Dichotomy of TNF Family Ligands Expression on Classical Dendritic Cells and Monocyte-derived Antigen Presenting Cells During Viral Infection

By

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Abstract

Tumor necrosis factor receptor (TNFR) superfamily members, including 4-1BB, OX40, GITR and CD27 contribute to T cell survival during viral infection. However, the cell types that provide the ligands have not been fully investigated. Here we use multiparameter flow cytometry to follow the induction of TNF family ligands on APC subsets during lymphoytic choriomeningitis (LCMV) clone 13 infection. We found that at day 2 post infection, type I interferon induced TNFSF ligands GITRL, 4-1BBL, OX40L, and CD70 predominantly on monocyte-derived APCs, whereas MHC II and B7 family ligands are highest on classical dendritic cells (cDCs) in the spleen. These findings shed light on the expression of TNF family ligands by different APC and suggest that the temporal and spatial regulation of these ligands may be important in determining immune control or pathology during viral infections.

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List of Abbreviations

4-1BB/L	TNFRSF/TNFSF member 9
α-DG	α-dystroglycan
Ag	Antigen
AKT	Protein kinase B
APC	Antigen presenting cells
Batf3	Basic leucine zipper transcription factor ATK-like 3
Bcl-xL	B cell lymphoma -xL
Bim	Bcl-2-like protein 11
Blimp-1	B lymphocyte-induced maturation protein 1
BTLA	B- and T-lymphocyte attenuator
CCR/L	C-C Chemokine receptor/ligand
CD	Cluster of differentiation
cDC	Conventional dendritic cells
CDP	Common DC precursor
cIAP	Cellular inhibitors of apoptosis
CSF1	Colony-stimulating factor 1
CSF1R	Colony-stimulating factor 1 receptor
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte Antigen-4
CX3CR/L1	CX3C-chemokine receptor/ligand 1
DCs	Dendritic cells
DD	Death domain
ERK	Extracellular signal-regulated kinase
F260L	Phenylalanine to leucine at position 260
Fcγ/εR1	Fragment crystallization region gamma/epsilon receptor-1
Flt3	Fms like tyrosine kinase 3
GITR/L	Glucocorticoid-induced TNFR-related protein / ligand
GP	Glycoprotein
HIV	Human immunodeficiency virus
HSC	Haematopoietic stem cell
HSV-1	Herpes simplex virus 1
ICOS	Inducible T cell costimulator
IFNGR	IFNγ receptor
IFN-I/R	Interferon α/β / receptor (aka type I IFN)
IFNγ	Interferon γ (aka type II IFN)
Ig	Immunoglobulin
IgSF	Immunoglobulin superfamily
IL	Interleukin
iregAPC	Immune regulatory antigen presenting cells

iregDC	Inducible regulatory DCs
IRF	IFN regulated factor
IRSE	IFN-stimulated response elements
ISGF	IFN-stimulated gene factor
ISGs	IFN-stimulated genes
JAK	Janus kinase
JNK	Jun-N-terminal kinase
K1079Q	Lysine to glutamine mutation at position 1079
LAG-3	Lymphocyte activation gene-3
LCMV	Lymphocytic choriomeningitis
LCMV Arm	Lymphocytic choriomeningitis armstrong strain
LCMV13	Lymphocytic choriomeningitis clone 13 strain
LNs	Lymph nodes
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinases
MDA5	Melanoma differentiation-associated protein 5
MerTK	Tyrosine protein kinase MER
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
moDC	Monocyte-derived DC
MPS	Mononuclear phagocyte system
mRNA	Messenger ribonucleic acid
mTORC1	Mammalian target of rapamycin
N176D	Asparagine to aspartic acid at position 176
NF-κB	Nuclear factor κB
NK	Natural killer Cell
NKT	Natural killer T Cell
NP	Neucleoprotein
OX40/L	TNFRSF/TNFSF member 4
p.i.	Post infection
PD-1/L1	Programmed cell death protein-1 / -ligand 1
pDC	Plasmacytoid DCs
PKCθ	Protein kinase C theta
PMN	Polymorphonuclear
PR8	Influenza A/Puerto Rico/8/1934 H1N1
pSTAT1	Phospho-STAT1
Rac2	Ras-related C3 botulinum toxin substrate 2
RdRp	RNA dependent polymerase
SAPK	Stress-activated protein kinase
SSC	Side-scattered light
STAT	Signal transducer and activator of transcription

TAP	Transporter associated with antigen processing
T-bet	(aka T box transcription factor 21)
TCID50	50% tissue culture infectious dose
TCR	T cell receptor
Tfh	T follicular helper cells
TG MF	Thioglycolate-elicited peritoneal macrophages
TGF	Transforming growth factor
Th1, 2, 17	T helper cell type 1, 2, 17
Tim-3	T cell immunoglobulin and mucin domain-containing protein 3
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNF/TNFSF	Tumor necrosis factor superfamily of receptors ligands
TNFR/TNFRSF	Tumor necrosis factor superfamily of receptors
TRAF	TNF receptor-associated factor
Treg	Regulatory T cells
TYK	Tyrosine kinase
zbtb46	Zinc finger transcription factor

Chapter 1

Introduction

This thesis focuses on investigating the expression of TNF family ligands on antigen presenting cell subsets over the course of a chronic viral infection. In this introduction, I will first introduce the viral model. I will then discuss T cell costimulatory molecules, the TNF superfamily ligands and antigen presenting cell subsets.

Part 1 LCMV:

1.1.1 LCMV infection in mice

Lymphocytic choriomeningitis (LCMV) infection is a popular mouse model to study acute and persistent viral infection. As LCMV is a natural rodent pathogen, it allows the study of virus-immune system interactions in the natural host. The non-lytic life cycle allows easy separation between viral pathological effect and host immunity (Oldstone, 2007). The shared major histocompatibility complex (MHC) I- and II-restricted epitopes between the acute Armstrong strain (LCMV Arm) and the persistent clone 13 strain (LCMV13) of LCMV make it possible for researchers to simultaneously investigate LCMV-specific T cell responses in both an acute and a chronic infection, respectively (Zhou *et al.*, 2012; Oldstone, 2013). Additionally, the similarities between adaptive immune responses to LCMV13 in mouse and human immunodeficiency virus (HIV) in human have made LCMV13 a cost-effective model to study parameters that are conserved across species (Wilson and Brooks, 2010; Youngblood *et al.*, 2012). Currently, studies conducted with LCMV13 have built the foundation of our understanding on persistent viral infection by characterizing host and pathogen dynamics and mechanisms of T cell exhaustion (Klenerman and Hill, 2005; Wilson and Brooks, 2010; Zehn and Wherry, 2015).

1.1.2. Biology of LCMV

As a member of the *Arenaviridae* family, LCMV is a negative strand RNA virus that has bi-segmented RNA genome. Each segment of the genome (L, 7.3kb; S, 3.5kb) encodes 2 viral gene products by using an ambisense coding strategy (De La Torre, 2009). The S RNA encodes the 63 kDa viral neucleoprotein (NP) and the 75 kDa precursoar of glycoprotein (GP), GPC. The

L RNA encodes the 200 kDa viral RNA dependent polymerase (RdRp) and a 11 kDa RING finger-containing Z protein (DeLa Torre, 2009). The GPC is post-translationally processed by cellular protease S1P into GP-1, GP-2, and SSP (Beyer *et al.*, 2003), where GP-1 and GP-2 are trafficked to the cell surface by SSP to form the spikes of the virion. The equally spaced spikes on the viral lipid envelope, especially GP-1, are responsible for recognition of host cellular receptor α-dystroglycan (α-DG) and subsequent viral entry via receptor-mediated endocytosis (Cao *et al.*, 1998; Eschli *et al.*, 2006). Z protein is important for the inhibition of RNA synthesis by the RdRp. In addition, Z protein serves as matrix protein for viral particle assembly and budding, making Z protein the main driver of viral exit (Perez *et al*., 2003; DeLa Torre, 2009).

LCMV Arm was the first isolated strain of LCMV from patients with aseptic encephalitis in 1933 by Armstrong and his colleagues. LCMV Arm in adult immunocompetent mice induces robust cytotoxic T lymphocyte (CTL) response that results in the resolution of an acute infection typically lasting for 2 weeks. LCMV13 was later isolated using serial passages of splenic homogenates from mice infected with LCMV Arm (Ahmed R *et al*., 1984). LCMV13 differs from LCMV Arm by 6 nucleotides and 3 amino acids (Ng *et al*., 2011; Sullivan *et al.*, 2011) and shows persistence with higher viral titres for over 2 months (Ahmed R *et al*., 1984). Mutation from phenylalanine to leucine at position 260 (F260L) on GP-1 was the most critical for the persistence of LCMV13 as it enhances the binding affinity to α-DG by 2-2.5 $\log_{10}$-fold thus enhances viral entry (Sullivan *et al.*, 2011). The lysine to glutamine mutation at position 1079 (K1079Q) on RdRp also contributes somewhat to the persistence of LCMV13 by increasing replicative capacity (Sevilla *et al.*, 2000; Bergthaler *et al.*, 2010; Ng *et al.*, 2011). Lastly, mutation of asparagine to aspartic acid at position 176 (N176D) on GP-1 doesn't appear to contribute to persistence (Sullivan *et al.*, 2011).

1.1.3 Preferential infection of APC for establishment of persistence

LCMV13's mechanism of persistence is a multifaceted process that stems from its preferential infection of antigen presenting cells (APC): $CD11b^{+}CD8\alpha^{-}$ and $CD11b^{-}CD8\alpha^{+}$ conventional dendritic cells (cDC) (Sevilla *et al.*, 2000; Ng and Oldstone, 2012), macrophages (Matloubian *et al.*, 1993), and plasmacytoid DCs (pDC) (Bergthaler *et al.*, 2010; MacAl *et al.*, 2012). pDCs are the first APC subset to be infected by LCMV13 within 12-24 hours of infection

(Bergthaler *et al.*, 2010; MacAl *et al.*, 2012), allowing virus to access and multiply in the white pulp and marginal zone of spleen. Notably, pDCs are well known to participate in establishing host type I interferon (IFN-I) antiviral responses in the initial hours of infection (Zuniga *et al.*, 2008; MacAl *et al.*, 2012; Y.Wang, Swiecki, *et al.*, 2012). From the splenic white pulp, LCMV13 with its higher affinity F260L mutation in GP-1 readily infects splenic dendritic cells (DCs) that are the main expressers of α-DG in the spleen (Sevilla *et al.*, 2000, 2003). Consequently, LCMV13 prevents splenic DCs from carrying out their role of antigen presentation to CD4 and CD8 T cells (Merad *et al.*, 2013; Guilliams *et al.*, 2014; Murphy *et al.*, 2016). In contrast to LCMV Arm, infection of cells with LCMV13 has also been reported to reduce the expression of MHC-I, MHC-II, CD40, CD80, CD86, and GITRL (Sevilla *et al.*, 2000, 2004; Clouthier *et al*., 2014), thus hindering the stimulatory capacity of DCs. By day 5 post infection (p.i.), nearly all APCs in the spleen are infected with the virus (MacAl *et al.*, 2012). As a result of unchecked replication in the splenic white pulp, LCMV13 infection typically leads to the destruction of the microarchitecture of secondary lymphoid organ that is important for innate and adaptive cell crosstalk (Muller *et al.*, 2002; Mueller and Germain, 2009). In essence, by directly infecting and disrupting APCs, LCMV13 is able to interfere with the host IFN-I response and antigen presentation to allow its replication in splenic white pulp. Ultimately, the overwhelming antigen (Ag) load, impaired DC function, and prolonged inflammatory state contribute to reduction in functional T cell responses, which rapidly exhausts both CD4 and CD8 T cells.

1.1.4 T cell exhaustion during LCMV13 infection

During an persistent infection such as LCMV13 of mice or HIV of human, the host immune system and the virus compete to establish control. Without effective means to clear Ag, the host CD4 and CD8 T cells are unable to form functional memory. In order to prevent the occurrence of immunopathology, T cells become exhausted at the cost of persistent infection (Mueller *et al.*, 2007, 2010; Yi *et al*., 2010).

CD8 T cell exhaustion happens in a hierarchical manner. First, T cells lose the ability to produce pro-inflammatory cytokines (IL-2 and TNF). IFNγ production is also affected, however, this occurs in the later stages of LCMV13 infection (Wherry *et al.*, 2003; Fuller *et al.*, 2004).

Later, there is also progressive impairment of cytotoxic function (Wherry *et al.*, 2007). Eventually, severely exhausted CD8 T cells are deleted by decreased responsiveness to IL-7 and IL-15 and increased expression of pro-apoptotic factors (Wherry *et al.*, 2004; Fuller *et al.*, 2005; Grayson *et al.*, 2006).

