 T cells while simultaneously reducing their capacity to form memory (Gerlach *et al*. 2010; Kaech and Cui 2012). Inhibitory and immunoregulatory pathways may be a mechanism by which CD8 T cells are able to limit accumulation of activating and inflammatory signals, thereby favouring the development of memory CD8 T cells. Therefore, we hypothesize that *the immunoregulatory pathways mediated by PD-1 and IL-10 signalling reduce CD8 T cell activation to favour memory development in the context of acute infection* with the following specific aims:

1. To examine the role of the inhibitory PD-1 signaling pathway on CD8 T cell memory progression
2. To examine the role of the IL-10/Mgat5 immunoregulatory loop on CD8 T cell memory progression

Chapter II - Memory precursor cells express higher levels of PD-1 than terminal effector cells, but PD-1 signalling does not regulate the size or functionality of the memory CD8 T cell pool generated following acute infection.

Materials and Methods

Mice:

C57BL/6 mice were originally purchased from Charles River Laboratories and bred in house. *Il10*$^{-/-}$ and *Lgals3*$^{-/-}$ mice were originally purchased from The Jackson Laboratory and bred in house. P14 TCR-transgenic mice were kindly provided by Dr. A. Lamarre (INRS-Institut Armand-Frappier) and OT-I TCR-transgenic mice were kindly provided by Dr. Connie Krawczyk (McGill University). Both P14 and OT-I mice have been previously described and were bred in house (Hogquist *et al*. 1994; Pircher *et al*. 1987). 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. Infected mice were housed in biocontainment level 2. 6-12-week-old mice of both sexes were used for all experiments.

Pathogens:

LCMV Armstrong was kindly provided by Dr. J. Harty (University of Iowa) and was propagated as described (Slifka and Whitton 2001; Smith *et al*. 2018). An attenuated strain of *L. monocytogenes* deficient in act-A (DPL1942) expressing ovalbumin (act-LM-OVA) was provided by Dr. J. Harty (Brundage *et al*. 1993; Harty and Bevan 1995). Mice were infected with $2x10^5$ plaque forming units (PFU) of LCMV Armstrong by intraperitoneal (i.p.) injection, or with $1x10^6$ CFU of freshly grown act-LM-OVA by i.v. injection.

Primary cells:

Ex vivo peptide stimulation of total splenocytes was performed in RPMI 1640 media supplemented with 1% penicillin and streptomycin and 5% heat inactivated FBS and titrated concentrations of peptide, either LCMV-specific epitopes GP_{33-41} or $NP_{396-404}$. *Ex vivo* expansion and activation of wild-type CD8 T cells as well as P14 TCR transgenic

(TCR-tg) cells was performed in Iscove's Modified Dulbecco's Medium supplemented with 1% penicillin and streptomycin, 80mM N-acetylglucosamine and 10% heat-inactivated FBS. Isolated splenocytes were cultured at 37°C with 5% CO_2.

Adoptive Transfer:

Naïve P14 TCR-tg CD8 T cells for adoptive transfer were either collected via splenic harvest and preparation of a single cell suspension or through submandibular bleeding. Erythrocytes were lysed using Vitalyse (Cedarlane, BioE) and 1-25 x 10^4 naïve P14 TCR-tg CD8 T cells were adoptively transferred into congenically mismatched recipients by i.v. injection. The day following transfer mice were infected with the appropriate strain of LCMV. Mice were either sacrificed at 8 days post-infection for analysis of splenic CD8 T cells or blood was collected via submandibular bleeding for longitudinal analysis of the CD8 T cell response. Blood was collected at approximately days 8, 10, 15, 20 and 30 post-infection for longitudinal studies. At the final timepoint mice were sacrificed for analysis of splenic CD8 T cells in addition to peripherally circulating CD8 T cells.

Staining:

All antibody or lectin stains were performed on 1-3 x 10^6 cells/well at 4°C unless otherwise indicated. At indicated timepoints spleens were harvested and single cell suspensions were prepared. Staining was performed in 96 well plates at a volume of 100ul of antibody stain solution per well. All antibody staining steps were performed in the presence of Fc block at a dilution of 1:100. Antibody surface stains were diluted to experimentally determined dilutions in FACS buffer for 20 minutes. This step was followed by fixation of cells with ICFix buffer (eBioscience) for 10 minutes. Samples which were stained with MHC-I tetramers were pre-incubated with 50ul of FACS buffer with a 1:50 dilution of Fc block. Following this 50ul of a 1:200 dilution of PE-conjugated tetramer for the LCMV epitopes GP_{33-41} or $NP_{396-404}$ (provided by Dr. Alain Lamarre's laboratory) respectively was added to each well for a final volume of 100ul and final dilutions of 1:100 and 1:400 for Fc block and tetramer respectively. Nuclear staining was performed using a FoxP3 staining kit from ThermoFisher. Cells were fixed and permeabilized in the dark for at least 30 minutes, then stained for at least 30 minutes in

1x permeabilization buffer. *Ex vivo* peptide stimulation was performed as follows: prepared splenocytes were incubated *ex vivo* with titrated concentrations of GP_{33-41} or $NP_{396-404}$ peptide in the presence of Brefeldin A (BFA). Cells were stimulated for 5½ hours at 37°C, 5% CO_2 and then stained for the production of cytokines by intracellular cytokine staining (ICS). ICS was performed following surface staining and fixation of cells; antibodies were diluted to the appropriate concentrations in 1x permeabilization wash buffer (eBiosciences) for 20 minutes.

Infection matched cell transfer:

1-5 x 10^4 naïve P14 TCR-tg CD8 T cells were injected i.v. into naïve congenically mismatched recipients; 1 day later mice were infected with LCMV Arm. At the same time naïve mice that had not received an adoptive transfer of P14 cells were also infected with LCMV Arm. On day 8 post-infection, spleens were harvested from mice which had received P14 cells, and transgenic cells were isolated by Thy1.1 or Thy1.2-PE positive-selection, accordingly. Briefly, cells were stained with the appropriate PE conjugated antibody at a dilution of 1:500 in the presence of FcBlock at a dilution of 1:100 and purified using anti-PE-magnetic separation according to standard AutoMACS protocols (Miltenyi Biotec). These cells were then transferred to congenically-mismatched but infection-matched recipients and followed until at least day 30 post-infection.

Branched N-glycan surface expression analysis:

Prepared cells were pre-treated to remove bound galectins with 50mM D-Lactose, as these may impair staining through steric hindrance. Cells underwent antibody surface staining and were then fixed as described above. Cells were then incubated with 50ug/mL biotinylated *Phaseolus vulgaris* Leukoagglutinin (PHA-L) for 5.5 hours at room temperature, washed and incubated with streptavidin conjugated with either PE or APC for 20 minutes and then washed before analysis by flow cytometry. Panels included two control samples which were stained as described which lacked either of PHA-L or streptavidin.

In vivo treatments:

For experiments using anti-PD-1 or anti-PD-L1 blocking antibodies: 1-5 x 10^4 naïve P14 TCR-tg CD8 T cells were injected into naïve congenically mismatched recipients; one day later mice were infected with LCMV Arm as described above. At days 3, 6 and 9 post-infection mice were treated with 200µg of rat-anti-PD-1, rat-anti-PD-L1, rat-IgG2b isotype control or PBS by i.p. injection. Cells were analyzed from blood samples collected at indicated timepoints and were harvested for splenic analysis at the final timepoint at least 30 days post-infection.

Gene expression analysis:

For RT-qPCR analysis, P14 cells were enriched by PE-selection as described above. RNA was then extracted using Trizol reagent and cDNA was generated with iScript reverse transcriptase (Bio-Rad) using 1µg of total RNA. Analysis of gene expression was conducted via RT-qPCR was then conducted using SensiFAST SYBR (Bioline). Expression levels of transcripts of interest were 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 indicated group (Livak and Schmittgen 2001).

Data analysis:

Data were analyzed using FlowJo and GraphPad Prism analysis software. Both packages are publicly available through public vendors. 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

PD-1 expression is higher on memory precursor cells than on terminal effector cells

To determine whether PD-1 signalling is involved in shielding MPC from activating signals to reduce the activation of a fraction of the total effector population to favour memory development, we first asked whether MPC express higher levels of PD-1 than their TEC counterparts within the same host. To address this question, TCR-transgenic P14 CD8 T cells, which express a TCR that is specific to the GP_{33-41} epitope of

lymphocytic choriomeningitis virus (LCMV), were adoptively transferred into congenically mismatched wild type (WT) mice. The day following adoptive transfer mice were infected with the Armstrong strain of LCMV (LCMV Arm) to induce an acute infection. Mice were sacrificed and spleens harvested at days 5, 8 and 10 post infection, timepoints were selected to correspond with before, during and after the peak of the CD8 T cell response, respectively. Total splenocytes were stained with CD127 and KLRG1 for TEC/MPC differentiation, TEC are $KLRG1^{HI}CD127^{LO}$ and MPC are $KLRG1^{LO}CD127^{HI}$, and expression of PD-1 on each of these subsets was analyzed via flow cytometry.

MPC showed significantly elevated expression of PD-1 when compared to TEC isolated from the same mice at all three time points, as measured by geometric mean fluorescence intensity (gMFI) of PD-1 staining (Figure II - 1A, 1B). It is important to note that the expression of PD-1 is much higher at 5dpi than either of the later time points. These results are expected since it is known that PD-1 expression is driven by stimulation of the TCR and at 5dpi the virus has not yet been cleared so antigen stimulation can still occur or is more recent (Ahn *et al*. 2018). Therefore, since MPC express more PD-1 than their TEC counterparts and that expression of PD-1 is regulated by TCR signalling (Ahn *et al*. 2018), we hypothesize that MPC are receiving a stronger initial TCR signal compared to their TEC counterparts, resulting in the observed increased relative expression of PD-1. In turn, PD-1 signalling may shield MPC from accumulation of further activating and inflammatory signals throughout the course of infection, thereby reducing their activation to favour memory development.

To ensure these results are not an artefact of either the P14 transgenic mouse model or restricted to viral infection models, the above experiment was repeated with a bacterial model of infection. Briefly, OT-I TCR-tg CD8 T cells which are specific for an epitope of ovalbumin (OVA) were adoptively transferred to congenically mismatched WT hosts and the following day the mice were infected with an $actA^{-/-}$ attenuated strain of *Listeria monocytogenes* expressing OVA (actA-LM-OVA). Mice were sacrificed and spleens harvested at 7dpi, which corresponds to the peak of the CD8 T cell response in this infection model. Splenocytes were again analyzed for expression of PD-1 using flow cytometry as described above. As seen in P14 TCR-tg cells, OT-I MPC express

significantly higher levels of PD-1 than do TEC from the same mice (Figure II - 1C, 1D). These results confirm that elevated expression of PD-1 on MPC compared to TEC subpopulations is generalizable to T cells of different specificities responding to either viral or bacterial infections. These results support our hypothesis that PD-1 expression on MPC may limit their activation to favour memory generation in the context of acute infection.

$NP_{396-404}$-specific CD8 T cells are more sensitive to their cognate antigen and express more PD-1 than GP_{33-41}-specific CD8 T cells.

We have shown that PD-1 expression is increased on MPC compared to TEC, and it has previously been shown that TCR signalling drives expression of PD-1 (Ahn *et al*. 2018). In order to examine whether the strength of antigenic signal received influences the capacity of CD8 T cells to form memory we utilized an endogenous model of CD8 T cell responses. This endogenous model allowed us to directly compare the capacity of two epitope-specific populations of CD8 T cell to form memory within the same mouse. This also allows us to validate that the results observed thus far are not an inherent artefact of the TCR transgenic systems used above. We compared two well-described endogenous populations of epitope-specific CD8 T cells: $NP_{396-404}$ (NP396) and GP_{33-41} (GP33) (Masopust *et al*. 2007). Prior to using this model we needed to validate that NP396-specific cells have increased sensitivity for their cognate antigen compared to GP33-specific cells, as described in the literature (Raué and Slifka 2009).

Antigen sensitivity of NP396- and GP33-specific populations was assessed as follows: WT mice were infected with LCMV Arm and sacrificed on day 8 post-infection. Total splenocytes were incubated with titrated concentrations of either $NP_{396-404}$ or GP_{33-41} peptide in the presence of brefeldin A (BrfA). Responding CD8 T cells were quantified by measuring the frequency of cells producing IFNγ as measured by intracellular cytokine staining (ICS) (Richer *et al*. 2013). Compared to GP33-specific CD8 T cells, the antigen-sensitivity of NP396-specific CD8 T cells was 6.6x higher based on the concentration of peptide needed to induce 50% of the maximum IFNγ production (effective concentration 50 [EC_{50}]) (Figure II - 2). This validates these populations as an

appropriate model with which to observe the effects of signal strength on PD-1 expression and memory progression within an identical inflammatory milieu.