Several transcription factors have been reported to be characteristic of exhausted T cells. Particularly, Eomes, T-bet, and Blimp-1 have been reported to play different roles in persistent infection versus acute infection. In acute infection, effector T cell transition to memory is signified by a decrease in T-bet expression and a simultaneous increase in Eomes levels (reviewed in (Kaech and Cui, 2012)). In contrast, in persistent infection, a small Eomes^{lo} T-bet^{hi} CD8 T cell population is the "stem-like" population that replenishes the most exhausted Eomes^{hi} T-bet^{lo} effector population (Paley *et al.*, 2012). On top of the opposing roles of Eomes and T-bet in persistent viral infection, Blimp-1 serves as the main driver of expression of co-inhibitory receptors such as PD-1, Tim-3 and LAG-3 in exhausted CD8 T cells (Shin *et al.*, 2009; Wherry, 2011). Of note, PD-1 is one of the most potent inhibitory signals to CD8 T cells. A single dose of PD-1 blockade, to interfere with PD-L1 binding from APC during LCMV13 infection, can revitalize exhausted CD8 T cell function and accelerate viral clearance (Barber *et al.*, 2006).

T cell exhaustion also occurs in the CD4 compartment during LCMV13 infection. CD4 T cell depletion experiments have demonstrated their importance during LCMV13 infection. Without CD4 T cells, severely exhausted CD8 T cells are quickly deleted, humoral immunity against LCMV is impaired, and infected mice suffer from life-long persistence of LCMV (Fung-Leung *et al.*, 1991; Battegay *et al.*, 1994; Ciurea *et al.*, 2001). Like exhausted CD8 T cells, exhausted CD4 T cells also express higher Eomes and Blimp-1 compared to T-bet (Crawford *et al.*, 2014). However, unlike progressive CD8 T cell exhaustion, exhausted CD4 T cells can still produce cytokines. Nevertheless, the cytokine production profile shifts from co-production of INFγ, TNFα, and IL-2 to IL-10 and IL-21 (Fahey *et al.*, 2011; Crawford *et al.*, 2014). This change in cytokine profile occurs because the expression of Blimp-1 during LCMV infection induces the up-regulation of co-inhibitory receptors (i.e. PD-1) to suppress the establishment of the Th1 response (Crawford *et al.*, 2014; Zehn and Wherry, 2015). Instead, T follicular helper cells (Tfh) take over the CD4 T cell compartment and produce IL-21 (Fahey *et al.*, 2011;

Crawford *et al.*, 2014). Without IL2, CD8 T cells cannot form long-live memory against LCMV infection (Williams *et al*., 2006; Bachmann *et al.*, 2007).

Other cytokines, such as type I IFNs, also play important roles in regulating exhaustion of T cells during LCMV13 infection (Snell *et al*., 2017). The function of IFN-I can be either stimulatory or inhibitory depending on timing. IFN-I plays important roles in early viral control, however, chronic production of IFN-I can lead to engagement of immunosuppressive programs (Teijaro *et al.*, 2013; Wilson *et al.*, 2013; Snell *et al*., 2017), as evidenced by the restoration of CD4 and CD8 responses and viral control with anti-IFNR blockade (Teijaro *et al.*, 2013; Wilson *et al.*, 2013).

1.1.5 Type I interferon responses during LCMV13 infection

Collectively, 13 subtypes of IFNα (1,2,4,5,6,7,8,10,13,14,16,17, and 21) and one IFNβ make up the family of IFN-I (Platanias, 2005). In the canonical signaling pathway, IFN-I binds to IFNAR, that is comprised of a high affinity subunit, IFNAR-2, and a low affinity subunit, IFNAR-1. Binding of IFN-I to IFNAR-2 induces the recruitment of IFNAR-1 and the dimerization of the receptor to allow potentiation of downstream signals through JAK1 and TYK2 (Ihle, 1995; Lamken *et al.*, 2005; Platanias, 2005). Subsequently, autophosphorylation of JAK induces the activation of classical JAK-STAT pathways induces a myriad of cellular functions. For example, the formation of a STAT1/STAT2 heterodimer through tyrosine phosphorylation and its association with IFN regulated factor (IRF) 9 forms the IFN-stimulated gene factor (ISGF) 3 complex that translocates into the nucleus to bind to IFN-stimulated response elements (IRSE) to activate the transcription of numerous IFN-stimulated genes (ISGs) (Platanias, 2005). Both IFNα and IFNβ subtypes of type I IFN are rapidly induced in the early stages of LCMV13 infection. Expression of IFN-I in serum peaks at day 1-2 p.i. followed by a rapid decline to undetectable levels after day 3-5 p.i. (Zuniga *et al.*, 2008; Walsh *et al.*, 2012; Teijaro *et al.*, 2013). Similar kinetics can be observed at the message level in the spleen for IFNα4 and β (Wilson *et al.*, 2013; Chang, 2016). However, the cellular sources of IFN-I during LCMV13 infection remain to be unclear. It was observed that in the absence of MDA5, IFN-I responses and viral control are severely impaired (Y.Wang *et al.*, 2012). Since, MDA5 is broadly

expressed in DC, Mo and other non-immune cell types, it suggests that possibly multiple sources contribute to the production of IFN-I during LCMV13 infection (Y.Wang, Swiecki, *et al.*, 2012; Snell and Brooks, 2015; Snell, McGaha and Brooks, 2017).

IFN-I has traditionally been known for antiviral ability as well as to stimulate DC to upregulate both MHC-I and -II to promote T cell responses (Guidotti and Chisari, 2001; Katze, He and Gale, 2002). The importance of IFN-I signaling for early viral control is clearly demonstrated by studies using deletion of the IFN-I negative regulator, OASL1, or administration of exogenous IFN-I at the onset of infection. In both instances, IFN-I signaling was shown to enhance viral control and clearance(Y.Wang, Swiecki, *et al.*, 2012). However, in recent years, the immunosuppressive role of IFN-I has also been observed during persistent viral infection (Snell and Brooks, 2015; Snell, McGaha and Brooks, 2017). IFN-I signaling was demonstrated to be required for T cells to upregulate co-inhibitory receptors (i.e. PD-1, Tim-3) and transcription factors associated with exhaustion (i.e. Eomes, Blimp-1) (Shin *et al.*, 2009; Paley *et al.*, 2012; Wilson *et al.*, 2013; Crawford *et al.*, 2014; Snell, McGaha and Brooks, 2017). In addition, IFN-I signalling has also been shown to impair *de novo* differentiation of Th1 CD4 T cells. Thus, interfering with Th1 help to CD8 T cells and the accumulation of Tfh during persistent viral infection (Fahey *et al.*, 2011; Crawford *et al.*, 2014; Snell, McGaha and Brooks, 2017). Furthermore, chronic IFN-I signaling induces immunosuppressive APC. After day 9 p.i., chronic IFN-I signaling directly enhances the immunosuppressive capacity of immunoregulatory (ireg) APC by inducing their expression of IL-10, PDL-1, CD95 (Fas), and CD39 (Cunningham *et al.*, 2016). These findings suggest the dual role of IFN-I in LCMV13 infection, where it can be either stimulatory or inhibitory depending on the stage of infection. While stimulatory at the onset of infection to promote antiviral immunity, in the chronic stages, IFN-Is are immunosuppressive, presumably to limit pathology.

1.1.6 Type II interferon responses during LCMV13 infection

In contrast to IFN-I, there is only one IFN-II, IFNγ. IFNγ is a markedly different cytokine than IFN-I, but it was originally classified in the IFN family due to its ability to interfere with viral infections. Like IFN-I, IFN-II also relies on a multichain receptor for its signaling. Binding of IFNγ to its receptor, IFNGR, induces a ligand-dependent rearrangement and dimerization of

the receptor subunits, IFNGR1 and IFNGR2 (Platanias, 2005). The dimerization is followed by the autophosphorylation of JAK1 and JAK2 that are constitutively associated with IFNGR1 and IFNGR2, respectively. In the canonical pathway, activation of JAK results in tyrosine phosphorylation of STAT1 to induce the formation of a STAT1 homodimer (Platanias, 2005). STAT1 homodimers translocate to the nucleus and bind to IFNγ activated site (GAS) elements that are present in the promoter of certain ISGs, thereby initiating the transcription of these genes (Platanias, 2005).

IFNγ plays a crucial role in immunity to viruses. Its importance stems from the ability to either directly inhibit viral replication or indirectly through its immunostimulatory effects. Notably, IFNγ can enhance the antigen presenting capacity by upregulating the expression of MHCI and II subunits, TAP1/2, invariant chain, and immunoproteasome subunits (Young and Hardy, 1995; Platanias, 2005). Cellular sources of IFNγ can be found in both the innate and adaptive immune systems. In the innate immune system, natural killer (NK) cells and natural killer T (NKT) cells constitutively express IFNγ mRNA to allow for rapid production and secretion of IFNγ upon infection thus contributing to early detection and destruction of viral infected host cells (Stetson *et al.*, 2003; Strengell *et al.*, 2003; Kronenberg, 2005; Schoenborn and Wilson, 2007). On the other hand, in the adaptive immunity compartment, naïve CD4 and CD8 T cells can gain the ability to effectively transcribe *ifng* and ramp up their IFNγ production over a few days post infection by a process that is dependent on their activation, proliferation, and differentiation (Schoenborn and Wilson, 2007). More specifically, through a complex process that involves upregulation of IFNγ-promoting transcription factor, chromatin remodeling of the *ifng* locus, and imprinting of epigenetic memory, naïve T cells differentiate into effector T cells and memory cells (Murphy and Reiner, 2002; Dong, 2006; Weaver *et al.*, 2006). Naïve CD8 T cells, by default, differentiate into IFNγ-producing CTL. Conversely, CD4 T cells can differentiate into several distinct effector lineages, of which Th1 produce large amounts of IFN-γ (Murphy and Reiner, 2002; Dong, 2006; Lohr, Knoechel and Abbas, 2006; Weaver *et al.*, 2006). During infection, the innate recognition of pathogens and production of IFNγ by NK and NKT cells in turn influences the differentiation of naïve CD4 and CD8 T cells to IFNγ-producing effector T cells.

In LCMV infections, IFNγ has been demonstrated to be important for viral control and clearance during acute infection by the Arm strain. Using treatment of mice with neutralizing monoclonal antibody to endogenous IFNγ during infection and administration of exogenous recombinant IFNγ before or at the same time as virus inoculation, Mosophidis et al. showed that IFNγ is crucial for inhibiting viral replication early on during LCMV Arm infection and for eventual viral clearance (Moskophidis *et al.*, 1994). Furthermore, in mice that are IFN-γ deficient, CTLs cannot clear the infection despite unimpaired cytotoxic abilities resulting in viral persistence (Bartholdy *et al.*, 2000). In contrast, there is some evidence that IFNγ contributes to viral persistence during LCMV13 infection. From the study conducted by Cunningham et al., it was shown that in IFNγ$R^{-/-}$ mice, the number of monocyte-derived DC (moDC) decreased 30-fold and iregDC decreased 60-fold compared to WT mice after day 9 p.i.. In addition, moDC differentiation was significantly reduced when naïve IFNγR-/- monocytes were adoptively transferred into WT mice followed by LCMV cl13 infection. Together, it was suggested IFNγ signaling is critical for the differentiation of naïve monocytes to iregAPC (Cunningham *et al.*, 2016).

In sum, both IFN-I and IFN-II are crucial for immunity against viral infections. The function of both types of cytokine can be either stimulatory or inhibitory depending on timing, nature of the infecting pathogen, and the cytokine milieu. The collaboration between the IFN systems has been demonstrated to play a key role in sustaining viral persistence in LCMV13 infection, where IFNγ induces the *de novo* development of iregAPC with immunosuppressive potential that are programed by chronic IFNβ signaling.

1.1.7 LCMV Summary

LCMV cl13 is a practical model to study persistent viral infection in its natural murine host. Multiple factors contribute to persistence, including mutations that enhance viral entry into APCs, the resulting higher viral load and T cell exhaustion, and finally, the chronic signaling and collaboration between IFN-I and IFN-II to further sustain immune suppression. It is an arms race between the host immune system and the virus to establish control. The result is a complicated process that many factors could play both stimulatory or suppressive roles depending on the stage of infection.