We have shown that NP396- specific cells are more sensitive to their cognate antigen than GP33-specific cells. It is known that TCR signalling drives the expression of PD-1, and as such we asked whether NP396-specific cells will express higher levels of PD-1 than their GP33-specific counterparts (Ahn *et al*. 2018; Raué and Slifka 2009). Additionally, we asked whether in both populations of epitope-specific cells MPC will express higher levels of PD-1 than their respective TEC counterparts. To address this, we again infected WT mice with LCMV Arm and sacrificed the mice at day 8 post infection, splenocytes were stained for surface expression of PD-1 and analyzed via flow cytometry. As expected based on our previous results, PD-1 expression was significantly higher in MPC compared to TEC in both NP396 and GP33-specific CD8 T cells (Figure II - 3A, 3B). Additionally, PD-1 expression was significantly higher in NP396-specific compared to GP33-specific CD8 T cells, for the total epitope-specific population as well as TEC and MPC sub-populations (Figure II – 3B). These results indicate that MPC consistently express higher levels of PD-1 compared to TEC isolated from the same mice and that this relationship is independent of the population's initial antigen sensitivity.

MPC are more sensitive to their cognate antigen than TEC

PD-1 expression is driven by TCR signalling and we have shown that cells which are more sensitive for their cognate antigen also express higher levels of PD-1 (Ahn *et al*. 2018). Since we have also shown that MPC express higher levels of PD-1 than do TEC isolated from the same mice, we asked whether MPC are more sensitive to their cognate antigen compared to TEC. To address this, P14 TCR-tg CD8 T cells were adoptively transferred into WT mice, the following day these mice were infected with LCMV Arm. Mice were sacrificed and spleens harvested at 8dpi. Antigen sensitivity was interrogated as described above. First, we asked whether *ex vivo* peptide stimulation decreased the expression of surface marker CD127, which we use to define TEC and MPC subpopulations. *Ex vivo* T cell stimulation has been shown to modulate the expression of certain surface receptors, such as the TCR (Crotzer *et al*. 2004; Lee *et al*.

1999). In line with these observations, *ex vivo* stimulation with cognate antigen resulted in a decrease in the gMFI of CD127 (Figure II - 4B). However, despite this change, the frequencies of TEC and MPC populations are not altered in stimulated groups compared to unstimulated surface stained controls from the same mice, thereby allowing us to compare the antigen sensitivity of the different effector CD8 T cell subsets (Figure II - 4A, 4C). P14 MPC were 1.8x more sensitive to their antigen compared to P14 TEC isolated from the same mice (Figure II - 4D, 4E). This shift in antigen sensitivity is relatively small compared to the differences observed between NP396 and GP33-specific endogenous CD8 T cell populations. As such, it is possible that the shift we observed, though statistically significant, is not biologically relevant. However, it is possible that downstream integration and amplification of the TCR signalling cascade may result in a more significant difference in the magnitude of signal received by TEC and MPC effector subpopulations. These data reveal that despite differentiating within the same inflammatory milieu, CD8 T cells with identical TCRs which are activated simultaneously do not necessarily receive identical signals.

PD-1 signalling does not play a functional role in CD8 T cell memory progression

Our data show that NP396-specific cells, which are more sensitive to their cognate antigen than GP33-specific, cells express higher levels of PD-1. Additionally, we have shown that MPC are more sensitive to their antigen and express higher levels of PD-1 than their TEC counterparts. Therefore, to determine whether antigen-sensitivity and PD-1 expression play a role in the memory progression of CD8 T cells, we asked whether NP396-specific CD8 T cells preferentially seed the memory pool in comparison to GP33-specific CD8 T cells. To address this question WT mice were infected with LCMV Arm and a longitudinal analysis was performed at days 8, 10, 15, 21 and 35 post infection. Blood was collected from the submandibular vein and epitope-specific populations were identified using MHC class I tetramers and analyzed via flow cytometry.

NP396-specific cells reached a significantly higher peak of effector expansion in terms of both frequency of epitope-specific cells compared to peripheral blood leukocytes (PBL) and numbers of cells than did GP33-specific CD8 T cells within the

same host. This difference was not statistically significant after 15dpi, although NP396-specific cells trended higher at all time points (Figure II - 5A, 5C). Direct comparison of the memory pool formed following contraction must take into consideration the magnitude of effector expansion, to correct for this we compared the percentage of cells surviving to memory time points after normalizing for the peak of expansion. Although the differences were not significant NP396-specific cells showed a trend to increased capacity to form memory compared to GP33-specific cells isolated from the same host (Figure II - 5B, 5D). These data suggest NP396-specific CD8 T cells have an enhanced capacity to form memory compared to GP33-specific cells. However, the relative proportion of NP396 and GP33-specific CD8 T cells at memory time points had high variability between individual mice. Furthermore, we observed a clear and statistically significant differences in antigen sensitivity (Figure II – 2) and expression of PD-1 (Figure II – 3) between these two populations, and the observed difference in the relative capacity of these populations to form memory is comparatively minor, and as such it is unclear whether PD-1 signalling directly plays a major role in regulating the capacity of CD8 T cells to form memory.

To more directly address whether PD-1 signalling plays a functional role in memory development, we employed an antibody blockade of PD-L1, the primary canonical ligand of PD-1 (Ahn *et al*. 2018; Brooks *et al*. 2008; David *et al*. 2019). WT mice were infected with LCMV Arm and received either 200ng PD-L1 blocking antibody or an isotype-matched control on days 3, 6 and 9 post infection via intraperitoneal injection. The memory development of NP396 and GP33-specific CD8 T cell populations were analyzed as described above. We observed a trending decrease in the frequency of NP396-specific cells of total PBL in the PD-L1 blockade group compared to the isotype control and this trend was preserved following correction for peak expansion (Figure II – 6A, 6C). However, we observed no significant difference in the frequency of GP33-specific CD8 T cells or the number of memory CD8 T cells formed in either epitope-specific population (Figure II -, 6B, 6D, 6E). Additionally, we observed no significant difference in the percentage of GP33-specific cells or number of either epitope-specific population surviving to memory time points when normalized to the peak of expansion (Figure II - 6C, 6F).

In addition to overall frequency and number of cells present at memory time points, we assessed the functionality of memory cells formed by measuring the production of effector cytokines IFNγ, TNFα and IL-2 in response to stimulation with cognate antigen via ICS. Polyfunctionality, or the ability of memory cells to produce multiple effector cytokines in response to TCR signalling is a hallmark of highly functional memory (Lelic *et al*. 2012; Park and Shin 2019). We found no difference in the frequency of responding cells producing IFNγ (Figure II - 6G), although mice which had received the PD-L1 blockade treatment trended towards increased expression of IFNγ on a per-cell basis as measured by gMFI of IFNγ expression (Figure II - 6H). This is expected since PD-1 signalling is known to decrease T cell activation and ability to respond to cognate peptide and similar results were reported by Ahn *et al* (Ahn *et al*. 2018). No differences were observed in the frequency or number of cells producing both IFNγ and TNFα between the blockade and control groups (Figure II - 6I). The PD-L1 blockade group represented a trending increase in the frequency of memory cells producing both IFNγ and IL-2 (Figure II – 6J) although this didn't reach statistical significance. These results strongly suggest that PD-1 signalling does not play a major functional role in the development of memory CD8 T cells in the context of acute infection.

In the above experiment, we utilized a blockade of PD-L1 to interrogate the role of PD-1 signalling in CD8 T cells during the response to an acute infection. While PD-L1 is the primary canonical ligand of PD-1, PD-L1 blockade on its own may be inadequate to ensure complete abrogation of PD-1 signalling. To account for this, the above longitudinal time course was repeated with a PD-1 neutralizing antibody. We observed no significant differences in the frequency, number or functionality of memory CD8 T cells formed in either epitope-specific population (Figure II - 7). Based on these collective results, we are able to conclude that although epitope-specific populations with enhanced affinity for their cognate antigen and expression of PD-1 exhibited a trend towards enhanced memory formation capacity, PD-1 signalling does not play a functional role in the development of memory CD8 T cells following acute infection.

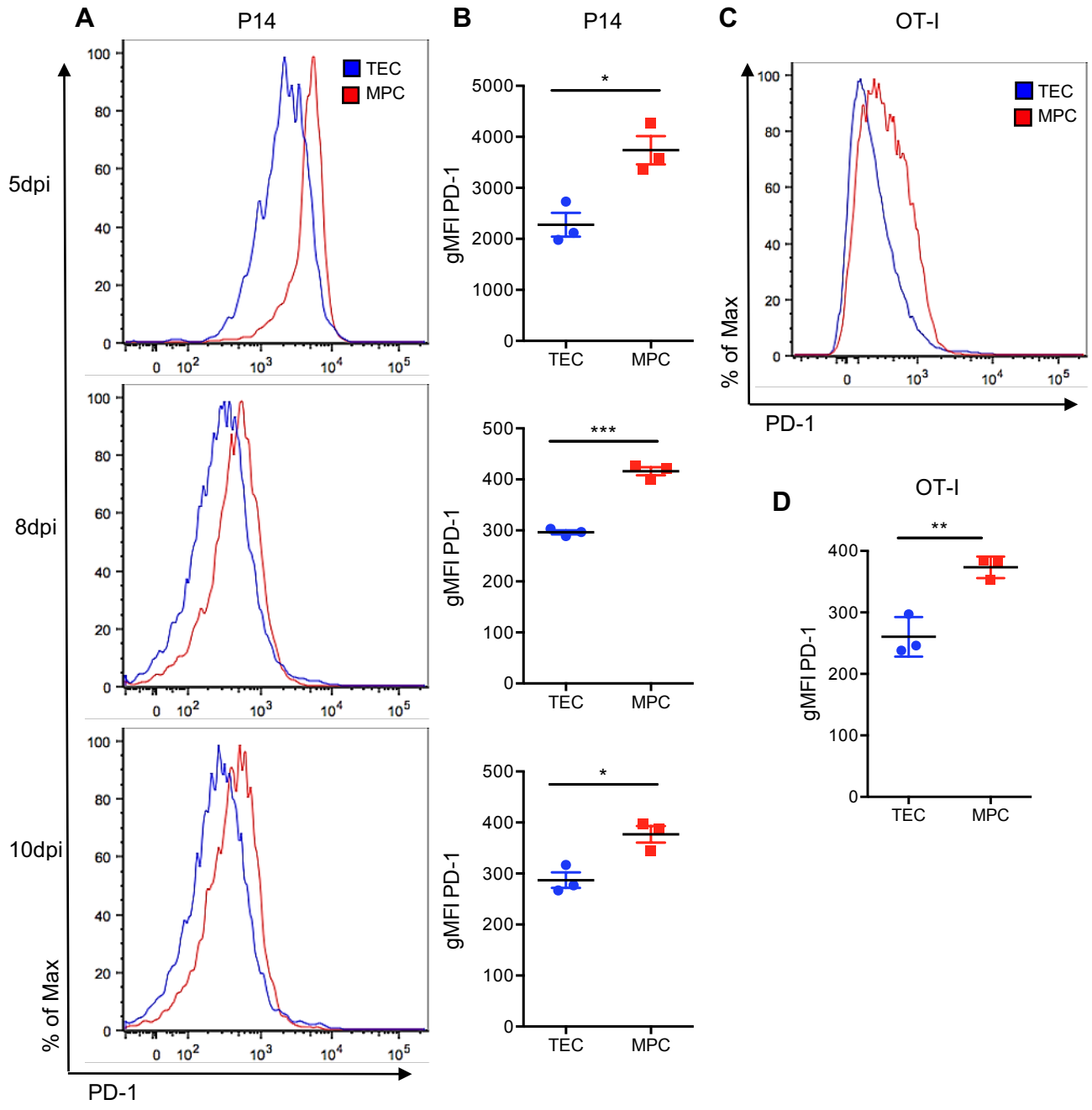

Figure II - 1. PD-1 expression is higher in MPC compared to TEC effector sub-populations. A) Representative histograms and B) dot plots quantifying the geometric mean fluorescence intensity of PD-1 on TEC and MPC P14 cell subsets at indicated time points. C) Representative histogram and D) dot plots quantifying the geometric mean fluorescence intensity of PD-1 on TEC and MPC on OT-I cell subsets. P14 data (Mean ± SEM) is representative of at least two independent experiments (n=3 per group). OT-I data (Mean ± SEM) is representative of one experiment (n=3 per group. P values determined using a two-tailed unpaired Student's t-test for each time point (* = $p<0.05$, ** = $p<0.01$ ***=$p<0.001$).