Part 2: T Cell Costimulation

1.2.1 T cell costimulation

T cells recognize cognate antigen presented by MHC molecules. After the discovery of T cell receptor (TCR) in the early 1980s, it was soon observed that TCR and antigen engagement alone was not sufficient to carry out T cell activation. In fact, a secondary costimulatory signal was necessary to provide contextual information for proper activation to prevent T cell unresponsiveness and anergy by TCR ligation alone (Jenkins, Ashwell and Schwartz, 1988; Mueller, Jenkins and Schwartz, 1989; Esensten *et al.*, 2016). The original 2 signal model of T cell activation has since been revised by several decades worth of work revealing the tremendous diversity in both T cell costimulatory and coinhibitory pathways that can fine-tune T cell fate following activation (Attanasio and Wherry, 2016; Esensten *et al.*, 2016). Most notably, many members of the immunoglobulin (Ig)-superfamily related to CD28 and tumor necrosis factor (TNF) receptor superfamily have been reported to participate in T cell co-signaling (Chen and Flies, 2013; Attanasio and Wherry, 2016; Esensten *et al.*, 2016). Many of these co-signaling receptors and ligands are tightly regulated in space and time, whereby waves of costimulatory or coinhibitory molecules are upregulated in succession to determine T cell fate. Thus, giving rise to the "tidal" model of T cell co-signaling.

1.2.2 T cell costimulation by CD28 and B7 family ligands

CD28 is the founding member of a subfamily of co-signaling molecules characterized by an extracellular variable immunoglobulin-like domain (Chen and Flies, 2013; Esensten *et al.*, 2016). Its importance in T cell activation quickly came under the spotlight following discovery of TCR. In 1986, a monoclonal antibody (mAb) against CD28, Tp44, was found to be able to induce Jurkat cell and primary human T cell activation along with immobilized TCR stimuli (Martin *et al.*, 1986; Weiss, Manger and Imboden, 1986; Esensten *et al.*, 2016). Furthermore, soluble CD28 antagonist treatment was demonstrated to induce T cell tolerance that prevented organ graft rejection (Lenschow *et al.*, 1992). On the other hand, the advent of CD28 agonists have been shown to be able to rescue T cells from tolerogenic state to possibly treat chronic viral infections (Attanasio and Wherry, 2016; Esensten *et al.*, 2016).

Over the last 2 decades, an increasing number of surface molecules have been discovered that share homology to CD28 and its ligands, B7-1 (CD80) and B7-2 (CD86). Other members of the subfamily include CTLA4, ICOS, PD1, PD1H, and BTLA (Chen and Flies, 2013). CD28 is expressed constitutively on mouse T cells, whereas other family members, such as CTLA4 and ICOS, are induced by cytokines like IL-2 in response to immunological events (Gray Parkin *et al.*, 2002; Rozanski *et al.*, 2011; Esensten *et al.*, 2016). Derived from a gene duplication event, ICOS also contributes to T cell activation like CD28. However, ICOS and CD28 receptors cannot substitute one another in function and bind to different ligands (Linterman *et al.*, 2009). On the other hand, CD28 and CTLA4 are highly homologous and compete for the same ligands (Linsley, Clark and Ledbetter, 1990; Engelhardt, Sullivan and Allison, 2006) but have opposing effects on T cell stimulation, where CD28 is costimulatory while CTLA4 is coinhibitory (Krummel and Allison, 1995; van der Merwe and Davis, 2003).

The complex biological effect of this subfamily is reflected by its complex binding behavior. The signaling of these receptors is in part regulated by the expression and availability of their ligands (Esensten *et al.*, 2016). While both CD80 and CD86 are induced by exposure to inflammatory environments, CD86 is rapidly induced on APCs by innate stimuli whereas CD80 is upregulated at later time points (Lenschow *et al.*, 1994; Sharpe and Freeman, 2002). In addition, CD86 has a relative preference for CD28, whereas CD80 binds very strongly to CTLA4 (van der Merwe and Davis, 2003). Thus, the sequential expression of CD86 followed by CD80 on APCs may function as a prototypical immune checkpoint to increase suppressive function of CTLA4 following CD28 costimulation (Walunas *et al.*, 1994; Krummel and Allison, 1995). In contrast to CD80 and CD86, ICOSL is widely and constitutively expressed (Linterman *et al.*, 2009). Thus, the opposing roles of CD28 and ICOS compared to CTLA4 allow this family of receptors and ligands to fine tune the immune response through competing pro-inflammatory and anti-inflammatory effects using their complex binding patterns and variance in expression.

CD28 is expressed on the cell surface as a glycosylated, disulfide-linked homodimer consisted of 220 amino acids and 44 kDa (Esensten *et al.*, 2016). Members of the family share similar features. The receptors typically contain a paired V-set immunoglobulin superfamily (IgSF) domains attached to a single transmembrane domain and cytoplasmic tail that contains signaling motifs (Carreno and Collins, 2002). The ligands, on the other hand, typically contain

single V-set and C1-set IgSF domains. The binding of the ligand to receptor is mediated by the MYPPPY motif within the V-set domains on the receptor (Metzler *et al.*, 1997; Evans *et al.*, 2005). Upon binding of CD80/CD86 to CD28, CD28 drives the pathways that increase T cells' glycolytic rate in order to generate energy for expansion and differentiation (Frauwirth *et al.*, 2002). CD28 costimulation has diverse effects on T cell function. First, CD28 participates in the early formation of immunological synapse that is necessary for productive TCR signaling by regulating the spatial localization of PKCθ (Huang *et al.*, 2002; Yokosuka *et al.*, 2008). Second, CD28 plays an important role in the remodeling of actin cytoskeletal to potentiate downstream TCR signaling (Tan *et al.*, 2014). More importantly, CD28 ligation leads to the production of key cytokines, chemokines, and survival signals including IL-2 and Bcl-xL (Watts, 2010). Therefore, CD28 costimulation is not merely an amplifier of TCR signals but also a driver of unique signals that control intracellular biochemical events from transcription, post-translational protein modification, to epigenetic changes that promote T cell growth and proliferation (Martin *et al.*, 1986; Bluestone, St.Clair and Turka, 2006; Bour-Jordan *et al.*, 2011; Esensten *et al.*, 2016).

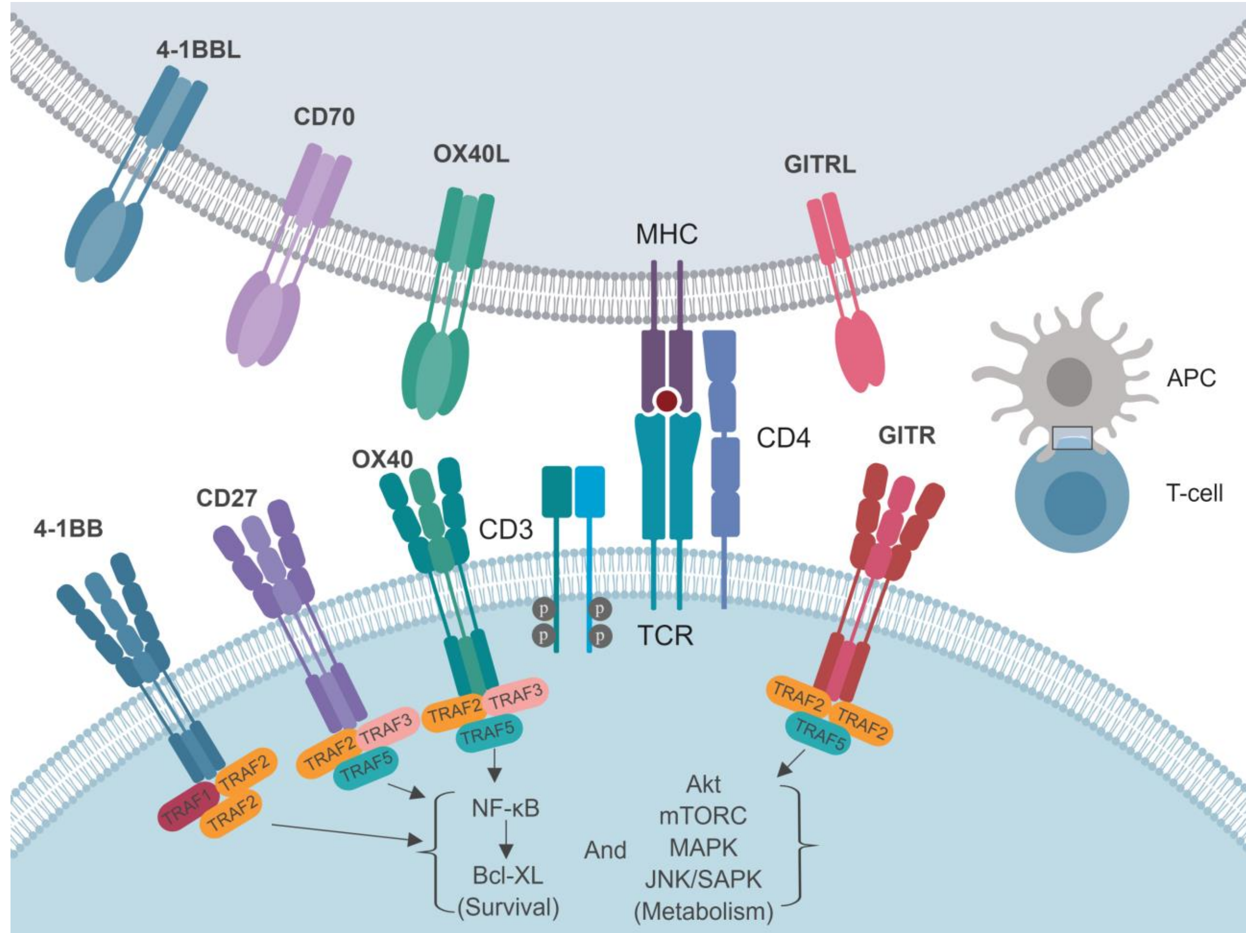
The discovery of a series of receptors and ligands that share significant homology with CD28 and its ligands have revealed a complex system of interactions wherein a single receptor, CD28, binds to multiple ligands and the ligands, CD80 and CD86, in turn can bind to multiple receptors including CTLA4 depending on the circumstances. This intricate network of receptor and ligand interactions, allow the Ig-superfamily receptor and ligands to serve as a very important checkpoint of T cell mediated immune response that can promote both pro-inflammatory and anti-inflammatory effects.

1.2.3 T cell Costimulation by TNF receptor superfamily

Following the initial effects of CD28-B7 interaction that lowers the threshold for T cell activation, the Tumor Necrosis Factor superfamily of receptors (TNFR/TNFRSF) and ligands (TNF/TNFSF) come in to play their role as key mediators of survival and accumulation of activated T cells (Watts, 2005). The TNFRs are type I transmembrane proteins defined by structural homology in their extracellular domains that share a conserved cysteine-rich motif (Locksley, Killeen and Lenardo, 2001; Bodmer, Schneider and Tschopp, 2002; Watts, 2005). The membrane bound (can be cleaved from the surface to make soluble forms) subset of TNF

superfamily ligands are type II cell surface glycoproteins that also share structural similarities in their extracellular domains and usually assemble into trimers (with the exception of murine GITRL being a dimer that associates through a unique C terminus tethering arm (Zhou *et al.*, 2008)) that enhances affinity of binding to receptors. The high affinity binding allows receptor clustering upon ligation which in turn initiates downstream signal transduction. The cytosolic signaling domain subdivides the superfamily into 3 subgroups, death domain (DD)-containing receptors, decoy receptors, and TNF receptor-associated factor (TRAF) binding receptors (Locksley, Killeen and Lenardo, 2001; Aggarwal, 2003; Dempsey *et al.*, 2003; Watts, 2005). This thesis focuses on the latter subgroup.

The TRAF binding receptors are typically costimulatory in nature and contain TRAF2 binding motifs consisting of the major conserved motif, (P/S/A/T)x (Q/E), or the minor motif, PxQxxD (Ye *et al.*, 1999). Six mammalian TRAF proteins have been identified (Dempsey *et al.*, 2003). All members of the subgroup can recruit TRAF2 with their shared binding motif, but they demonstrate differences in recruitment of other TRAF proteins(Arch, Gedrich and Thompson, 1998; Aggarwal, 2003). Upon receptor ligation, TRAFs are recruited to the receptor's cytoplasmic tail to form trimers and interact with each other to potentiate downstream signaling pathways. Aggregation of TRAFs induce genes associated with cell survival. For instance, both TRAF2 and TRAF5 induce the activation of nuclear factor κB (NF-κB), which lead to the transcription of Bcl-$_x$L, c-flip, cellular inhibitors of apoptosis (cIAP1 and 2), and even TRAF1(Karin and Lin, 2002). TRAF2 also recruits and activates mitogen activated protein kinases (MAPK) 3 and 4, leading to activation of jun-N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 MAPK cascades(Aggarwal, 2003; Dempsey *et al.*, 2003). In summary, TRAFs link the costimulatory TNFR family members to NF-κB and stress kinase signaling resulting in enhancement of cell survival, cytokine production, and proliferation of T cells. In this thesis, 4 members of TNF superfamily ligands will be investigated: GITRL, 4-1BBL, CD70, and OX40L, with emphasis on GITRL and 4-1BBL (**Figure I**).



(**Figure I.** TNF receptor signaling)

GITR and GITRL

GITR or CD357, is the 18th member of the TNF receptor superfamily (TNFRSF18). GITR is expressed on a spectrum of both immune and non-immune cell types, including B cells, T cells, NK cells, and polymorphonuclear (PMN) leukocytes (Clouthier, Zhou and Watts, 2014), with highest basal level on $CD25^{+}Foxp3^{+}$ regulatory T cells (Tregs) (McHugh *et al.*, 2002; Shimizu *et al.*, 2002). Typically, GITR expression is upregulated upon inflammation and infection. For instance, GITR is expressed at basal level on naïve and memory T cells and is upregulated with activation (Kanamaru *et al.*, 2004). On the other hand, the expression of the cognate ligand to GITR, GITRL, has been less well studied. Nevertheless, like GITR, GITRL is also induced upon inflammation and infection on APCs, however, it is almost undetectable in steady state (Clouthier, Zhou and Watts, 2014; Chang, 2016).

GITR utilizes TRAF2 and TRAF5 to recruit cIAP1/2 and activate the canonical NFκB pathway (Snell *et al.*, 2010, 2011). Stimulation of GITR has also been shown to activate other pro-survival signaling cascades such as the MAPK and mTORC1 pathways (Clouthier *et al.*, 2015). Together, the induction of these pathways results in a co-stimulatory response in T cells that upregulate the expression of activation makers, cytokine production (e.g. IL-2, TNFα, and IFNγ) and pro-survival factors (e.g. Bcl-$_x$L) (Tone *et al.*, 2003; Stephens *et al.*, 2004; Igarashi *et al.*, 2008; Snell *et al.*, 2010; Clouthier *et al.*, 2015). GITR activation is important for CD4$^+$ and CD8$^+$ T cell differentiation and accumulation to control both acute and chronic viral infections (Snell *et al.*, 2010; Clouthier, Zhou and Watts, 2014; Clouthier *et al.*, 2015; Pascutti *et al.*, 2015).

In the context of LCMV infection, using global GITR-knockout mice, Clouthier et al. showed that mice had impaired viral control as evident by the increased in T cell exhaustion and decreased number of LCMV-specific CD8 T effector cells, leading to higher viral load in both the acute and chronic stages of infection without GITR costimulation (Clouthier *et al.*, 2015). In parallel, Pascutti et al. discovered that mice with constitutively expressed GITRL on B cells have enhanced CD8 T cell response to LCMV13 infection and superior control of viral load (Pascutti *et al.*, 2015). Furthermore, using mixed bone marrow chimera studies, Clouthier et al. revealed that the *in vivo* effect of GITR costimulation is largely CD4 T cell intrinsic. Where in the absence of GITR, the accumulation of IL-2$^+$ IFNγ$^+$ Th1 CD4 helper cells was significantly impaired (Clouthier *et al.*, 2015). In addition, neutralization of IL-2 at day 4-6 p.i. abrogated the costimulation effect of GITR on CD8 T cell expansion (Clouthier *et al.*, 2015). Thus, the authors postulated that GITR costimulation enhances CD4 help through IL-2 production to promote other immune responses during LCMV infection.

4-1BB and 4-1BBL

4-1BB (CD137, TNFRSF9) is a TNFR superfamily family member that is expressed on multiple cell types of the hematopoietic lineage. Most notably, 4-1BB is known to be transiently expressed on CD4 and CD8 T cells following activation (Shuford *et al.*, 1997; Kim *et al.*, 2003; Dawicki and Watts, 2004). It can also be expressed on activated B cells, NK cells, APCs, as well as endothelium and epithelium cells (Melero *et al.*, 1998; Pauly *et al.*, 2002; Drenkard *et al.*, 2007; Vinay *et al.*, 2012; Ward-Kavanagh *et al.*, 2016). On the other hand, its ligand, 4-1BBL is

mainly expressed on professional APCs (Watts, 2005; Wortzman *et al.*, 2013; Ward-Kavanagh *et al.*, 2016) by Toll-like receptor (TLR) signaling (Wang *et al.*, 2009). Upon ligand binding, 4-1BB recruits TRAF1 and TRAF2 (Arch, Gedrich and Thompson, 1998; Jang *et al.*, 1998; Saoulli *et al.*, 1998). Structural analysis of the TRAF N domains suggests that the physiological complex that recruits cIAPs consists of 1 TRAF1 and two TRAF2 molecules (Zheng *et al.*, 2010). This signalling adaptor complex potentiates signaling through the NF-κB, AKT (protein kinase B), p38 MAPK and ERK pathways (Kim, Kwack and Lee, 2000; Lee *et al.*, 2013; Oussa, Soumounou and Sabbagh, 2013), leading to the increased expression of survival genes Bcl-2, Bcl-$_{x}$L, and Bfl-1 and decreased expression of pro-apoptotic Bim (Kim, Kwack and Lee, 2000; Sabbagh *et al.*, 2013; Wortzman *et al.*, 2013).

4-1BB signaling was shown to be an important costimulatory pathway for T cells early but not late during chronic viral infections. During the first week of LCMV13 infection, 4-1BBL$^{-/-}$ mice showed impaired accumulation of virus specific CD8 T cells and higher viral loads (C.Wang *et al.*, 2012). However, this early effect of 4-1BBL on virus specific CD8 T cells is lost after day 8 p.i. as evident by the failure of anti-4-1BB agonist to improve the T cell response at the later stages, despite the persistent expression of 4-1BB on the antigen-specific T cells. Further investigation revealed that in the chronic stage of LCMV13 infection, TRAF-1 is degraded in antigen-specific CD8 T cells in a transforming growth factor (TGF)-β-dependent manner (C.Wang *et al.*, 2012). Nevertheless, early administration of 4-1BB agonist in combination with IL-7, a key cytokine for T cell survival and memory, showed beneficial effects on preventing T cell exhaustion during LCMV13 infection (C.Wang *et al.*, 2012). In addition, treatment with 4-1BB agonist in combination with PD-1:PD-L1 pathway blockade also increased the number, cytolytic activity, and cytokine production of virus-specific CD8 T cells better than PD-1:PD-L1 blockade alone during LCMV infection (Vezys *et al.*, 2011). Thus, 4-1BB is a unique costimulatory signaling that has been reported to predominantly affect CD8 T cell response and is regulated by the desensitization of its signaling adaptor, TRAF-1.

CD27 and CD70

CD27 signaling is tightly regulated by the availability of its ligand, CD70 (Nolte *et al.*, 2009). During LCMV Arm infection, CD27 is expressed on naïve T cells and maintained

throughout the course of the infection. However, CD70 is barely detected on $CD11c^{+}$ MHC II^{+} DCs, with a peak expression at day 2 p.i. before returning to baseline by day 6 p.i. (Kuka *et al.*, 2013). In comparison, during LCMV WE infection, CD70 is more highly expressed on T cells and B cells than APCs, peaking at day 8 p.i. and day 15 p.i., respectively (Matter *et al.*, 2006). Furthermore, $CD70^{-/-}$ mice or treatment of CD70 blocking antibody during acute LCMV infections resulted in decreased in effector CD8 T cell function and a delay in viral clearance (Penaloza-MacMaster *et al.*, 2011; Munitic *et al.*, 2013). By contrast, the main source of CD70 during LCMV13 infection is yet to be reported. However, from the study conducted by Penaloza-MacMaster et al., blockade of CD70 resulted in increased CD8 T cell response (Matter *et al.*, 2006; Penaloza-MacMaster *et al.*, 2011). Thus, the effect of CD27 signaling is context-dependent depending on the persistence of the pathogen.

OX40 and OX40L

In contrast to GITR and CD27, OX40 (CD134, TNFRSF4) is not expressed on naïve T cells, but is rapidly induced upon T cell activation (Croft, 2010). During LCMV13 infection, OX40 is induced to higher levels on CD4 T cells than on CD8 T cells and persists to day 21 p.i. (Boettler *et al.*, 2012). OX40L is detected on $CD11c^{+}$, $CD11b^{+}$, and $F4/80^{+}$ APC, with a peak at day 4 p.i. before returning to baseline by day 7 p.i. (Boettler *et al.*, 2012). OX40 signaling also has a more profound effect on CD4 T cells than CD8 T cells. Most evidently, in $OX40^{-/-}$ mice, the Tfh response, germinal center formation, and humoral response were severely impaired (Boettler *et al.*, 2012). Furthermore, adoptive transfer of $OX40^{+/+}$ or $OX40^{-/-}$ CD4 SMARTA showed that OX40 is important for T cell survival rather than proliferation and direct antiviral function shown by increased expression of pro-survival Bcl-2 and Bcl-xL in $OX40^{+/+}$compared to $OX40^{-/-}$ (Boettler *et al.*, 2012).

1.2.4 Summary - T cell costimulation

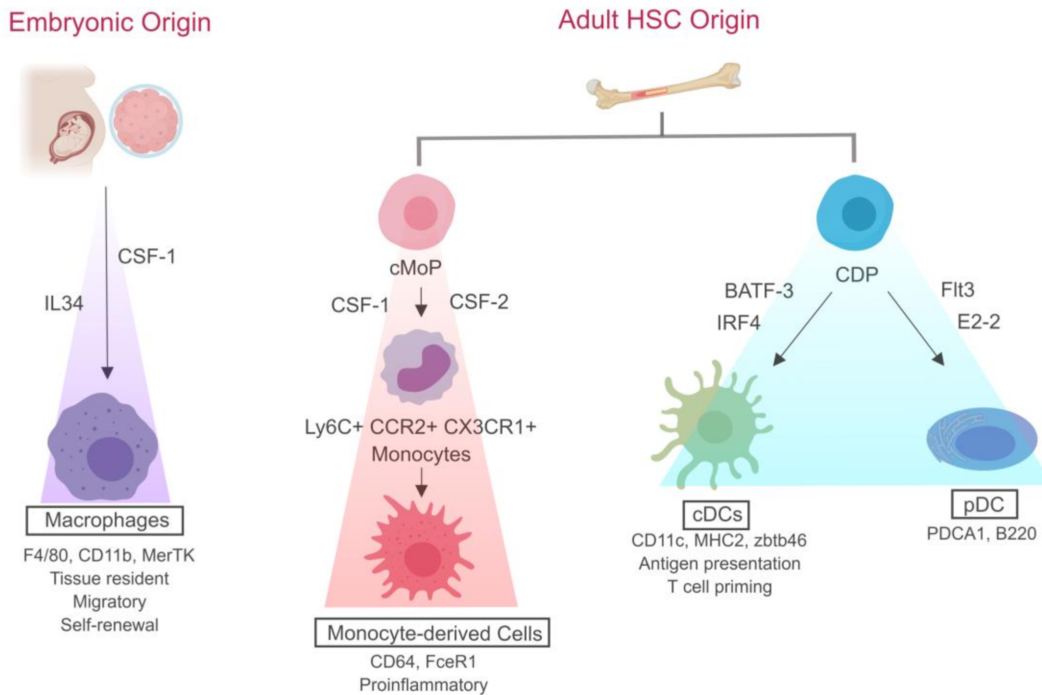
In summary, T cell costimulatory receptors play key roles in regulating immune responses to viral infections. Signaling of costimulation can be controlled by low and transient ligand expression, transient receptor expression, receptor competition for ligands, or

desensitization of signalling by loss of a signaling adaptor. Both Ig superfamily and TNF superfamily remain attractive therapeutic targets for intervention in chronic viral infections. Further investigation on the timing, dosage, and mechanism of interaction is required to provide highly tailored approaches for control of chronic viral infection.

Part 3: Antigen Presenting Cells

1.3.1 Intro to antigen presenting cells and the mononuclear phagocyte system

The mononuclear phagocyte system (MPS) includes dendritic cells (DCs), monocytes, and macrophages that exhibit a wide array of functions during immune responses, among which is their ability to present antigen and provide costimulation to T cells (Shi and Pamer, 2011; Guilliams *et al.*, 2014; Sprangers, Vries and Everts, 2016). Over a century ago, Metchnikoff – the father of cellular immunity – was the first person to observe the capabilities of the phagocytes and established the phagocyte system (reviewed in Gordon et al., (Gordon, 2008)). By conducting studies spanning from echinoderm amoebocyte to vertebrates, Metchnikoff observed cells that he termed macrophages and microphages (now know as polynuclear leukocytes), and learned that phagocytosis is more than a mere ability of cells to engulf foreign microorganism but it is also an active defense mechanism, thus giving rise to the concept of innate immunity. Over the years, with the advent of new discoveries paralleled with new technologies that enabled further assessments of the cells (i.e. multiparameter flow cytometry, lineage tracing), many distinct DC, monocyte, and macrophage subsets have been identified (Merad *et al.*, 2013; Ginhoux and Jung, 2014). Currently, the MPS encompasses three broad families of cells: haematopoietic stem cell (HSC)-derived common DC precursor (CDPs)-derived DCs (Schraml *et al.*, 2013), bone marrow-derived monocytes, and embryonic-derived macrophages that are capable of self-renewal and are independent of blood monocytes (Ginhoux *et al.*, 2010; Schulz *et al.*, 2012; Yona *et al.*, 2013) (**Figure II**).