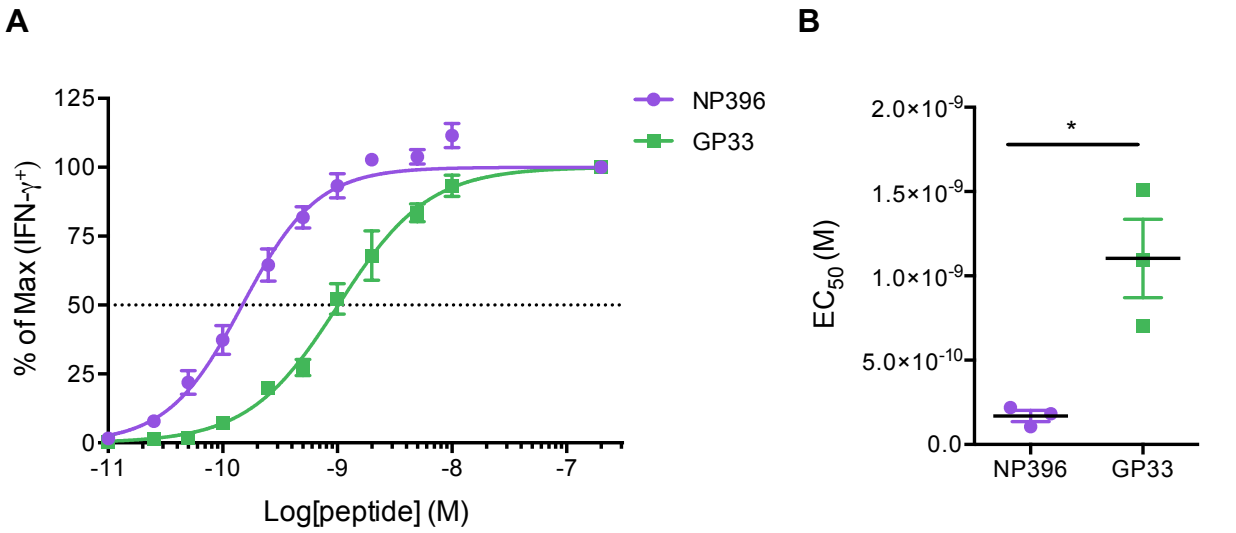

Figure II - 2. NP396-specific endogenous CD8 T cells are more sensitive to stimulation with their cognate antigen than are GP33-specific cells. A) Comparison of antigen sensitivity between $NP_{396-404}$ and GP_{33-41}-specific CD8 T cells at the peak of effector response to LCMV Arm. Data are normalized to the proportion of $IFN\gamma^+$ cells at saturating peptide concentration (200 nM). B) dot plot quantification of each population's EC_{50} (M) value. Data (Mean ± SEM) is representative of at least two independent experiments (n=3 per group). P-values determined using a two-tailed unpaired Student's t-test (* = p<0.05).

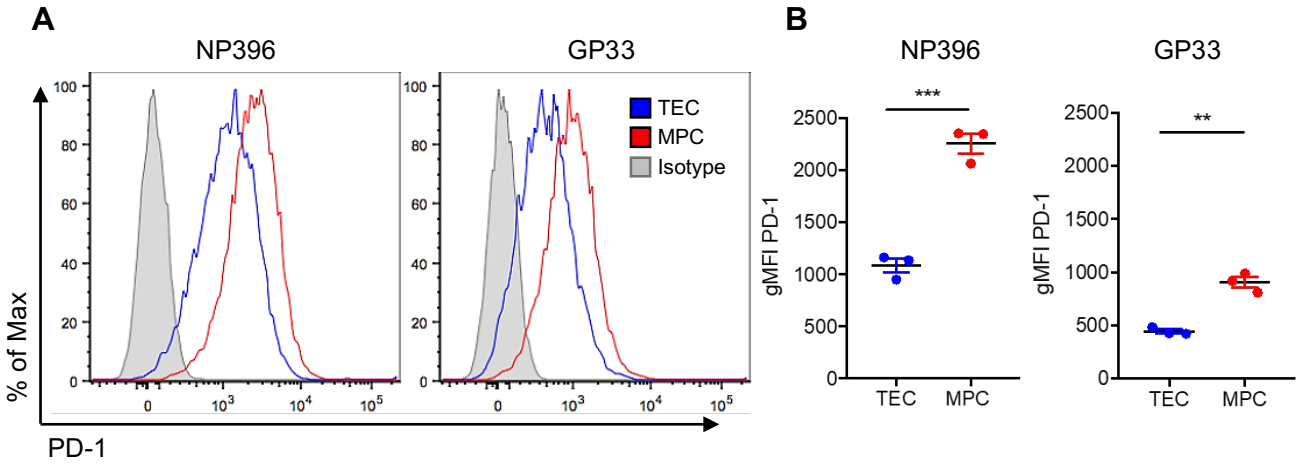

Figure II - 3. PD-1 expression is higher in NP396-specific cells compared to GP33-specific cells and MPC compared to TEC populations. A) Representative histograms and B) dot plots quantifying the geometric mean fluorescence intensity of PD-1 on TEC and MPC cells for either NP396- or GP33-specific endogenous CD8 T cells. Data (Mean ± SEM) are representative of at least two independent experiments (n=3 per group). P values determined using a two-tailed unpaired Student's t-test (** = p<0.01, *** = p<0.001, **** = p<0.0001)

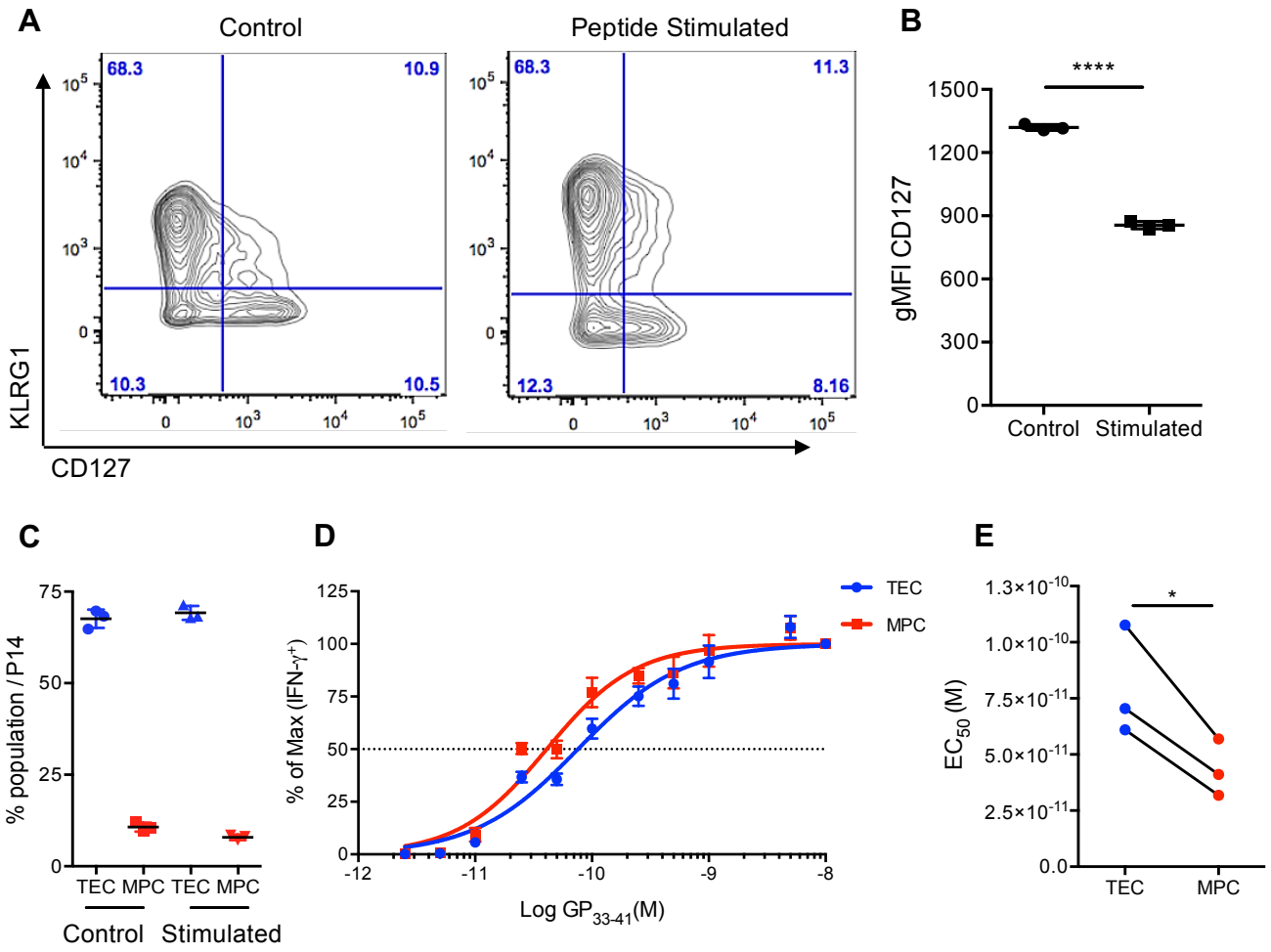

Figure II - 4. MPC are more sensitive to their cognate antigen than are TEC at the peak of the CD8 T cell response. A) representative of KLRG1 and CD127 gating on control and stimulated samples. B) Dot plots quantifying the geometric mean fluorescence intensity of CD127 on control and stimulated samples. C) Dot plots quantifying the relative TEC and MPC frequencies between control and stimulated samples. D) Comparison of antigen sensitivity between TEC and MPC P14 effector sub-populations at the peak of effector response to LCMV Arm. Data are normalized to the proportion of $IFN\gamma^+$ cells at saturating peptide concentration (10 nM). E) dot plot quantification of each population's EC_{50} (M) value. Data (Mean ± SEM) is representative of at least two independent experiments (n=3 per group). P values determined using a two-tailed unpaired Student's t-test (* = $p<0.05$).

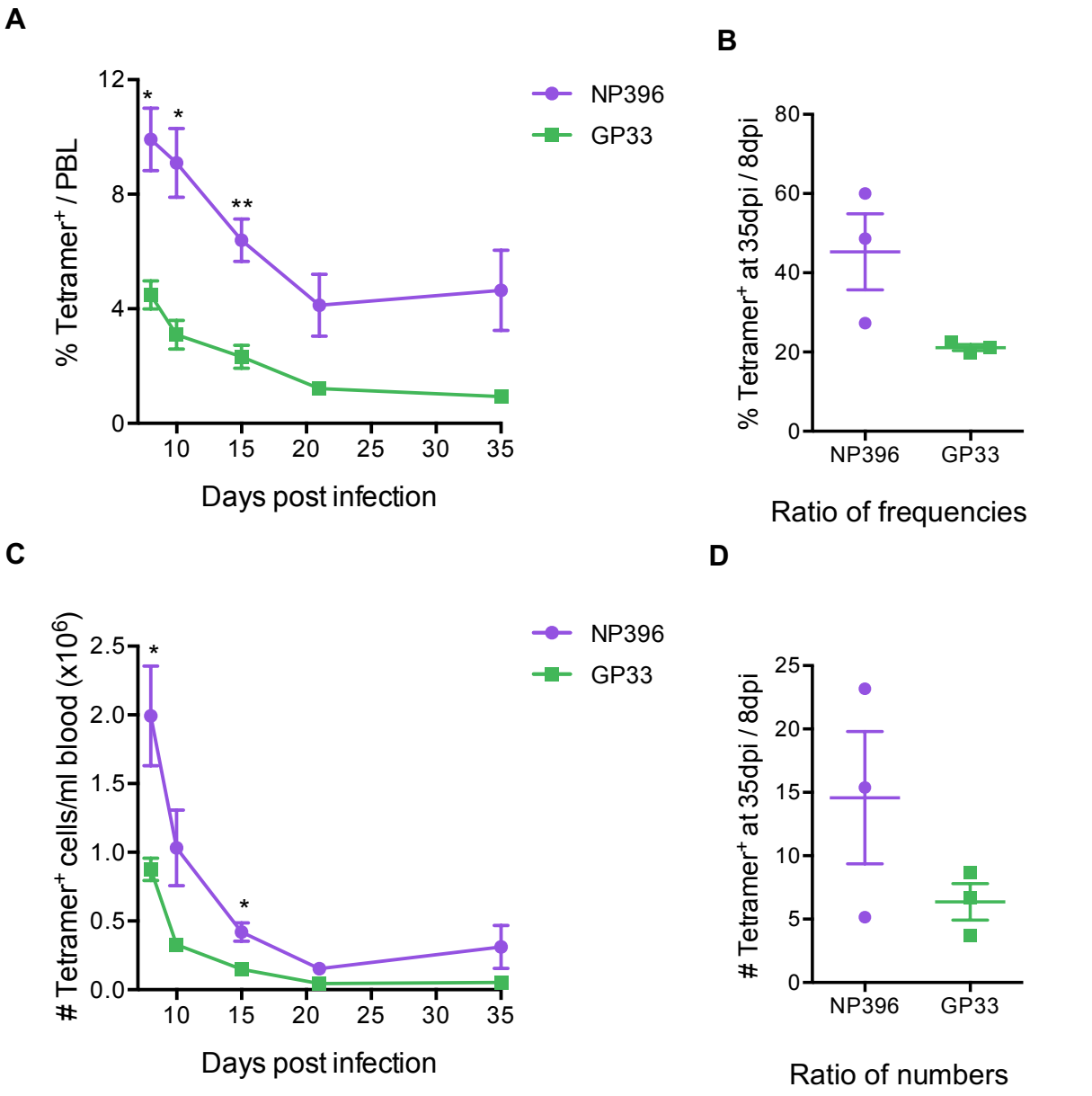

Figure II - 5. NP396 -specific CD8 T cells trend towards increased memory potential compared to GP33-specific cells following infection with LCMV Arm. A) Frequency of $NP_{396-404}$ or GP_{33-41} tetramer$^+$ of total endogenous CD8 T cells of PBL. B) Dot plot quantification of percentage of 35dpi:8dpi frequencies of epitope-specific populations C) Number of NP396-404 tetramer+ CD8 T cells/ml blood D) Dot plot quantification of percentage of 35dpi:8dpi numbers of epitope-specific populations. Data (Mean ± SEM) are representative of at least two independent experiments (n=3 for per group). P values determined using a two-tailed unpaired Student's t-test for each time point with no correction for multiple tests (* = $p<0.05$, ** = $p<0.01$).