(**Figure II**. APC lineage (Inspired by and modified from Figure 2 of (Guilliams *et al.*, 2014)))

1.3.2 CDPs-derived dendritic cells

Derived from their haematopoietic precursor CDPs, cDCs serve as the sentinel of sensing environmental stimuli for the immune system. cDCs are located throughout the body in nonlymphoid and lymphoid tissues in the steady state allowing them to constantly examine and acquire blood and tissue antigens. Upon encounter and phagocytosis of antigen, cDCs move to T cell zones of lymph nodes (LNs) and spleen to present captured antigen to T cells and to prime naïve T cell responses (Banchereau and Steinman, 1998). Through these processes, cDCs shape the adaptive immunity to foreign environmental cues. On the cell surface, cDCs constitutively express the haematopoietic markers, CD45, MHC II, and CD11c and lack T cell, B cell, NK cell, and granulocyte markers. However, with emerging understanding of their origins, differentiation, and function, more markers have been identified to classify a wide array of subsets of cDCs. A few transcription factors have been identified to regulate development cDCs including basic leucine zipper transcription factor ATK-like 3 (Batf3) (Hildner *et al.*, 2008), the zinc finger transcription factor (zbtb46) (Satpathy *et al.*, 2012), and STAT3 of the Flt3 signaling pathway (Laouar *et al.*, 2003). cDCs represent 1% - 5% of cells in the tissues depending on the organ and

consist of 3 major subsets: $CD8^+$ lymphoid tissue cDCs, $CD103^+CD11b^-$ nonlymphoid tissue cDCs, and $CD11b^+$ nonlymphoid tissue cDCs (Merad *et al.*, 2013).

$CD8^+$ cDCs and $CD103^+$ cDCs share the same origin, transcriptional profile, and function (del Rio *et al.*, 2010). Both $CD8^+$ cDCs and $CD103^+$ cDCs are positioned effectively in ideal locations to filter environmental and tissue antigens. $CD8^+$ cDCs are located in the marginal zone of spleen tissue and subcapscular sinus of LNs to allow efficient migration into the T cell zones after antigen encounter (Qiu *et al.*, 2009). $CD103^+$ cDCs are located at the interface of nonlymphoid tissues and environment and migrate to the T cells zones of draining LN (Helft *et al.*, 2010). *Ex vivo* studies using $CD8^+$ cDCs and $CD103^+$ cDCs purified after antigen inoculation *in vivo* have revealed their superior antigen presentation ability and priming potential to $CD8^+$ T cells over other cDC subsets (Hochrein *et al.*, 2001; Belz *et al.*, 2007; Hildner *et al.*, 2008; Bedoui *et al.*, 2009; Helft *et al.*, 2010; Mashayekhi *et al.*, 2011). Batf3-deficient mice that specifically lack $CD8^+$ cDCs and $CD103^+$ cDCs are unable to induce a sufficient CD8 T cell immunity against West Nile virus or Influenza virus (Hildner *et al.*, 2008). Furthermore, $CD8^+$ cDCs also express more genes related to MHC I presentation (Dudziak *et al.*, 2007) and have higher production of IL-12 (Hochrein *et al.*, 2001; Mashayekhi *et al.*, 2011) and IL-15 (Mattei *et al.*, 2001) that are important for differentiation of cytotoxic CD8 T cells (Pulendran, 2004). $CD8^+$ cDCs also demonstrate higher efficiency than $CD11b^+$ cDCs in processing and loading exogenously acquired antigen onto MHC I molecules to allow cross presentation of antigen. Mechanistically, splenic $CD8^+$ cDCs express high levels of Rac2 that limits the protease activity of the phagosome (Savina *et al.*, 2009), and high levels of adipose differentiation-related protein that provides a source of oxidative stress to destabilize phagosomal membranes and thus leading to the release of antigens into the cytosol and favoring cross-presentation of exogenous antigens (Bougnères *et al.*, 2009; Merad *et al.*, 2013). Similarly, lung and dermal $CD103^+$ cDCs also have been reported to have superior cross-presentation potential than $CD11b^+$ cDCs (Heath and Carbone, 2009). $CD8^+$ cDCs and $CD103^+$ cDCs also play important role in the activation of CD4 T cells. $CD8^+$ cDCs are the main producer of IL-12 to help Th1-polarization in the spleen (Maldonado-López *et al.*, 1999) and provide the cognate antigen to CD4 T cell to induce CD8 effector response and memory during herpes simplex virus 1 (HSV-1) infection (Smith *et al.*, 2004). On the other hand, $CD103^+$ cDCs control the induction of pathogen-specific IFNγ-producing CD4 T cell during in the skin during *Candida albican* infection (B. Igyarto *et al.*,

2011). Both cDCs are also involved in central and peripheral tolerance through negative selection of developing thymocytes, deletion of self-reactive T cells, the induction of antigen-specific Treg (Klein *et al.*, 2009; Hsieh, Lee and Lio, 2012).

CD11b^{+} cDCs in nonlymphoid tissues are comprised of a mixture of tissue cDCs and macrophages that are not yet very well distinguished in the literature (Merad *et al.*, 2013). Similar to CD8^{+} and CD103^{+} cDCs, CD11b^{+} cDCs can sense foreign antigen (Luber *et al.*, 2010), participate in the activation of CD4 and CD8 T cells and peripheral tolerance (Bonasio *et al.*, 2006; Kim and Braciale, 2009). However, in contrast to CD8^{+} and CD103^{+} cDCs, CD11b^{+} cDCs have a more predominate role in MHC II presentation with higher expression levels of genes coding for MHC II antigenic pathways (Dudziak *et al.*, 2007). Furthermore, *in vivo* delivery of antigen to CD11b^{+} cDCs in the spleen revealed that CD11b^{+} cDC is more efficient at MHC II presentation to CD4 T cells in the steady state (Dudziak *et al.*, 2007). Upon subcutaneous vaccination, CD11b^{+} dermal cDCs are the main subset of APC to drive the accumulation of antigen-specific CD4 T cell response (Kastenmüller *et al.*, 2011). Nevertheless, conditional deletion model of CD11b^{+} DC is still lacking from the literature, limiting our understanding of their contribution to CD4 T cell priming *in vivo* (Merad *et al.*, 2013).

There are other subsets of DCs that have varying functions and morphology. Most notably, pDCs, that accumulate mainly in the blood and lymphoid tissues and enter the LN through blood circulation upon inflammation. pDCs are identified by the low expression of MHC II and CD11c in steady state and the expression of surface marker PDCA-1. Upon viral infection, pDCs produce large amounts of IFN-I (Reizis *et al.*, 2011). Tissue migratory DCs are the nonlymphoid tissue DCs that migrated to the peripheral draining LNs via the lymphatics (Randolph, Angeli and Swartz, 2005). The migration to peripheral draining LN is controlled by CC-chemokine receptor 7 (CCR7), as evident by the lack of tissue migratory DCs in CCR7$^{-/-}$ mice (Ohl *et al.*, 2004). In steady state, tissue migratory DCs have higher expression of MHC II and lower expression of CD11c than resident DCs. However, upon inflammation, migratory DCs enter the LNs and undergo a process called DC maturation leading to dramatic transformation of the tissue migratory DCs, resulting in the production of inflammatory cytokines and upregulation of costimulatory molecules (such as B7 family ligands) that drive adaptive immunity (Reis E Sousa, 2006).

1.3.3 Monocyte-derived APCs

Monocytes are a subset of circulating leukocytes that can be recruited to the tissue where they differentiate into a wide array of macrophages and DCs (Auffray, Sieweke and Geissmann, 2009; Shi and Pamer, 2011). Cells derived from monocytes represent an important link between inflammatory conditions and the adaptive immune response. They are a heterogenous population that is different in size, phenotype, and function. Originating in the bone marrow, after detection of circulating pathogenic molecules, monocytes emigrate out to the blood stream, mediated by CCR2 and the induction of CC-chemokine ligand 2 (CCL2) on bone marrow stromal cells. Monocytes constitute 4% of the total leukocyte population in mice and 10% in humans (Ginhoux and Jung, 2014). Mouse monocytes in the bloodstream can be divided into 2 functionally distinct subsets based on their expression of Ly6C, CCR2, and CX3C-chemokine receptor 1 (CX3CR1) (Ziegler-Heitbrock, 2014).

The $Ly6C^{+}CCR2^{high}CX3CR1^{low}$ subset is the inflammatory monocyte that represents 2-5% of circulating white blood cells in an uninfected mouse. These cells are rapidly recruited to the sites of inflammation upon infection (Metschnikoff, 1887; Serbina *et al.*, 2008; Jakubzick *et al.*, 2013; Tamoutounour *et al.*, 2013). At the site of infection, further differentiation divides $Ly6C^{+}$ monocyte into subsets that can express CD11c and MHC II like cDCs or F4/80 and tyrosine protein kinase MER (MerTK) like macrophages (Guilliams *et al.*, 2014). More recently, CD64 and the high affinity IgG receptor, FcεR1, expression on these monocyte derived inflammatory APCs have been observed, adding to their inflammatory profile (Langlet *et al.*, 2012; Plantinga *et al.*, 2013; Segura and Amigorena, 2013). On the other hand, the $Ly6C^{-}CCR2^{low}CX3CR1^{high}$ subset is the patrolling monocyte that mostly remains in the vasculature (Auffray *et al.*, 2007). Intravital microscopy studies have revealed that they adhere and migrate along the luminal surface of endothelial cells that line blood vessels (Auffray *et al.*, 2007).

The mobility and the ability to traffic to the site of infection are crucial to the function of monocytes in promoting immune responses. CCR2 deficient mice have a severe reduction in $Ly6C^{+}$ inflammatory monocyte recruitment to sites of inflammation (Kurihara *et al.*, 1997). On the other hand, deletion of CX3CR1 results in reduction of patrolling $Ly6C^{-}$ monocytes (Auffray *et al.*, 2007). In addition, CX3CR1 signaling also promotes the survival of $Ly6C^{-}$ monocytes under both steady and inflammatory conditions (Landsman *et al.*, 2009).

During viral infection, monocytes have been demonstrated to provide both beneficial and detrimental effects. CCR2 deficient mice have demonstrated decreased immunopathology and mortality during Influenza infection (Dawson *et al.*, 2000). However, delayed clearance of the virus was also demonstrated due to insufficient priming of virus-specific CD8 T cells. Interestingly, adoptive transfer of Ly6C$^+$ monocytes into CCR2-deficient mice restores CD8 T cell response and facilitates viral clearance (Aldridge *et al.*, 2009). These studies suggest that monocytes have an essential role in microbial immune defense but at the same time they can also contribute to tissue destruction during some viral infections.

1.3.4 Embryonic-derived macrophages

Macrophages are derived from embryonic progenitors including fetal monocytes and yolk sac derived macrophages (Ginhoux and Jung, 2014). During fetal development, macrophages spread throughout the body via the blood stream giving rise to tissue-resident macrophages that are capable of self-renewal throughout life. The development of macrophages is highly dependent on colony-stimulating factor 1 receptor (CSF1R), the receptor for the cytokines colony-stimulating factor 1 (CSF1) and IL-34 (Greter *et al.*, 2012; Y.Wang, Szretter, *et al.*, 2012). These cytokines support the survival and differentiation of most macrophages. Typically, macrophages express surface markers including F/480 and MerTK (Gautiar *et al.*, 2012). Tissue-resident macrophages have been involved in various functions ranging from tissue development, tissue repair, homeostasis, and defense against pathogens, fibrosis, and even cancer (reviewed in Wynn et al. (Wynn, Chawla and Pollard, 2013)).

1.4 Outstanding Questions for the Regulation of TNF Costimulation During Chronic LCMV13 Infection

As reviewed above, TNFRs have diverse effects in control of chronic LCMV infection. Using a series of mixed adoptive transfer and bone marrow chimera experiments, Clouthier et al., showed that the role GITR signaling plays on T cell accumulation and effector function is mainly CD4 T cell intrinsic and IL-2 dependent (Clouthier *et al.*, 2015). Furthermore, Clouthier et al., have also shown that GITR is upregulated upon LCMV13 infection and sustained into the chronic stage and that CD8 T cells are capable of responding directly to exogenous DTA-1 treatment (Clouthier, Zhou and Watts, 2014). These findings suggest that GITR remains functional even in the chronic exhausted state of immune response, and that the regulation of GITR signaling is in large controlled by the availability of its ligand, GITRL. Thus, this insight prompted us to investigate further in regard to when and where these TNF superfamily ligands are expressed and how they are regulated.

A previous study from the lab had shown preferential GITRL expression on $CD11b^+$ $F4/80^+$ APC at the onset of LCMV13 infection (Clouthier, Zhou and Watts, 2014). However, the heterogeneity and plasticity of these APCs are very complex and require further investigation and delineation. How and when different APC subtypes contribute these factors is incompletely understood. In addition to the identification of the specific APC subset that express these ligands, how the expression of these ligands is regulated during chronic viral infection also remains unclear. Lastly, tissue specific difference between lymphoid and nonlymphoid organs also remains unclear.

1.5 Thesis Synopsis

When I began my MSc in 2015, Yu-Han (Frank) Chang in our laboratory had just worked out the 11-color flow cytometry panel that delineated the inflammatory monocyte-derived APCs from cDCs and other tissue resident macrophages. His work demonstrated that inflammatory APCs are the predominate expressors of GITRL throughout the 21-day period of LCMV13 infection. Subsequently, he demonstrated in a series of *in vitro* and *in vivo* experiments that a complex relationship between IFN-I response and LCMV13 infection underpins the *in vivo* GITRL regulation across all APCs investigated. In collaboration with Frank, we were able to show that GITR signaling occurs post T cell priming and is provided by inflammatory APCs separately from cDCs during LCMV13 infection (Chang, 2016). Thus, these findings made it clear to us that further investigation is needed to delineate the specific cell types that prime T cells or provide TNF family signal.

I started my thesis by focusing on the 4 TNF family ligands (GITRL, 4-1BBL, CD70, and OX40L) using the panel that Frank had designed, and explored the heterogeneity of different APCs' expression. Expanding the analysis from Franks initial analysis of GITRL, we discovered dichotomous expression of TNF and B7 superfamily ligands on inflammatory APCs and cDCs's respectively. This dichotomy of expression was also confirmed not only in the spleen during LCMV13 infection, but also in the lung tissue and mediastinal lymph node. This division of labor was the focal point that made us hypothesize the "Signal 4" of T cell activation that is important for the accumulation and survival of primed T cells. In subsequent studies, we confirmed the importance of IFN-I in regulating not only the uniform upregulation of the TNF family ligands on inflammatory APCs, but also the upregulation of B7 family ligands on cDCs. In addition, to expand the analysis on the regulatory mechanism of expression of TNF family ligands, I also investigated the role of IFNγ and LPS in TNF family ligand induction.

Together, this thesis looks to expand our current understanding on the expression kinetics of TNF and B7 superfamily ligands across different APC subsets and provides detailed insight on the *in vitro* and *in vivo* expression and regulation of these ligands.

Chapter 2

Materials and Methods

Materials and methods are modified from as reported in "Chang YH, Wang KC, Chu KL, Clouthier DL, Tran AT, Torres Perez MS, Zhou AC, Abdul-Sater AA, Watts TH. Dichotomous Expression of TNF Superfamily Ligands on Antigen-Presenting Cells Controls Post-priming Anti-viral CD4(+) T Cell Immunity. Immunity. 2017 Nov 21;47(5):943-958".

EXPERIMENTAL MODEL AND SUBJECT DETAILS

LCMV13

LCMV13 from Dr. M. Oldstone and LCMV13-GFP from Dr. J. C. de la Torre were propagated on BHK cells and assayed by focus forming assay, as described (Clouthier *et al.*, 2015). $2x\ 10^6$ ffu of LCMV13 was injected intravenously per mouse in the infected groups where indicated. LCMV13-GFP was added to *in vitro* thioglyocolate elicited macrophage cultures at various MOI as described in figures.

Influenza A/PR8

Influenza A/Puerto Rico/8/1934 H1N1 (PR8) was grown in eggs. 50% tissue culture infectious dose (TCID50) was determined by MDCK assays (Cottey, Rowe and Bender, 2001). $5x\ 10^5$ TCID50 was given intra-nasally per mouse for infection.

Mice

Age-matched (6-10 weeks old) female mice were used in all experiments performed. CD45.2 wildtype mice (C57BL/6NCrl) were used in all experiments presented**.** Amongst each genotype of mice, assignments to experimental groups were random throughout. All animals were housed under spf conditions in the Division of Comparative Medicine at the Terrence Donnelly Centre for Cellular and Biomolecular Research (University of Toronto). Animal protocols (Protocol #: 20011176 and 20011642) were approved by the animal care committee at the University of Toronto in accordance with the Canadian Council on Animal Care.

Resources and Reagents

Detailed chemical resources and reagents can be found in **Table 1**.

Table 1. Resource Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-CD16/32 (93)	eBioscience	Cat. 14-0161-82
Anti-B220 (RA3-6B2)-PerCP-eF710	eBioscience	Cat. 47-0452-82
Anti-FcεR1 (MAR-1)- PE-Cy7	eBioscience	Cat. 25-5898-82
Anti-NK1.1 (PK136)- PE, APC-eF80	eBioscience	Cat. 47-5941-82 Cat. 47-5941-82

Anti-PDCA-1 (eBio927)-APC, FITC	eBioscience	Cat. 17-3171-82 Cat. 11-3172-82
Anti-CD11c (N418)-AF700, APC	eBioscience	Cat. 56-0114-82 Cat. 17-0114-82
Anti-CD11b (M1/70)- FITC	eBioscience	Cat. 11-0118-42
Anti-F4/80 (BM8)-APC-eF780	eBioscience	Cat. 47-4801-82
Anti-MHCII (I-A/I-E M5/114.15.2)- eF450, PE	eBioscience	Cat. 48-5321-82 Cat. 12-5321-82
Anti-CD8α (53-6.7)-APC	eBioscience	Cat. 17-0081-82
Anti-CD103 (2E7)-APC	eBioscience	Cat. 17-1031-82
Anti-CD95 (15A7)-APC	eBioscience	Cat. 17-0951-82
Anti-CD39 (24DMS1)-PE	eBioscience	Cat. 12-0391-82
Anti-CD80 (16-10A1)-PerCP-eF710, Biotin	eBioscience	Cat. 46-0809-42 Cat. 13-0801-82
Anti-PD-L1 (MIH5)-PerCP-eF710	eBioscience	Cat. 46-5983-42
Anti-CD86 (GL-1)-APC, FITC, Biotin	eBioscience	Cat. 17-0862-82 Cat. 11-0862-82 Cat. 13-0862-82
Anti-Ly-6C (HK1.4)-APC-eF780, PE-Cy7	eBioscience	Cat. 47-5932-82 Cat. 25-5932-82
Anti-CD19 (eBio1D3)-Biotin	eBioscience	Cat. 13-0193-82
Mouse IgG2a (eBM2a)-Biotin	eBiosicence	Cat. 13-4724-83
Anti-CD3 (17A2)-BV605	BioLegend	Cat. 100237
Anti-CD3 (2C11)-Biotin	This paper	N/A
Anti-CD19 (6D5)-BV605, Biotin	BioLegend	Cat. 115539
Anti-B220 (RA3-6B2)-BV605	BioLegend	Cat. 103243
Anti-CD64 (X54-5/7.1)-BV711, APC	BioLegend	Cat. 139311 Cat. 139306
Anti-CD70 (FR70)-Biotin	BioLegend	Cat. 104603
Anti-OX40L (RM134L)-Biotin	BioLegend	Cat. 108803
Anti-Ly-6G (1A8)-APC	BioLegend	Cat. 127613
Anti-CX3CR1 (SA011F11)-APC, PE	BioLegend	Cat. 149007 Cat. 149006
Anti-GITRL (MIH44)-PE	BD	Cat. 563541
Anti-zbtb46 (U4-1374)-PE	BD	Cat. 565832
Anti-OX40 (OX86)-Biotin	BD	Cat. 550977
Anti-MerTK (108928)-PE	R&D	Cat. FAB5912P
Anti-CCR2 (475301)-PE	R&D	Cat. FAB5538P

Anti-LCMV NP-Biotin (1.1-3) (flow cytometry)	Dr. M. Buchmeier, University of California	N/A
Anti-Mouse 4-1BBL (19H3)-Biotin	Purified from hybridoma provided by Dr. R. Miller, Emory University, and labeled using Molecular probes N-hydroxy succinimidyl biotin	N/A
Anti-IFNAR1 antibody (MAR1-5A3)-Unconjugated/Functional Grade	Bio X Cell	Cat. BE0241
Anti-IFNGR antibody (GR-20)-Unconjugated/Functional Grade	Bio X Cell	Cat. BE0029
Mouse IgG1 isotype-Unconjugated	Bio X Cell	BE0083
Rat IgG2a (2A3) isotype – Unconjugated	Bio X Cell	BE0089
Rat IgG isotype-Unconjugated	Jackson Laboratory	Cat. 012-900-002
Bacterial and Virus Strains		
Lymphocytic Choriomeningitis Virus Clone 13 (LCMV13)	Dr. M. Oldstone, Scripps Research Institute	N/A
LCMV13-GFP	Dr. J. C. de la Torre, Scripps Research Institute (Emonet et al., 2009)	N/A
Influenza A/PR8	Originally obtained from Dr. B. Barber, University of Toronto	N/A
Chemicals, Peptides, and Recombinant Proteins		
Fixable viability dye eF506	eBioscience	Cat. 65-0866-14
Streptavidin-APC (flow cytometry)	eBioscience	Cat. 17-4317-82
Streptavidin-PE (flow cytometry)	eBioscience	Cat. 12-4317-87
Streptavidin-BUV395 (flow cytometry)	BD	Cat. 564176
Murine IFNβ	PBL Assay Science	Cat. 12405-1
Murine IFNα4	PBL Assay Science	Cat. 12115-1

Murine IFNγ	Peprotech	Cat. 315-05
Brewer Thioglycollate	Sigma-Aldrich	Cat. B2551-500G
BD Cytofix	BD	Cat. 554655
O-Phenylenediamine	Sigma-Aldrich	Cat. P9029-50G
TRIzol	Invitrogen	Cat. 15596018
Collagenase IV	Invitrogen	Cat. 17104019
Percoll	GE healthcare	Cat. 17089101
Critical Commercial Assays		
Foxp3 Transcription Staining Buffer Set	eBioscience	Cat. 00-5523-00
EasySep Biotin Positive Selection Kit	StemCell Technologies	Cat. 18559
RT^2 First Strand Kit	Qiagen	Cat. 330401
RT2 SYBR Green qPCR Mastermix	Qiagen	Cat. 330502
Mouse Type I Interferon Response RT^2 Profiler PCR Array	Qiagen	Cat. 330231
Experimental Models: Organisms/Strains		
C57BL/6NCrl	Charles River Laboratories	Cat. 027
Software and Algorithms		
RT^2 Profiler PCR Array Data Analysis	Qiagen	N/A
Prism v6	GraphPad	N/A
BioRender	BioRender Inc.	N/A

METHOD DETAILS

Flow Cytometry

Freshly isolated spleens were processed through 70µm cell strainers to create single cell suspension. Following perfusion, mediastinal lymph nodes and lung tissues were first digested with collagenase IV and DNase I for 45min at 37 °C on a shaker prior to the use of cell strainers. All samples, where indicated, were then treated with Fc block for 10 minutes at 4°C followed by surface staining for 30 minutes at 4°C, except for MerTK and GITRL, which were stained at room temperature for 30 minutes between Fc block and the remaining surface staining. Samples from infected mice were fixed in 4% paraformaldehyde or BD Cytofix following surfacing staining. Intracellular staining, where applicable, was conducted with 30 min incubation at 4°C (with the exception of anti-LCMV NP (1.1-3) at room temperature) following surface staining described above and permeabilization with Foxp3 Transcription Factor Staining Buffer Set. See Key Resources Table for the complete list of antibodies used for flow cytometry staining (i.e.

surface and intracellular). Data were acquired on a Fortessa X20 and LSRII Fortessa with FACSDiva software. Data analyses were performed using FlowJo v10.

In vivo IFNAR-1 Blockade and IFN*β* Infusion

For *in vivo* IFNAR-1 blockade, 500μg of αIFNAR-1 antibody or IgG1 isotype were injected intraperitoneally at day -1 and day 0 (total of 1mg/mouse) prior to LCMV13 infection. Alternatively, for IFNβ infusion experiments, a single dose of 25,000U IFNβ was injected intravenously at either day 5 or day 8 p.i

In vivo IFNGR Blockade

For in vivo IFNGR blockade, 500ug of aIFNGR antibody or IgG2a isotype were injected intraperitoneally at day 1, 2, and 3 (total of 1.5mg/mouse) post LCMV13 infection for day 5 p.i. readout.