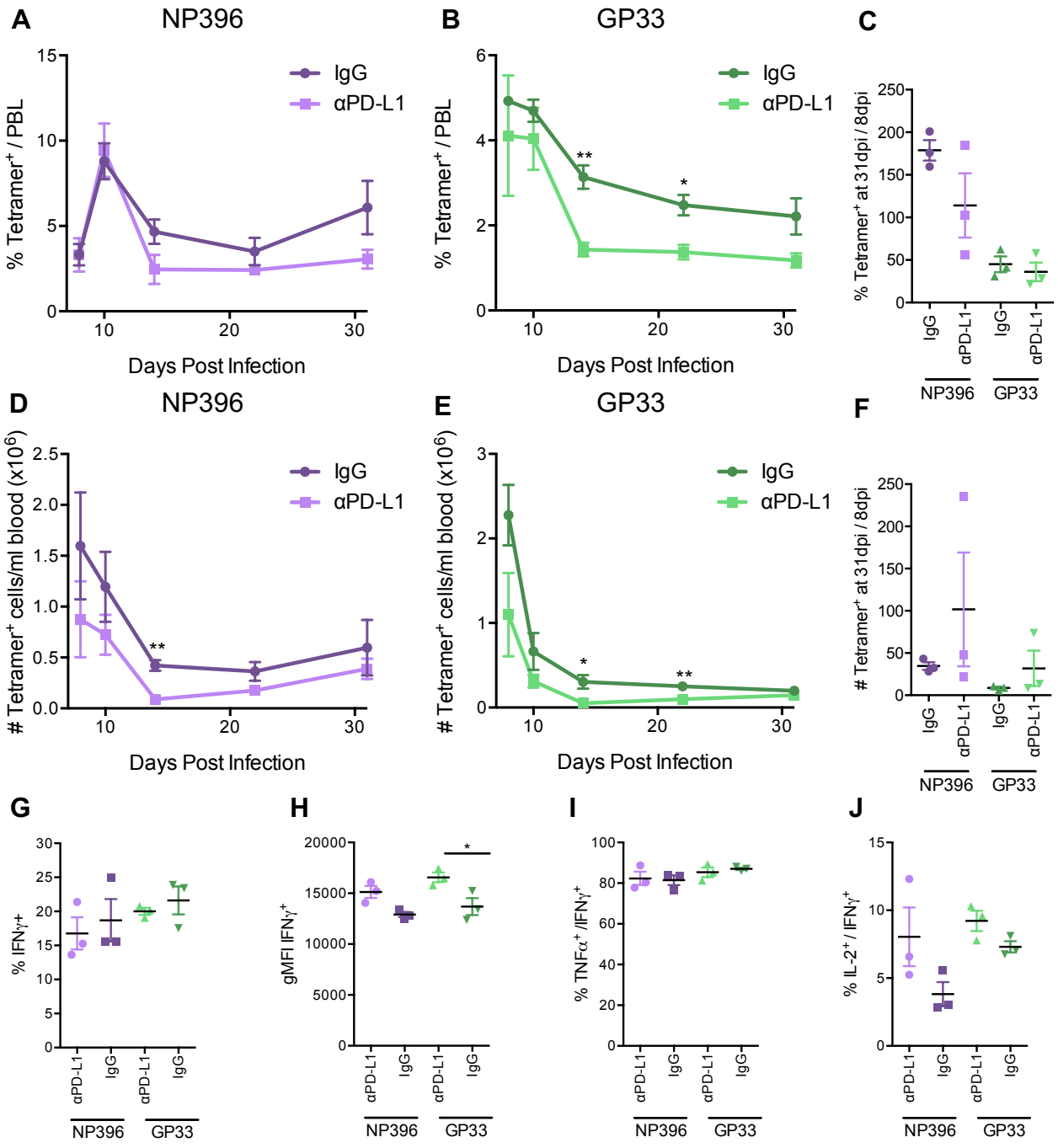
A
NP396
% Tetramer+ / PBL
IgG
αPD-L1
Days Post Infection
B
GP33
% Tetramer+ / PBL
IgG
αPD-L1
Days Post Infection
C
% Tetramer+ at 31dpi / 8dpi
IgG
αPD-L1
IgG
αPD-L1
NP396
GP33
D
NP396
Tetramer+ cells/ml blood (x10^6)
IgG
αPD-L1
Days Post Infection
E
GP33
Tetramer+ cells/ml blood (x10^6)
IgG
αPD-L1
Days Post Infection
F
Tetramer+ at 31dpi / 8dpi
IgG
αPD-L1
IgG
αPD-L1
NP396
GP33
G
% IFNγ+
αPD-L1
IgG
αPD-L1
IgG
NP396
GP33
H
gMFI IFNγ+
αPD-L1
IgG
αPD-L1
IgG
NP396
GP33
I
% TNFα+ /IFNγ+
αPD-L1
IgG
αPD-L1
IgG
NP396
GP33
J
% IL-2+ / IFNγ+
αPD-L1
IgG
αPD-L1
IgG
NP396
GP33

Figure II - 6. αPD-L1 blockade does not significantly alter frequencies, numbers or functionality of endogenous tetramer$^+$ CD8 T cells that survive to memory time points following infection with LCMV Arm. Frequencies of A) $NP_{396-404}$- specific cells over PBL B) GP_{33-41}-specific cells over PBL, Numbers of D) $NP_{396-404}$- specific cells E) GP_{33-41}-specific cells. Dot plot quantification of percentage of 31dpi:8dpi C) frequencies and F) numbers of epitope-specific populations G) Dot plots quantifying the frequency of cells producing IFNγ , H) the geometric mean of fluorescence intensity of IFNγ of IFNγ$^+$ cells, dot plots quantifying the frequency of IFNγ$^+$ cells also producing I) TNFα and J) IL-2. Data (Mean ± SEM) are representative of two independent experiments (n=3 for per group). P values for A,B,D, and E determined using a two-tailed unpaired student's t-test for each time point (* = $p<0.05$, ** = $p<0.01$). P values for C,F,G,H,I, and J determined using one-way ANOVA with Tukey's post hoc analysis of multiple comparisons (* = $p<0.05$).

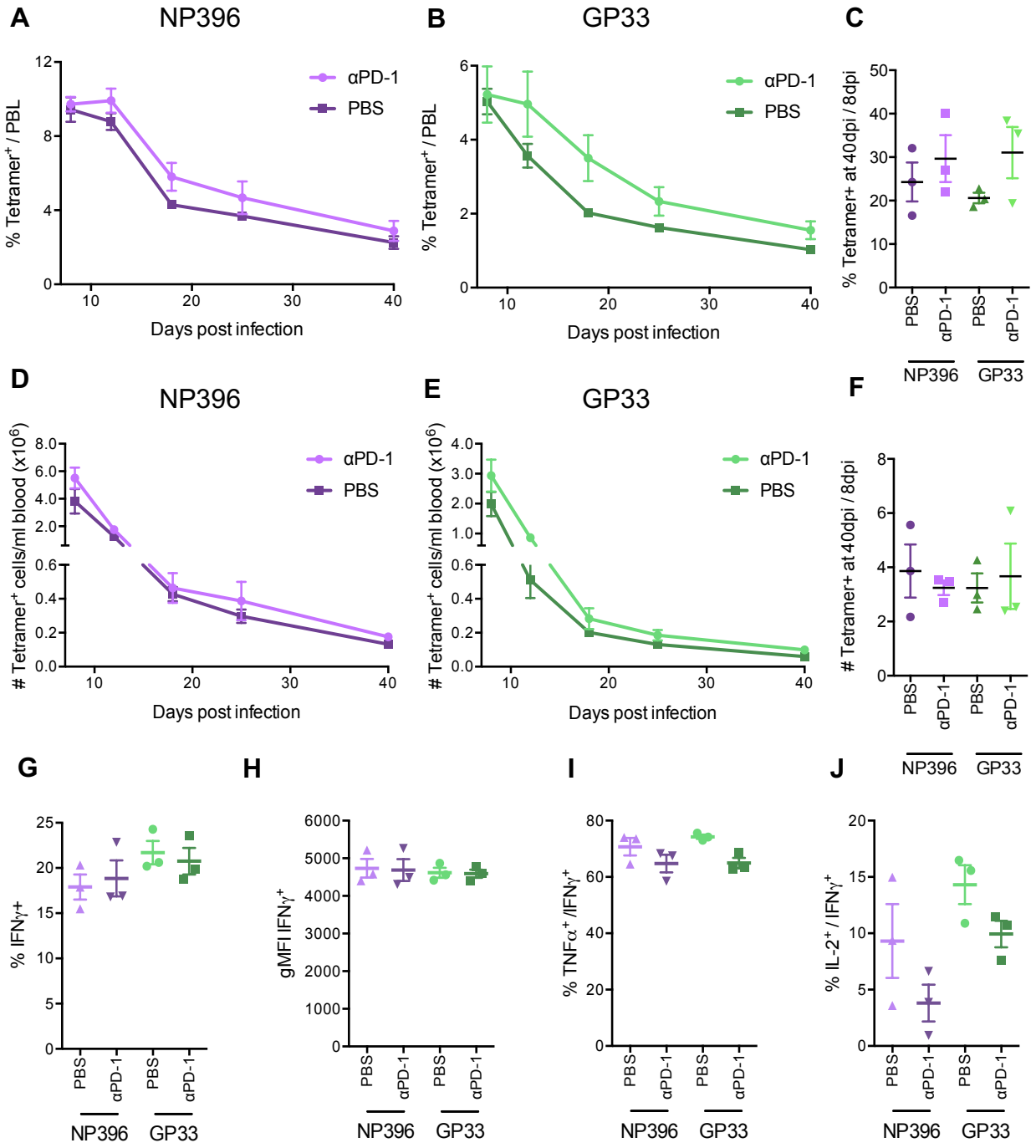
A
NP396
% Tetramer+ / PBL
Days post infection
αPD-1
PBS
B
GP33
% Tetramer+ / PBL
Days post infection
αPD-1
PBS
C
% Tetramer+ at 40dpi / 8dpi
PBS
αPD-1
PBS
αPD-1
NP396
GP33
D
NP396
Tetramer+ cells/ml blood (x10^6)
Days post infection
αPD-1
PBS
E
GP33
Tetramer+ cells/ml blood (x10^6)
Days post infection
αPD-1
PBS
F
Tetramer+ at 40dpi / 8dpi
PBS
αPD-1
PBS
αPD-1
NP396
GP33
G
% IFNγ+
PBS
αPD-1
PBS
αPD-1
NP396
GP33
H
gMFI IFNγ+
PBS
αPD-1
PBS
αPD-1
NP396
GP33
I
% TNFα+ /IFNγ+
PBS
αPD-1
PBS
αPD-1
NP396
GP33
J
% IL-2+ / IFNγ+
PBS
αPD-1
PBS
αPD-1
NP396
GP33

Figure II - 7. αPD-1 blockade does not significantly alter frequencies, numbers or functionality of endogenous tetramer$^+$ CD8 T cells that survive to memory time points following infection with LCMV Arm. Frequencies of A) $NP_{396-404}$- specific cells over PBL B) GP_{33-41}-specific cells over PBL, Numbers of D) $NP_{396-404}$- specific cells E) GP_{33-41}-specific cells. Dot plot quantification of percentage of 31dpi:8dpi C)frequencies and F) numbers of epitope-specific populations G) Dot plots quantifying the frequency of cells producing IFNγ , H) the geometric mean of fluorescence intensity of IFNγ of IFNγ$^+$ cells, dot plots quantifying the frequency of IFNγ$^+$ cells also producing I) TNFα and J) IL-2. Data (Mean ± SEM) are representative of two independent experiments (n=3 for per group). P values for A, B, D, and E determined using a two-tailed unpaired student's t-test for each time point (* = $p<0.05$, ** = $p<0.01$). P values for C, F, G, H, I, and J determined using one-way ANOVA with Tukey's post hoc analysis of multiple comparisons (* = $p<0.05$).