In vitro thioglycolate-elicited Macrophage Assays

Mice were injected intraperitoneally with 3% Brewer Thioglycolate medium aged at room temperature for at least 3 months. Thioglycolate elicited macrophages were harvested with 10% FBS-PBS at 4 days post-injection and cultured in complete media supplemented with combinations of IFNα4, IFNβ, IFNγ, αIFNAR-1 antibody, αIFNGR antibody, IgG1 isotype control, IgG2a isotype control, cycloheximide, and LCMV13-GFP as indicated in the figures.

IFN-I Response RT^2-PCR Array

Total splenocytes pooled from 20 wildtype mice at day 1 p.i. were depleted of T and B cells with biotinylated αCD3 and αCD19 antibodies using EasySep Biotin Positive Selection Kit. pDC, inflammatory APC and cDC subsets were sorted from samples by flow cytometry. RNA was extracted using RNAeasy Mini Kit and converted into cDNA using RT^2 First Strand Kit. Samples were then prepared with RT2 SYBR Green qPCR MasterMix and read using Mouse Type I Interferon Response RT^2 Profiler PCR Array with CFX384 Touch™ Real-Time PCR Detection System. Analyses were performed on the RT^2 Profiler PCR Array Data Analysis system. CT threshold was set at 40 cycles; gene expression was normalized by arithmetic mean to 5 housekeeping genes- *Actb, B2m, Gapdh, Gusb, Hsp90ab1*. 63 of 96 genes analyzed remained following selection and are presented in ClusterGram**.**

STATISTICAL ANALYSIS

Statistical analyses and graphs were generated using GraphPad Prism v6. Paired or unpaired two tailed, non-parametric student's t-tests were applied in these graphs as detailed in their respective figure legends. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *and* **** $p < 0.0001$ were applied.

Chapter 3

Results

Part I. Dichotomous expression of TNF family ligands during viral infection

Part I of this thesis section was previously reported in "Chang YH, Wang KC, Chu KL, Clouthier DL, Tran AT, Torres Perez MS, Zhou AC, Abdul-Sater AA, Watts TH. Dichotomous Expression of TNF Superfamily Ligands on Antigen-Presenting Cells Controls Post-priming Anti-viral CD4(+) T Cell Immunity. Immunity. 2017 Nov 21;47(5):943-958" and is reproduced here with only minor changes. Figures 1, 2A, 4A-C were also included in Yu-Han Chang's MSc thesis, 2016, but are included again here for completeness. Figures 1-5 were a collaboration between Yu-Han Chang and myself, with additional help from Kuan-Lun Chu on Figure 3B.

3.1.1. Dichotomous expression of B7 and TNF family ligands on classical DCs versus monocyte-derived inflammatory APCs

Previous work from our lab had shown that GITRL was detected on F4/80^{+} CD11b^{hi} cells early on during LCMV13 infection peaking at day 2 p.i. (Clouthier, Zhou and Watts, 2014). Nevertheless, many different subsets of cDCs, monocyte-derived APCs, and macrophages express both F4/80 and CD11b (Segura and Amigorena, 2013). Therefore, when Chang started his Master's thesis, he set out to delineate the subsets of APCs that express GITRL. Using flow cytometry, he devised a 11-parameter panel that allowed him to separate the 6 major subsets of APCs during LCMV13 infection (**Figure 1A**). After gating out T and B cells, which were reported to express minimal levels of GITRL (Clouthier, Zhou and Watts, 2014), CD64 and the high affinity IgE receptor, known as FcεR1, were used to distinguish monocyte-derived inflammatory APCs from cDCs and macrophages (Langlet *et al.*, 2012; Plantinga *et al.*, 2013; Segura and Amigorena, 2013). Inflammatory APCs were further divided into CD11b^{+} CD11c^{+} (inflammatory DCs) or CD11b^{hi} CD11c^{-} (inflammatory macrophages) (**Figure 1A**). Gating on the non-inflammatory populations, cDCs (MHC II and CD11c positive) were identified as CD8α^{+} and CD8α^{-} cDCs (Guilliams *et al.*, 2014). F4/80 and CD11b were used to identify red pulp macrophages and F4/80^{-} CD11b^{+} myeloid cells within the non-inflammatory populations (**Figure 1A**). To confirm the identities of these APC subsets, scatter parameter, transcription factors and surface markers were also used in separate panels (**Figure 1B**). Higher granularity (SSC) was seen on inflammatory macrophages and red pulp macrophages over the DCs. cDCs were different from the inflammatory APCs by showing the signature transcription factor zbtb46 (Satpathy *et al.*, 2012; Murphy *et al.*, 2016) (**Figure 1B**). Inflammatory macrophages were distinguished from inflammatory DCs by the expression of the macrophage marker, MerTK (Gautiar *et al.*, 2012) (**Figure 1B**). As reported in the literature, monocyte-derived inducible regulatory DCs (iregDC) (PD-L1^{hi} CD39^{+} CD95^{+}) accumulate with LCMV13 infection (Wilson *et al.*, 2012; Cunningham *et al.*, 2016). However, we did not observe CD39 nor CD95 expression amongst the 6 subsets investigated (**Figure 1B**). Moreover, inflammatory APCs did not show preferential expression of the inhibitory ligand, PD-L1, (**Figure 2A**), which also distinguished them from iregDC (Wilson *et al.*, 2012; Cunningham *et al.*, 2016).

Looking at the subsets of APCs, we observed dichotomous expression of TNF family ligands versus B7 family ligands on inflammatory APCs versus cDCs. At day 2 post LCMV13 infection, cDCs showed higher mean fluorescent intensity (MFI) of MHC II, CD80 and CD86 when compared to inflammatory APCs and red pulp macrophages (**Figure 2A**). In contrast, inflammatory APCs showed higher MFI of TNF family ligands, GITRL, 4-1BBL, CD70, and OX40L than cDCs (**Figure 2B**). Using separate panels, we showed that pDCs, NK cells, and neutrophils expressed only minimal levels of the 4 TNF family ligands compared to inflammatory APCs at day 2 p.i. (**Figure 2C,D,E**).

To further investigate the dichotomous expression of TNF versus B7 family ligands and provide additional validation of the results from the 11-color panel described above, we employed a simpler gating strategy and co-stained cells for costimulatory molecules, MHC II, and Ly6C, a monocyte-specific marker (**Figure. 3**). Similar to the results shown in figure 2, a dichotomy in costimulatory molecule expression was observed between $CD80^{hi}$ versus $CD80^{int}$ $GITRL^{hi}$ subsets. $CD80^{hi}$ APC had the highest MFI of MHC II and CD86 after LCMV13 infection but low expression of Ly6C and TNF family ligands, showing similar characteristics of cDCs. Conversely, $GITRL^{hi}$ APC had the highest MFI of Ly6C as well as 4-1BBL, OX40L and CD70 but lower expression of MHC II, CD80 and CD86, showing characteristics of inflammatory APCs (**Figure 3A**).

To determine whether this dichotomy is a general feature of viral infections, we also turned our attention to TNF family ligand expression on APC subsets in the lung and draining (mediastinal) lymph node (mLN) during influenza A/Puerto Rico/8/1934 H1N1 (PR8) viral infection. Using the same co-staining panel, $GITRL^{hi}$ cells had higher MFI for Ly6C and 4-1BBL compared to $CD80^{hi}$ cells and $CD80^{hi}$ cells expressed higher CD86 and MHC II than the $GITRL^{hi}$ cells in the mLN during influenza infection (**Figure 3B**). These findings demonstrate that there is differential expression of B7 and TNF family proteins between cDCs and inflammatory APCs during the early stages of both a chronic and an acute viral infection.

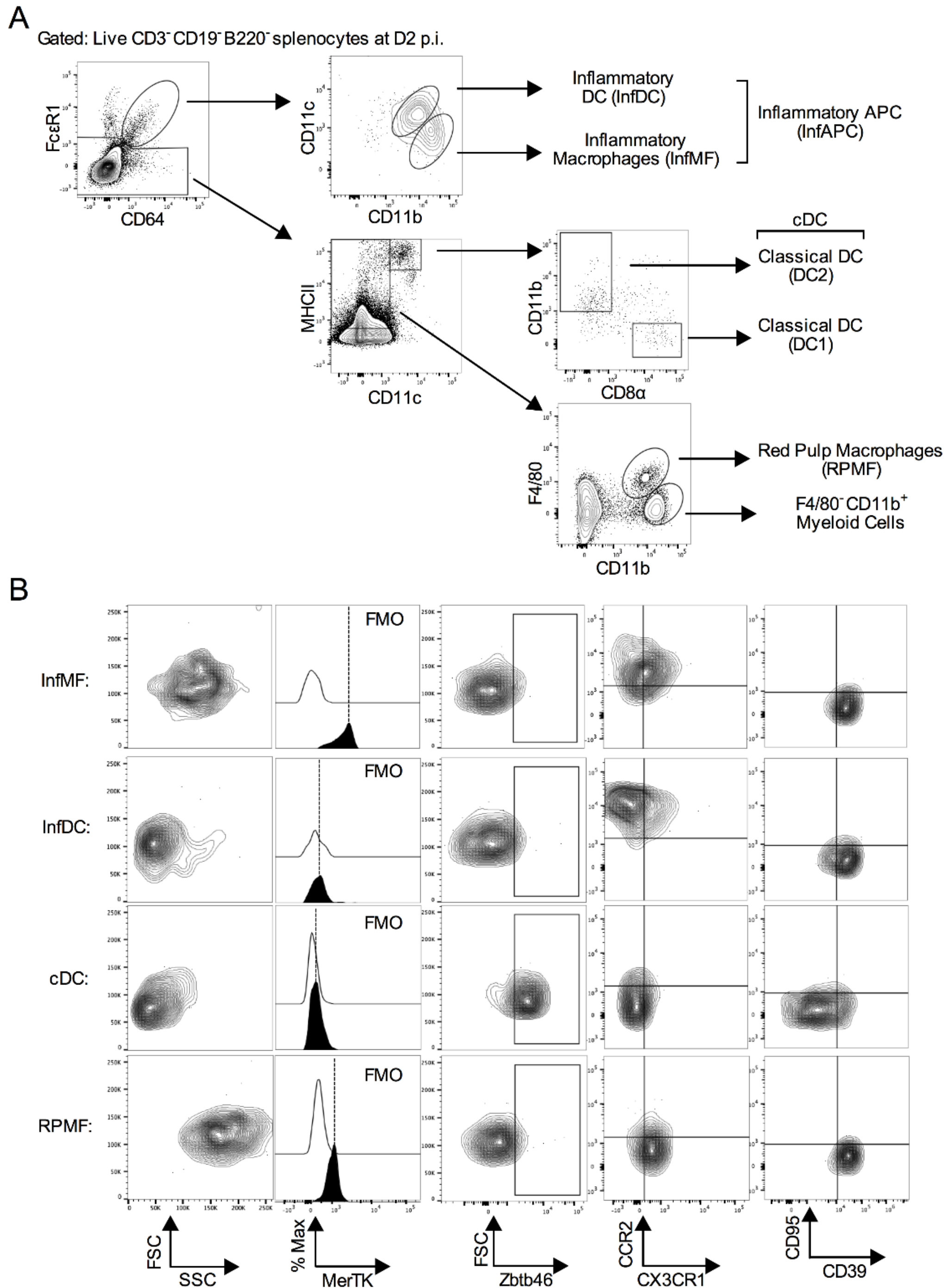
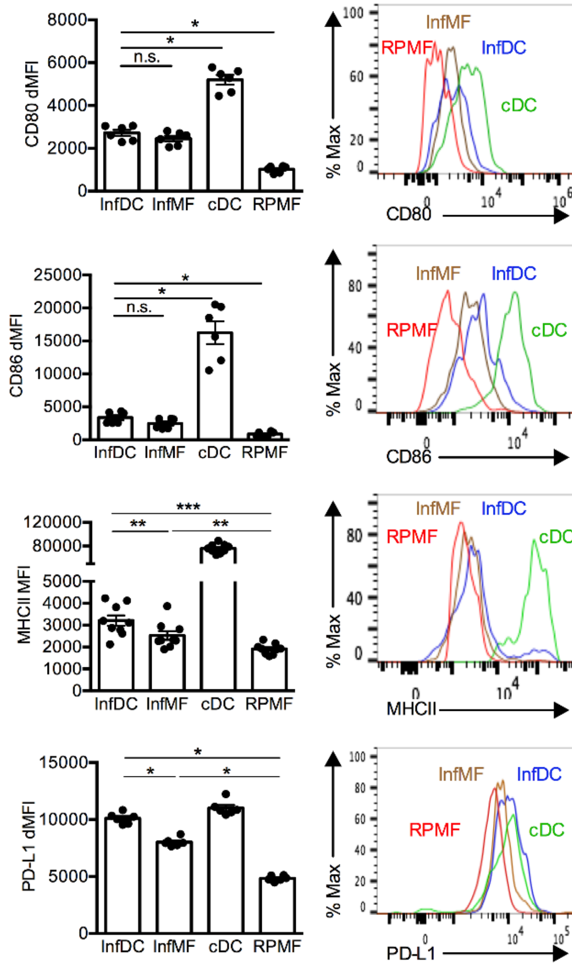
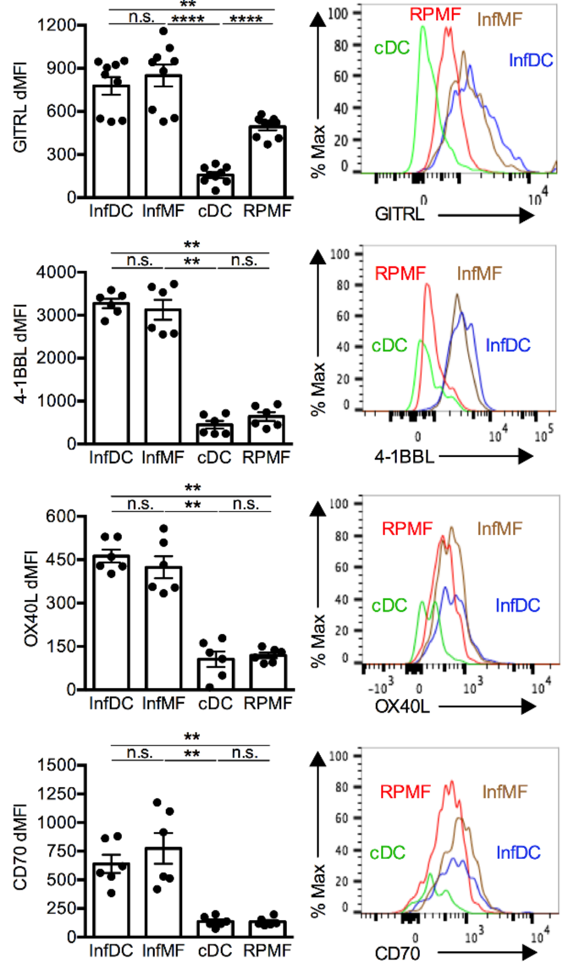
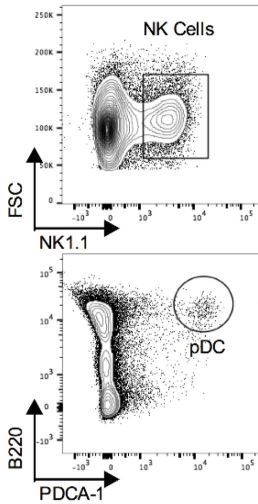
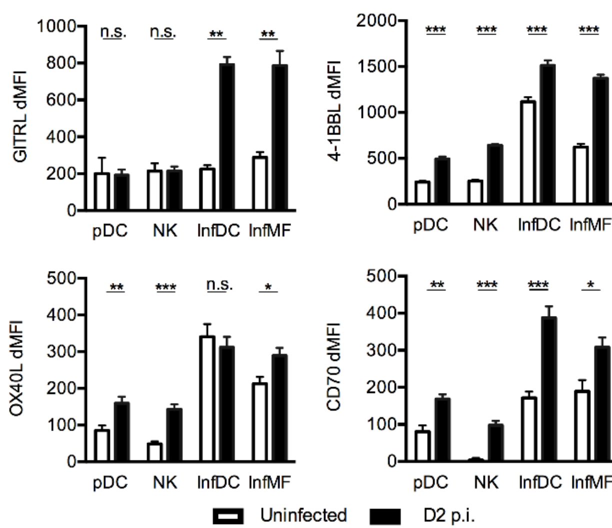