Chapter III - Memory precursor cells express higher levels of *Mgat5* and have higher levels of -glycan branching than terminal effector cells, but galectin binding to N-glycan branching during CD8 T cell contraction does not regulate the size or functionality of the memory CD8 T cell pool generated following acute infection.

Results

Mgat5 gene expression and N-glycan branching is elevated in MPC compared to TEC

To determine whether Gal3 binding to N-glycan branching is involved in shielding MPC from activating signals, we first asked whether MPC express higher levels of *Mgat5* than TEC within the same host during infection. Unfortunately, a reliable murine antibody for Mgat5 is not currently commercially available. Therefore, we evaluated gene expression levels of *Mgat5* in TEC and MPC. Briefly, P14 TCR-tg cells were adoptively transferred into congenically mismatched WT mice, and infected with LCMV Arm the following day. At the peak of effector CD8 T cell response, 8dpi, mice were sacrificed, and spleens harvested. TEC and MPC effector subpopulations were isolated using fluorescence-activated cell sorting (FACS) and cDNA was generated from the extracted RNA. and gene expression was analyzed using RT-qPCR. The RNA used in these experiments was generated by Stefanie Valbon, another graduate student in the Richer lab. We found that *Mgat5* expression is 4-fold higher in MPC compared to TEC isolated from the same host (Figure III - 1A). These results indicate that Mgat5-modified N-glycans may be involved in shielding MPC to favour memory development.

Although we observed increased *Mgat5* gene expression in MPC compared to TEC, mRNA transcript levels do not necessarily correlate with protein levels or levels of enzymatic activity. Therefore, we next asked whether cell-surface N-glycan branching was increased on MPC compared to TEC. Mgat5 generates a unique branch point on complex glycosidic structures which can be detected using the reagent phytohemagglutinin-L (PHA-L), a lectin isolated from *Phaseolus vulgaris*. PHA-L is known to bind specifically to extensions of Mgat5-generated N-glycan branch sites (Morgan *et al*. 2004). To assess relative levels of N-glycan branching on MPC and TEC, we adoptively transferred P14 TCR-tg cells into WT mice and infected with LCMV Arm

the following day. Mice were sacrificed and spleens harvested at 8dpi and total splenocytes were stained with PHA-L. We observed a small but statistically significant increase in N-glycan branching on MPC compared to TEC isolated from the same host (Figure III - 1B, 1C). These results are in line with our hypothesis and suggest that Mgat5-modified N-glycans may be selectively limiting the activation of MPC to favour memory development.

Galectin-3 deficiency does not regulate memory transition of CD8 T cells

We have shown that N-glycan branching as measured by PHA-L staining is increased on MPC compared to TEC from the same host at the peak of the effector response. IL-10 signalling during chronic infections has been demonstrated to directly increase expression of the glycosyltransferase Mgat5, and furthermore, IL-10 signalling during contraction of effector CD8 T cells has been shown to be critical for memory CD8 T cell generation (Laidlaw *et al*. 2015; Smith *et al*. 2018). During chronic infections, increased N-glycan branching is not sufficient to reduce CD8 T cell antigen sensitivity, and antigen sensitivity is only reduced as a result of Gal3 binding to extensions of Mgat5-modified N-glycans (Smith *et al*. 2018). Therefore, we asked whether deficiency of Gal3 during CD8 T cell contraction impact CD8 T cell memory generation. If this mechanism is involved in shielding MPC to favour memory development, we anticipate that Gal3 deficiency will reduce the size of the long-lived memory pool generated following acute infection.

To answer this question, we first adoptively transferred P14 TCR-tg cells into congenically mismatched WT mice, and infected the following day with LCMV Arm. At 8dpi, mice were sacrificed, and spleens were harvested. P14 cells were enriched using anti-PE-magnetic separation and were then adoptively transferred into infection-matched recipients which were either WT or *Lgal3*$^{-/-}$, which are deficient in Gal3. This model allows us to specifically interrogate the role of Gal3 binding during CD8 T cell contraction on memory transition, since all P14 cells were activated and expanded within an identical environment. Transferred P14 cells were longitudinally tracked with serial bleeding of hosts to an early memory timepoint of at least 30dpi to observe the contraction of CD8 T cells. We observed no difference in the frequency or number of

total P14 cells transferred to *Lgal3*$^{-/-}$ hosts compared to WT hosts (Figure III - 2A, 2B). Furthermore, there was no difference in the frequency or number of TEC (Figure III - 2C, 2D), or MPC (Figure III - 2E, 2F) P14 cells transferred to *Lgals3*$^{-/-}$ compared to WT hosts at any timepoint during contraction.

In addition to the size and composition of the memory pool generated, we also analyzed the functionality of splenic memory cells. Briefly, total splenocytes were incubated with GP_{33-41} peptide in the presence of brefeldin A and production of effector cytokines IFNγ, TNFα and IL-2 was measured using intracellular cytokine staining. We found no significant difference in the frequency of total P14s producing IFNγ (Figure III - 3A), and additionally saw no difference in the production of IFNγ on a per cell basis, as measured by the geometric mean of fluorescence intensity (gMFI) (Figure 10B, 10C). Additionally, we did not observe any differences in the frequency of IFNγ^{+} cells also expressing TNFα or IL-2 (Figure 10D, 10G), there were also no differences in the production of TNFα or IL-2 on a per cell basis as measured by gMFI (Figure III - 3E, 3F, 3H, 3I). Based on these results, we concluded that although *Mgat5* expression and N-glycan branching as measured by PHA-L staining is elevated in MPC compared to TEC, Gal3 binding during CD8 T cell contraction does not significantly alter the size or functionality of the memory pool formed following acute infection, and therefore Gal3 binding, at least during contraction, does not regulate CD8 T cell memory development.

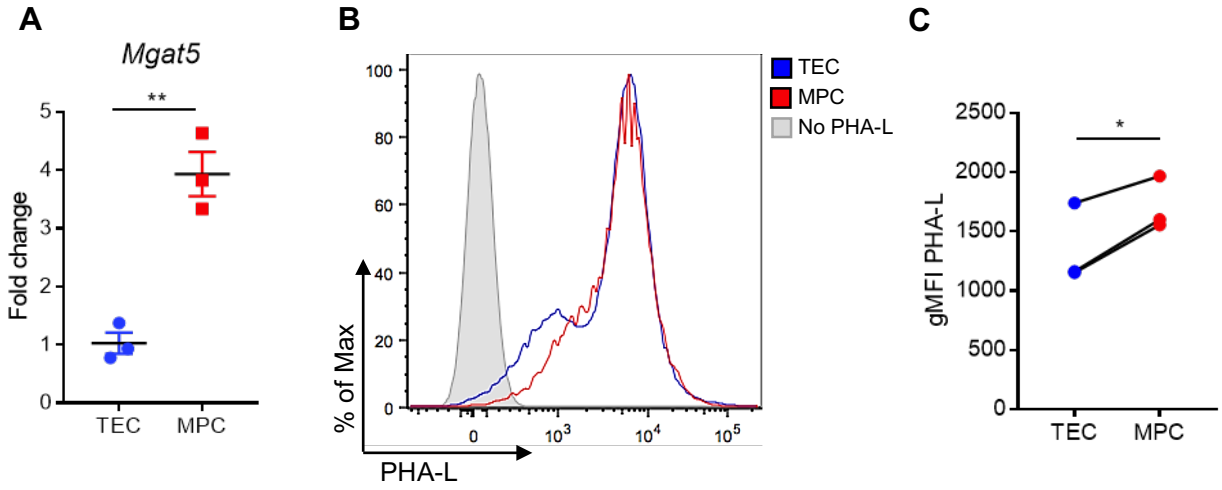

Figure III - 1. *Mgat5* expression and N-glycan branching is higher in MPC compared to TEC effector sub-populations. A) RT-qPCR analysis of *Mgat5* expression in day 8 effector P14 cells comparing MPC to TEC. B) Representative histograms and C) dot plot quantifying the geometric mean fluorescence intensity of PHA-L on TEC and MPC P14 cell subsets at 8dpi. Data (Mean ± SEM) is representative of at least two independent experiments (n=3 per group). P values determined using a two-tailed unpaired Student's t-test (* = $p<0.05$, ** = $p<0.01$).

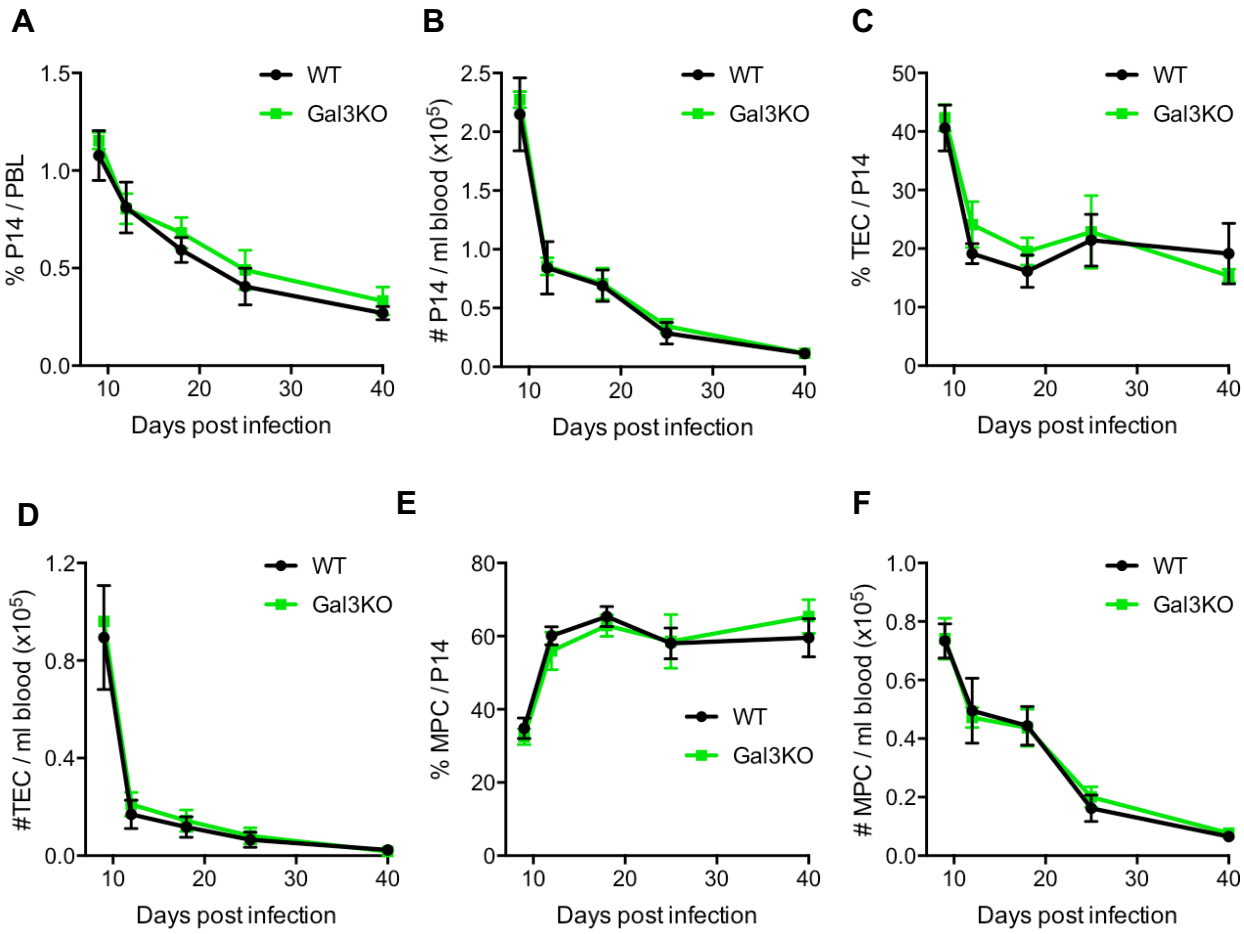

Figure III - 2. Galectin-3 deficiency during CD8 T cell contraction does not impact the size of the memory CD8 T cell pool following acute infection. Comparison of identically activated P14 TCR-tg cells contracting in either WT or *Lgals3*$^{-/-}$ hosts. A) frequency and B) number per ml blood of total P14 cells. C) frequency and D) number per ml blood of P14 TEC cells. E) frequency and F) number per ml blood of P14 MPC cells. Data (Mean ± SEM) is representative of one experiment (n=3 per group). P values determined using a two-tailed unpaired Student's t-test for each time point.

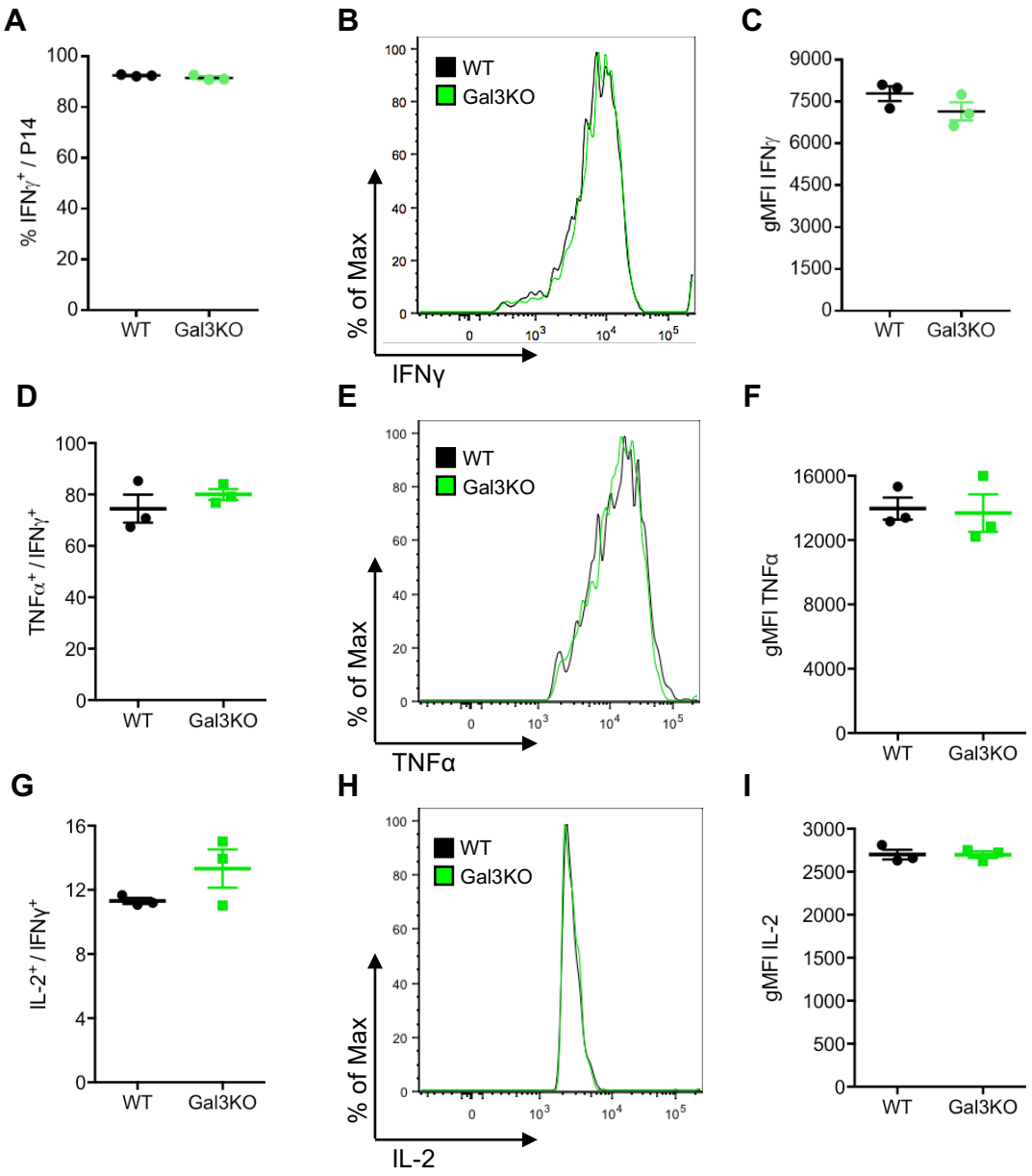

Figure III - 3. Galectin-3 deficiency during CD8 T cell contraction does not impact the functionality of the memory CD8 T cell pool following acute infection. Comparison of identically activated P14 TCR-tg cells contracting in either WT or *Lgals3*$^{-/-}$ hosts. A) frequency of P14 cells expressing IFNγ following stimulation with cognate peptide. B) histogram representative of IFNγ expression and C) dot plot quantification of IFNγ gMFI. Frequency of P14 IFNγ^{+} cells also expressing D) TNFα. E) histogram representative of TNFα expression and F) dot plot quantification of gMFI of TNFα. Frequency of P14 IFNγ^{+} cells also expressing G) IL-2. H) histogram representative of IL-2 expression and I) dot plot quantification of gMFI of IL-2. Data (Mean ± SEM) is representative of one experiment (n=3 per group). P values determined using a two-tailed unpaired Student's t-test.

Discussion

The generation of memory CD8 T cells following the resolution of acute infections provides enhanced host protection against pathogen reinfection. Selection of effector cells which go on to seed the long-lived memory pool is not stochastic, and it has been demonstrated that certain signals such as antigenic stimulation of the TCR and signalling from the inflammatory milieu are key players in the fate decision of effector CD8 T cells. However, the exact mechanisms underlying this critical fate decision remain to be fully elucidated. The memory potential of any given effector cell appears to be balanced against its effector functionality, where signals which enhance effector activation and function in turn reduce the pluripotency of effector cells, reducing their ability to survive contraction and transition to a memory phenotype (Gerlach *et al*. 2010; Kaech and Cui 2012; Stemberger *et al*. 2007a). Herein, we hypothesized that inhibitory and immunoregulatory signalling pathways such as PD-1 and IL-10 are mechanisms by which a fraction of the total pool of effector CD8 T cells may be shielded from activating signals, reducing their activation to favour memory development during CD8 T cell responses to acute infections.

Collectively, we demonstrated that MPC display enhanced expression of PD-1, *Mgat5* and surface levels of N-glycan branching compared to their TEC counterparts. However, PD-1 signalling during CD8 T cell activation and expansion did not result in significant differences in the size or functionality of the memory pool and furthermore, Gal3 binding to surface N-glycan branching during CD8 T cell contraction did not alter the size or functionality of the memory pool formed. It is important to note that our experiments are limited in the times examined: PD-1 signalling during activation and expansion and Gal3 binding during contraction. Our data strongly indicate that neither of these pathways regulate CD8 T cell memory capacity, although it is possible that examination of other time points may offer better insight into the role these pathways play in CD8 T cell memory generation. It is also possible that these pathways do not function alone, but rather are one piece of an integrative network of signals which together regulate CD8 T cell memory generation and that disruption of any signal node of this network is not sufficient to dramatically impact CD8 T cell memory formation. Despite these caveats, our data indicate the presence of underlying differences in the

signals received by TEC and MPC effector subpopulations and identification of these signals may offer crucial insights into the mechanisms which ultimately regulate the fate decision of individual effector CD8 T cells.

It has been demonstrated that PD-1 signalling in CD8 T cells results in SHP-2 dependent dephosphorylation of the CD28 co-stimulatory receptor (Hui *et al*. 2017; Kamphorst *et al*. 2017; Krueger and Rudd 2017). CD28 signalling enhances TCR signal transduction, but also provides additional proliferative and survival signals which are critical for CD8 T cell activation and function (Hui *et al*. 2017; Kamphorst *et al*. 2017; Krueger and Rudd 2017). It is unlikely that inhibition of CD28 co-stimulation is the complete explanation of PD-1 mediated inhibition of CD8 T cell activation. It is important to understand the mechanisms underlying regulation of PD-1 expression as well as PD-1-mediated CD8 T cell inhibition, since these pathways may be important differences underlying the fate decision between TEC and MPC and may contribute to the capacity of each of these populations to form memory CD8 T cells.

PD-1 mediated inhibition of CD8 T cells is known to be sustained in tumour microenvironments where ligands of PD-1 are present but ligands of CD28 are absent (Mizuno *et al*. 2019). Thus, in this context, re-invigoration of exhausted CD8 T cells in response to PD-1 signalling blockade must be occurring in a CD28-independent manner, indicating that PD-1 inhibition is not limited to CD28 signalling. In fact, recent evidence suggests that PD-1 signalling may directly inhibit TCR signals in the absence of CD28 signalling (Mizuno *et al*. 2019). Although PD-1 signalling reduces CD8 T cell activation during acute infections, herein we have demonstrated that PD-1 signalling alone does not regulate formation of memory CD8 T cells. However, expression of PD-1 is known to be regulated by antigenic stimulation, and therefore may be a useful marker of activated T cells and may be therapeutically useful to identify pathologic activation of CD8 T cells. Although it was known that TCR signalling induced expression of PD-1 we have further demonstrated that the magnitude of PD-1 expression, as measured by gMFI, correlates positively with antigen sensitivity and therefore signal strength of CD8 T cells (Ahn *et al*. 2018). These data indicate that PD-1 expression may be useful as a readout of relative TCR signalling and therefore CD8 T cell activation, which may

correlate to disease occurrence or progression, such as has been demonstrated during rheumatoid arthritis (RA) (Cho *et al*. 2012).

In fact, it has been demonstrated that CD8 T cells expressing PD-1 are present at significantly higher frequencies in the blood and synovial fluid (SF) of patients with RA compared to healthy controls (Cho *et al*. 2012; Li *et al*. 2014). Interestingly, increased presence of CD8 T cells in the SF of patients with RA is correlated with improved disease scores, indicating that the presence of these cells may play a vital role in regulating the inflammatory milieu of the SF (Cho *et al*. 2012; Li *et al*. 2014). This may be explained by the decreased expression of cytolytic molecules perforin and granzyme B on PD-1^+ CD8 T cells compared to PD-1^- CD8 T cells. Furthermore, it has been demonstrated that CD8 T cells from the SF of RA patients express more of the immunoregulatory cytokine IL-10 (Cho *et al*. 2012), which may further contribute to regulation of CD8 T cell-mediated damage to the joint tissues. Thus, PD-1 expression on CD8 T cells may be a useful therapeutic marker of disease severity in autoimmune diseases such as RA.

It is important to note that environment greatly impacts both the expression of PD-1 and functionality of CD8 T cells. As such, care must be taken when using CD8 T cell surface marker expression profiles as therapeutic markers. These studies noted dramatic differences in expression of PD-1 and IL-10 in CD8 T cells isolated from the SF and blood of the same RA patients (Cho *et al*. 2012; Li *et al*. 2014). These results indicate that analysis of peripheral CD8 T cells may not offer an accurate representation of disease status, especially if the analyzed CD8 T cells are not exposed to the diseased environment. As such, if CD8 T cell surface expression profiling is to be used as a therapeutic marker of disease, care should be taken to ensure that the CD8 T cells analyzed are comparable to those found within the diseased tissue.

Recent work by Ahn *et al.* demonstrated that PD-1 expression during CD8 T cell activation occurs downstream of TCR signalling, and its expression is dependent on signalling by the transcription factor NFAT (Ahn *et al*. 2018). As such, TCR signalling may be involved in maintaining expression of PD-1 throughout the course of the CD8 T cell response; however, it has also been shown that induction of PD-1 expression during CD8 T cell activation is dependent on Notch1 signalling (Mathieu *et al*. 2013).

Expression of Notch1 has been shown to increase rapidly following CD8 T cell activation, although this expression is transient and is maintained for only 48 hours post infection (Palaga and Minter 2013). Notch1 signalling occurs simultaneously to CD8 T cell activation and inhibition of Notch1 was shown to significantly decrease expression of PD-1 independently of CD8 T cell activation as measured by expression of CD44 (Mathieu *et al*. 2013). Notch1 signalling alone was demonstrated to be insufficient to induce PD-1 expression, and thus it is hypothesized that T cell activation is required to open the chromatin before Notch1 is able to bind, inducing PD-1 expression (Mathieu *et al*. 2013). Thus, PD-1 expression is regulated independently by both Notch and TCR signalling. Our findings that MPC express higher levels of PD-1 than TEC indicate that MPC are receiving stronger signals than their TEC counterparts, and therefore MPC are likely to receive enhanced Notch and TCR signalling compared to TEC.

We have demonstrated that MPC express higher levels of PD-1 compared to TEC and that PD-1 signalling alone does not regulate generation of memory CD8 T cells. Therefore, if differences in PD-1 expression between TEC and MPC are driven by differences in Notch signalling received by these two groups, we might anticipate that pathways both up and downstream of Notch signalling in CD8 T cells may play key roles in regulating CD8 T cell memory development. Notch signalling is a widely conserved regulator of binary fate decisions in many cell types (Mathieu *et al*. 2013; Palaga and Minter 2013). Four Notch receptors exist, although only Notch1 and Notch2 have been reported to be expressed on T cells (Backer *et al*. 2014). Furthermore, there are two classes of ligand which bind to notch receptors, the jagged ligands and the delta-like ligands (Backer *et al*. 2014). The effects of Notch signalling in CD8 T cells have been demonstrated to be dependent on not only which Notch receptor signalling occurs through, but also by the ligand to which Notch binds (Palaga and Minter 2013). Additionally, Fringe proteins Lunatic, Maniac and Radical alter the glycosylation state of the Notch receptor, which in turn alters the affinity of Notch for its various ligands (Palaga and Minter 2013). Ligands of Notch proteins tend to be membrane-bound, which indicates that cell-cell contact may play a role in Notch signalling in CD8 T cells. Therefore, since Notch1-mediated expression of PD-1 occurs rapidly following T cell activation, it is likely that CD8 T cells receive Notch1 signalling from contact with APC

during activation. Thus, it is possible that TEC and MPC progenitors receive different signals during activating interactions with APC and that differences in these signals received ultimately influences their capacity to form memory.

As such, it is pertinent to know whether APCs express Notch ligands following activation. In fact, activation of APCs in response to *in vivo* infection, activation with LPS or treatment with the RNA analog R-848, which is a mimic of RNA viruses, have all been demonstrated to increase expression of Notch ligands Delta-like ligand 1 (Dll1) and Jagged 1 (Backer *et al*. 2014). Activated APCs secrete type I IFNs and signalling by type I IFNs in turn induces Notch1 upregulation in CD8 T cells in an mTORC1-dependent manner (Backer *et al*. 2014). Induction of Notch1 expression has also been demonstrated to be enhanced by T-bet, antigen and IL-2 signalling (Backer *et al*. 2014).

Notch signalling in CD8 T cells has been shown to be critical for acquisition of CD8 T cell effector functions, and as such, inhibition of Notch signalling has been demonstrated to regulate both proliferation and IFNγ production in activated CD8 T cells (De Sousa *et al*. 2019; Palaga *et al*. 2003). It has also been demonstrated that expression of approximately 40% of the TEC-specific transcriptome is dependent on notch signalling and a nearly equivalent fraction of the MPC-specific transcriptome is upregulated in the absence of Notch signalling, indicating that Notch signalling actively promotes expression of TEC-associated genes while repressing MPC-associated genes (Backer *et al*. 2014). In fact, conditional knock out of both Notch1 and Notch2 on CD8 T cells results in a complete abrogation of the TEC effector subpopulation (Backer *et al*. 2014). These data indicate that differences between TEC and MPC interactions with APC have the potential to influence CD8 T cell effector fate.

CD8 T cell activation occurs following interaction with an APC in the T cell zone of lymphoid organs such as lymph nodes (Jung *et al*. 2010). It has previously been demonstrated that TEC and MPC effector subpopulations home to different niches in the spleen during and following resolution of infection; MPC are found primarily within the WP of the spleen, and TEC primarily within the RP (Jung *et al*. 2010). BLIMP-1 and T-bet are transcription factors which are critical drivers of TEC function and differentiation, and both have been demonstrated to selectively inhibit chemotaxis of effector CD8 T cells into the WP, where MPC have been shown to mature (Jung *et al*.

2010). BLIMP-1 and T-bet have also been shown to drive expression of receptors promoting chemotaxis of CD8 T cells to RP as well as those involved in targeting of effector cells to peripheral sites of infection (Jung *et al*. 2010). Furthermore, it has been demonstrated that CD8 T cells lacking expression of both Notch1 and Notch2, which do not generate any TEC, preferentially home to tissues which are inaccessible to blood-borne antibodies, such as WP of lymphoid tissues (Backer *et al*. 2014). Therefore, Notch signalling drives expression of transcription factors critical for acquisition of TEC function while simultaneously promoting differential homing of TEC and MPC within lymphoid tissues. This indicates a potential role for signals found in the WP in regulating the development of memory CD8 T cells.

Additionally, homing of CD8 T cells is may be influenced by glycosylation of tissue homing receptors and ligands. Very few mammalian proteins lack glycosylation and it has been demonstrated that receptors involved in tissue homing and trafficking, such as those involved in peripheral lymphocyte rolling and tethering are particularly influenced by glycosylation (Pereira *et al*. 2018). We observed enhanced expression of the glycosyltransferase *Mgat5* as well as increases in Mgat5-mediated N-glycan branching on MPC compared to TEC. It is possible that the differences we observed are representative of global changes in N-glycan branching which ultimately regulate this fate-specific tissue homing. Our observation that MPC had enhanced N-glycan branching compared to TEC may offer insights into intrinsic differences in TEC and MPC tissue homing. This may implicate N-glycan branching on CD8 T cells as a mechanism of tissue homing separate from the regulation of CD8 T cell antigen sensitivity observed in the context of chronic infection (Smith *et al*. 2018). Further investigation of N-glycan branching, global glycosylation and surface glycan modifications may offer further insights into signalling and localization differences between TEC and MPC CD8 T cells which may regulate their capacity to generate long-lived memory cells.

Furthermore, differences in glycosylation between TEC and MPC may indicate that binding of glycoproteins by carbohydrate-binding proteins, such as lectins and galectins are fundamentally different between these two populations. Previous work has shown that Gal3 binding to N-glycan branching on CD8 T cells reduces CD8 T cell antigen

sensitivity during chronic infections (Smith *et al*. 2018). We demonstrated that Gal3 binding during CD8 T cell contraction does not regulate memory generation of CD8 T cells; however, we did not investigate lectin or galectin-mediated regulation at other timepoints, such as during CD8 T cell activation or expansion, and we did not evaluate binding of CD8 T cells by other carbohydrate-binding proteins.

Galectins are a family of soluble and ubiquitously expressed proteins with carbohydrate-binding domains. Most galectins possess only one binding site, but many are multivalent (Boscher *et al*. 2011). Gal3 is the only chimera-type mammalian galectin, and forms unique multivalent homo-pentameric quaternary structures (Smith *et al*. 2018). We have shown Gal3 binding during CD8 T cell contraction does not regulate memory formation. However, Gal3 binding to CD8 T cells at other time points such as during activation and expansion may play a role in regulating CD8 T cell activation, function and memory transition (Boscher *et al*. 2011; Fortuna-Costa *et al*. 2014). Furthermore, other multivalent galectins may be involved in regulating the activation and memory transition of effector CD8 T cells during responses to infection. Tandem-repeat galectins possess two carbohydrate recognition domains and this class of galectins is comprised of galectins 4, 6, 8, 9 and 12, and although all contain two binding sites, these are not necessarily identical (Boscher *et al*. 2011). Tandem-repeat galectins may play a role in mediating interactions between cell-surface receptors, or even interactions at an intercellular scale and as such, these should be evaluated for a potential role in mediating CD8 T cell memory development. In addition to galectin binding, mechanisms underlying differences in global glycosylation should be evaluated.

It has been demonstrated that effector and memory CD8 T cells utilize different metabolic profiles, with effector cells relying most heavily upon aerobic glycolysis for ATP production and memory cells relying on fatty acid oxidation (Buck *et al*. 2016). Furthermore, effector and memory CD8 T cells have been demonstrated to possess different mitochondrial structures associated with these metabolic profiles (Buck *et al*. 2016). The metabolic profile and fuel choice of effector CD8 T cells is known to be associated with effector function, and has been demonstrated to impact CD8 T cell differentiation (Buck *et al*. 2016; Buck *et al*. 2015; van der Windt and Pearce 2012). Differences in metabolic profiles and fuel choice will ultimately skew the nutrients and

macromolecule precursors available within individual cells, which may impact their proliferation, function and differentiation. In fact, metabolic switching from oxidative phosphorylation to aerobic glycolysis in CD4 T cells has been shown to be a key regulator of CD4 fate decision through starvation of N-glycan branching pathways (Laidlaw *et al*. 2015). As such, analysis of global glycosylation patterns, metabolic profiles and binding of N-glycans by tandem-repeat galectins may offer insights into the mechanisms which regulate TEC and MPC diversification.

Since glycosylation is known to influence the localization of CD8 T cells, the environment to which TEC and MPC cells localize during immune responses may also influence the differentiation and memory potential of CD8 T cells. The architecture of lymphoid tissues is highly complex and contains a wide variety of both immune and stromal cell types. As such, differences in signals received by TEC and MPC may not originate exclusively from interactions with APC, but through zone-specific signals provided by stromal cells which are specific to either RP or WP. The WP is home to fibroblastic reticular cells which provide critical signals for the activation and function of CD8 T cells (Yu *et al*. 2017). Fibroblastic reticular cells are key producers of IL-7 (Jung *et al*. 2010), which is a critical signal for the long-term maintenance of naïve and memory CD8 T cells (Kaech *et al*. 2003). Expression of the high affinity chain of the IL-7 receptor, CD127, is well known to be a requirement for development of long-lived memory CD8 T cells. However, expression of CD127 alone is not sufficient to force memory development of CD8 T cells, and abundance of IL-7 has been demonstrated to be the limiting factor in CD127-driven memory formation (Hand *et al*. 2007). Thus, MPCs which preferentially home to the WP of lymphoid tissues may have improved access to IL-7 signalling and therefore preferentially be maintained to memory timepoints. Furthermore, fibroblastic reticular cells have been demonstrated to actively repress CD8 T cell activation and proliferation through secretion of nitric oxide (NO) and prostaglandin E_2 (PGE_2) (Yu *et al*. 2017). As such, signals received from the stromal cells which populate the WP of lymphoid tissues may play a key role in regulating CD8 T cell memory formation.

Fibroblastic reticular cell signalling in the white pulp has been demonstrated to regulate the activation and proliferation of CD8 T cells during responses to infection.

Fibroblastic reticular cells are stromal cells which are primarily located within the T cell zone of lymphoid tissues and have been demonstrated to potently raise the activation threshold of CD8 T cells (Yu *et al*. 2017). *In vitro* activation of CD8 T cells cultured in the presence of fibroblastic reticular cells showed significantly reduced expression of activation markers CD69 and CD44, and additionally had reduced down-regulation of CD62L, a lymphoid tissue homing marker which is expressed on naïve and memory cells but not on activated effector CD8 T cells (Yu *et al*. 2017). Cells activated in the presence of fibroblastic reticular cells also had decreased production of effector cytokines IFNγ and TNFα, and required 100 fold higher concentration of antigen to reach equivalent phosphorylation of Zap70 compared to cells activated without fibroblastic reticular cells (Yu *et al*. 2017). These data indicate that homing of MPC to the WP of lymphoid tissues may shield these MPC from activating and inflammatory signals present during infection, thereby favouring memory development.

Interestingly, our findings indicate that MPC are more sensitive to their cognate antigen compared to TEC. This contradicts the significant fibroblastic reticular cell-mediated inhibition of TCR signalling capacity. It is possible that MPC compensate for this inhibition by increasing expression of the TCR and associated downstream signalling molecules which may result in enhanced antigen sensitivity of MPC compared to TEC following homogenization of the splenic tissue, which eliminates spatial segregation of TEC and MPC populations. This may result in a relative increase in antigen sensitivity for MPC compared to TEC upon elimination of fibroblastic reticular cell-mediated inhibition. It is also possible that TEC and MPC populations are exposed to different levels of inflammatory cytokines during immune responses due to different accessibility of blood-borne molecules to RP and WP respectively (Backer *et al*. 2014; Richer *et al*. 2013). Inflammatory signals have been demonstrated to influence the antigen sensitivity of CD8 T cells and this is another potential mechanism that may describe the differences we observed between the antigen sensitivities of TEC and MPC isolated from the same host during infection.

Though residence of MPC in WP as a mechanism of preferential shielding is an attractive model of CD8 T cell diversification, it fails to resolve our findings that MPC express more PD-1 and are more sensitive to their cognate antigen in comparison to

TEC. Seemingly even more contradicting is the idea that Notch1 signalling drives both TEC differentiation and PD-1 expression. If this is true, we would expect expression of PD-1 on TEC to be higher than on MPC, however our data show the opposite, that MPC express higher levels of PD-1 than do TEC. These data can be resolved through examination of T-bet, a transcription factor which drives acquisition of TEC identity.

T-bet expression is induced in CD8 T cells in response to type I IFN signalling, and its expression has been shown to enhance expression of Notch1, which is also expressed in CD8 T cells in response to type I IFN signalling (Backer *et al*. 2014). In addition to driving the acquisition of TEC function, T-bet has also been demonstrated to repress expression of PD-1 (Kao *et al*. 2011). Furthermore, though PD-1 expression during CD8 T cell activation can be attributed to Notch1 signalling, sustained expression is likely due to TCR-mediated NFAT signalling, since Notch1 expression occurs only transiently during the first 48 hours of infection (Ahn *et al*. 2018; Mathieu *et al*. 2013). This corresponds with our observation that at 8dpi, which is the peak of effector response, NP396-specific CD8 T cells express more PD-1 than GP33- specific cells, which have a relatively lower sensitivity for their cognate antigen. Thus, enhanced IFN signalling in TEC compared to MPC may drive expression of T-bet, which represses expression of PD-1. Therefore, the relatively increased expression of PD-1 we observed on MPC compared to TEC may be a result of differential exposure to type I IFN signalling between these two populations.

Signalling by type I IFNs have also been demonstrated to greatly influence many aspects of CD8 T cell activation, function and differentiation. Thus, differences in type I IFN signalling received by TEC and MPC progenitors may alter their capacity to form memory CD8 T cells. Type I IFN signalling, along with IL-12 signalling during acute infections have both been demonstrated to enhance expression of CD25, the high affinity chain of the IL-2 receptor, on CD8 T cells (Starbeck-Miller *et al*. 2014). Subsequent IL-2 signalling during effector expansion was shown to prolong cell division and was critical for optimal accumulation of effector CD8 T cells (Starbeck-Miller *et al*. 2014). Furthermore, type I IFN signalling has been demonstrated to increase antigen sensitivity of CD8 T cells (Richer *et al*. 2013), which may further enhance their TCR signalling. It is important to note that increasing TCR signalling also results in enhanced

proliferation of CD8 T cells, and TCR and IL-2 signals together synergize to enhance expression of Notch1 in CD8 T cells (Backer *et al*. 2014). Together, these data suggest a complex feed-forward mechanism of TEC differentiation that is ultimately driven by Notch1-mediated integration of both antigenic and inflammatory signals. As such, our findings that MPC express higher levels of PD-1 than TEC may ultimately be a result of differences in initial IFN signals received. Differences in IFN signalling are likely to synergize with other signals, such as TCR and Notch signalling to direct the fate of individual effector CD8 T cells. Further investigations into strength of signals received by TEC and MPC progenitors and the effects of these signalling pathways on lymphoid tissue localization as well as relative capacity to seed the long-lived memory pool should be pursued.

During responses to chronic infections, the effector CD8 T cell pool becomes dysfunctional and hyporesponsive, a state which is known as CD8 T cell exhaustion. Similar to responses to acute infections, the effector pool during chronic infections undergoes a phase of contraction and forms a pool of memory-like cells (Wherry 2011). However, these memory cells retain an exhausted phenotype and are incapable of undergoing cytokine-dependent homeostatic proliferation, which is a key hallmark of memory CD8 T cells (Wherry 2011). Instead, these memory-like cells are solely dependent on antigen stimulation for long-term survival (Wherry 2011). While it has been demonstrated that chronic antigen stimulation alone is sufficient to drive the development of CD8 T cell exhaustion, it is possible that dysfunctional memory cells generated during chronic infections are due in part to disruption of TEC and MPC spatial segregation within the lymphatic tissue (Bucks *et al*. 2009). Lymphoid organs such as the lymph nodes have been demonstrated to undergo an architectural collapse during responses to chronic infections, and these collapses have been demonstrated to significantly modify the tissue architecture as well as modulating signals received by associated immune cells (van Grevenynghe *et al*. 2008).

Therefore, our findings that TEC and MPC possess differences in expression of PD-1 and *Mgat5*, and display differences in Mgat5-mediated N-glycan branching and antigen sensitivity indicate that there are critical differences in the signals these populations receive, which may ultimately regulate the capacity of individual effector

CD8 T cells to form memory. Review of the literature suggests that segregation of TEC and MPC effector subpopulations within the lymphoid tissues may occur as a result of differences in type I IFN signalling (Jung *et al*. 2010; Yu *et al*. 2017). Future studies should aim to confirm TEC and MPC localization to the RP and WP of lymphoid organs, respectively. This may be done using microscopy or histological staining methods. Furthermore, expression and function of other inhibitory immune checkpoint receptors such as LAG3 and CTLA-4 should be evaluated in the context of acute infection, as these pathways are independent of and may synergize with PD-1 signalling to regulate memory transition of CD8 T cells (Dyck and Mills 2017).

Differential homing of TEC and MPC within lymphoid tissues has also been demonstrated to be influenced by both type I IFN and Notch1 signalling (Backer *et al*. 2014; Palaga *et al*. 2003; Palaga and Minter 2013). Both IFN and Notch1 signalling are known to drive acquisition of TEC identity in addition to modulating tissue localization. In fact, enhanced Notch 1 signalling has been demonstrated to downregulate receptors associated with WP chemotaxis, promoting movement of TEC into RP and the periphery (Backer *et al*. 2014; Palaga *et al*. 2003). Additionally, differences in metabolic profiles, fuel choice, global glycosylation and binding of carbohydrate-binding proteins other than Gal3, such as tandem-repeat galectins, may further influence tissue localization and contribute to CD8 T cell memory formation following responses to acute infections (Buck *et al*. 2016; van der Windt and Pearce 2012). Further investigations should quantify global differences in glycosylation between TEC and MPC effector subpopulations, for example, using lectin staining. Furthermore, differences in galectin binding should be analyzed using flow cytometric analysis or microscopy techniques.

Fibroblastic reticular cells are stromal cells within the WP of lymphoid tissues that have been shown to significantly increase the activation threshold of CD8 T cells (Yu *et al*. 2017), and thus may shield the MPC which preferentially home to the WP by reducing their activation and preserving their pluripotency and memory potential (Jung *et al*. 2010). Although we did not observe PD-1 signalling and N-glycan branching to directly regulate CD8 T cell memory formation, differences in expression of these pathways between MPC and TEC may be considered as read outs of strength of signals received through the TCR, Notch receptors or IFNAR, and integration of these

signals may ultimately regulate the fate of effector CD8 T cells during responses to acute infections. Future studies should aim to quantify differences in expression of TCR, Notch1, Type I IFN receptor (IFNAR) and phosphorylation of downstream signalling molecules, such as Zap70 as a readout of TCR signal strength, between TEC and MPC. These may be quantified using RT-qPCR, western blot and flow cytometric analysis. Furthermore, interactions between CD8 T cells and APCs or stromal cells within lymphoid tissues should also be assessed and quantified using microscopy and live-imaging techniques. Cumulatively, these experiments would provide critical insight into the mechanisms of localization, strength of signal and cell-cell interactions of CD8 T cells during responses to acute infections.

Chapter IV Conclusions and future directions

Inhibitory and immunoregulatory pathways play a key role in regulating the activation of CD8 T cells during responses to infections (Ahn *et al*. 2018). During chronic infections, increased stimulation of immunoregulatory pathways, such as PD-1 and IL-10 signalling, contributes to the development of CD8 T cell exhaustion (Brooks *et al*. 2008; Smith *et al*. 2018). Furthermore, PD-1 and IL-10 signalling during acute infections have been shown to play a critical role in limiting immune-mediated damage to host tissue and loss of inhibitory signalling has been shown to lead to the development of spontaneous immunopathologies (Ahn *et al*. 2018; Rojas *et al*. 2017). Activating and inflammatory signals provoke activation, expansion and differentiation of responding CD8 T cells. It has been suggested that the accumulation of activating and inflammatory signals throughout the course of infection guides the fate decision of effector CD8 T cells along a gradient, with higher accumulation driving a more terminally differentiated TEC-like phenotype in direct opposition to memory potential (Kaech and Cui 2012). Inhibitory pathways work in opposition to activating ones, dampening the activation of CD8 T cells. The direct contribution of inhibitory and immunoregulatory signalling pathways in regulating memory CD8 T cell transition remained a significant gap in our understanding of immunoregulatory signalling during responses to acute infections.

Herein we demonstrate that although PD-1 and *Mgat5*-mediated N-glycan branching pathways are increased in MPC compared to TEC effector sub-populations; these pathways do not play a role in mediating CD8 T cell memory development in the context of acute infections. It is possible that our interrogation of the role of PD-1 signalling in CD8 T cell memory development overlooked collaborative inhibitory signalling of PD-1 and other inhibitory receptors such as LAG3 or CTLA-4. Future studies should evaluate the role of other inhibitory signalling pathways in addition to PD-1 signalling for their impacts on regulating CD8 T cell memory generation in the context of acute infections. Specifically, expression of immune checkpoint receptors such as these should be characterized on TEC and MPC subpopulations, and their role in CD8 T cell memory progression can be interrogated as we described above. Furthermore, the transcriptional programmes which control expression of these molecules should be

examined in detail to identify the signalling pathways which regulate individual immune checkpoint blockades to identify pathways with potential therapeutic value in the development of CD8 T cell therapies.

We showed that the PD-1 receptor, *Mgat5* and N-glycan branching as measured by PHA-L staining were elevated in MPC compared to TEC. Furthermore, we demonstrated that endogenous CD8 T cell populations specific for the $NP_{396-404}$ epitope of LCMV, which are more sensitive to their cognate antigen than endogenous cells specific for the GP_{33-41} epitope of LCMV also express higher levels of PD-1. These data suggest that magnitude of PD-1 expression during CD8 T cell responses to acute infections may pertain to the sensitivity of responding cells for their cognate antigen and may correlate with their relative level of activation. Therapeutically, presence of PD-1^+ CD8 T cells may be a valuable marker of CD8 T cell activation and may be therapeutically valuable for identifying or tracking the progress of autoimmune diseases such as rheumatoid arthritis.

Gal3 binding to Mgat5-generated N-glycan branching has been demonstrated to decrease antigen sensitivity of CD8 T cells in the context of chronic infections (Smith *et al*. 2018). Our evaluation of this mechanism in regulating CD8 T cell memory development was limited to the period of CD8 T cell contraction, and future studies should evaluate the role of binding by Gal3 during CD8 T cell activation and expansion. This could be accomplished using adoptive transfer models as described above, which compare CD8 T cell memory generation in WT and *Lgals3*$^{-/-}$ hosts. Additionally, although Gal3 is the only chimera-type galectin found in mammals, other galectins, such as multivalent tandem-repeat galectins should be examined for a potential role in the regulation of CD8 T cell activation (Boscher *et al*. 2011). Differences in metabolic profiles, fuel choice, and global differences in glycosylation should also be analyzed. This could be interrogated using knock-out mouse models, treatment with galectin binding inhibitors to abrogate galectin binding to N-glycan branching, and lectin staining to interrogate galectin binding. Treatments with L-lactose have been shown to effectively revitalise antigen sensitivity of CD8 T cells during chronic infection, although more stable lactose analogs with longer physiological half-lives are a preferable treatment option (Smith *et al*. 2018).

TEC and MPC effector sub-populations have been demonstrated to occupy different spaces within the lymphatic tissue during and following resolution of infections (Jung *et al*. 2010). Our findings that MPC express higher amounts of PD-1 than do TEC may be explained by preferential MPC localization to the white pulp of lymphoid tissues, where they receive signals involved in initiating and maintaining expression of PD-1, but not signals which drive TEC differentiation while decreased expression of PD-1. Stromal cells in the WP of lymphoid tissues may provide the inhibitory signals required to reduce activation of MPC to favour memory development during responses to acute infections (Yu *et al*. 2017). Additionally, WP stromal cells may provide the signals critical for long-term survival of memory CD8 T cells (Jung *et al*. 2010). Future studies should aim to confirm this localization of TEC and MPC within lymph tissues. Additionally, interaction of CD8 T cells with APC and stromal cells should be characterized using live imaging or microscopy techniques. Furthermore, the impact of glycosylation in regulating differential homing of TEC and MPC CD8 T cells within lymphoid tissues should be investigated, as this could be a potential mechanism whereby to improve the impact of therapies which aim to improve CD8 T cell memory formation, such as vaccinations.

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