Figure 1 ***Splenic APC subsets during LCMV13 infection can be delineated by multiparameter flow cytometry*****.** (A) Gating strategy for classical DCs (cDCs), red pulp macrophages (RPMFs), F4/80$^-$ CD11b$^+$ myeloid cells and inflammatory APC (InfAPC) subsets, including both the inflammatory DCs (InfDCs) and inflammatory macrophages (InfMFs), is shown. Representative flow cytometry plots are shown pre-gated on Live CD3$^-$ CD19$^-$ B220$^-$ splenocytes from D2 p.i. (B) Splenic APC subsets from D2 p.i. were compared by flow cytometry for side scatter (SSC), MerTK, Zbtb46, CCR2, CX3CR1, CD39 and CD95.


A
CD80 dMFI
InfDC InfMF cDC RPMF
n.s.
% Max
CD80
CD86 dMFI
CD86
MHCII MFI
MHCII
PD-L1 dMFI
PD-L1


B
GITRL dMFI
InfDC InfMF cDC RPMF
n.s.
% Max
GITRL
4-1BBL dMFI
4-1BBL
OX40L dMFI
OX40L
CD70 dMFI
CD70


C
Gated: Live CD3⁻CD19⁻ splenocytes
NK Cells
FSC
NK1.1
pDC
B220
PDCA-1


D
GITRL dMFI
4-1BBL dMFI
OX40L dMFI
CD70 dMFI
pDC NK InfDC InfMF
n.s.
Uninfected
D2 p.i.

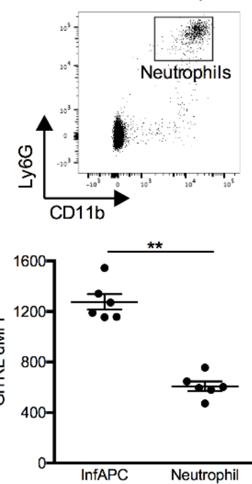

E
Gated: CD3⁻CD19⁻ B220⁻splenocytes
Neutrophils
Ly6G
CD11b
GITRL dMFI
InfAPC
Neutrophil

Figure 2. ***Antigen presenting cell subsets show dichotomous expression of CD28 and TNFR family ligands.*** (A-B): Splenic APC subsets from D2 p.i. with LCMV13 were analyzed by flow cytometry using the gating strategies described in Figure 1A for (A) CD80, CD86, MHCII, and PD-L1 (B) GITRL, 4-1BBL, OX40L and CD70. Right: representative histograms. Left: summary plots. pDC and NK cells were (C) identified from $CD3^-$ $CD19^-$ splenocytes and (D) analyzed for GITRL, 4-1BBL, OX40L and CD70 expression using flow cytometry. (E) Neutrophils were identified from $CD3^-$ $CD19^-$ $B220^-$ splenocytes at D2 p.i and analyzed for GITRL expression by flow cytometry. Data in (A-E) represent mean ± SEM with 6-9 mice pooled from 2-3 independent experiments. Two-tailed, non-parametric paired t-test was used for statistical analysis for all experiments. dMFI refers to the MFI for the specific antibody stain minus the FMO control.

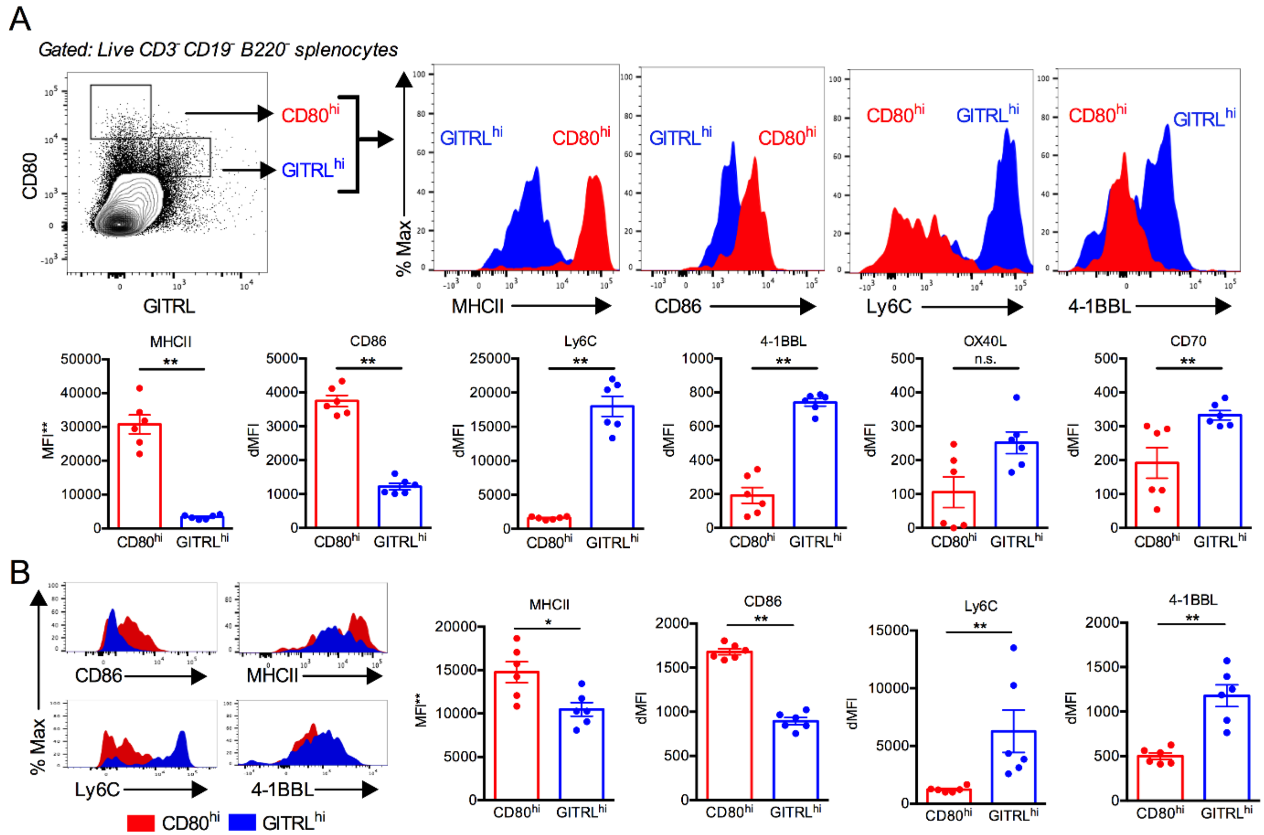


Figure 3. ***Dichotomous expression of CD28 and TNFR family ligands in both LCMV13 and Influenza A/PR8 viral infections.*** (A) Splenic APC subsets from D2 p.i. with LCMV13 were analyzed by flow cytometry where GITRL^{hi} and CD80^{hi} subsets from CD3^{-} CD19^{-} B220^{-} splenocytes were analyzed for MHCII, CD86, Ly6C, 4-1BBL, OX40L and CD70. Data are displayed as mean ± SEM, from 6 mice pooled from 2 independent experiments. (B) Mice were infected intranasally with Influenza A/PR8 and draining LNs analyzed on D3 p.i. Gating on CD80^{hi} or GITRL^{hi} populations was conducted as in 3A. Representative histograms are shown at left and summary data shown at right for 6 mice pooled from 2 independent experiments. dMFI refers to the MFI for the specific antibody stain minus the FMO control. MFI** reports the raw MFI for MHC II stain. Two-tailed, non-parametric paired t-test was used for statistical analysis for all experiments.

3.1.2. IFN-I coordinately regulates TNF family ligand expression during LCMV13 infection

Focusing on GITRL, we next analyzed the kinetics of expression. GITRL protein peaked at day 2 p.i., with the highest expression on the two inflammatory APC subsets before declining to below baseline by day 8 p.i. Furthermore, *Ifna, Ifnβ1*, and *Isg*15 showed a similar expression kinetics that slightly preceded *Tnfsf18 message* (encoding GITRL) at day 1 p.i., suggesting that GITRL could be regulated by IFN-I (Chang, 2016). Thus, in order to test this hypothesis, we incubated thioglycolate-elicited peritoneal macrophages (TG MF) in various conditions to investigate IFN-dependent induction of GITRL *in vitro*. IFNβ and IFNα4 each induced dose-dependent GITRL surface expression (**Figure 4A**), dependent on IFN-I receptor (IFNAR1) binding (**Figure 4B**). Also, washout of IFN-I from the cultures led to a rapid decline in GITRL levels (**Figure 4C**). To extend the analysis, we also measured 4-1BBL, OX40L and CD70. All three TNF family ligands were also inducible by IFN-I (**Figure 4D**), and the induction was also dependent on IFNAR1 (**Figure 4E**).

To determine whether IFN-I was required for LCMV-mediated induction of the TNF family ligands *in vivo*, mice were treated with isotype control or anti-IFNAR1 blocking antibody at days -1 and 0 p.i. and GITRL protein level was measured at its peak (day 2 p.i.). The MFI of all 4 TNF family ligands was significantly blocked by anti-IFNAR1 treatment *in vivo* across most APC subsets, most prominently on inflammatory APCs (**Figure 5A**). Expression of MHC II, CD80, and CD86 was also dependent on IFNAR during LCMV13 infection (**Figure 5B**). However, in contrast to TNF and B7 family ligands, MHC II expression was enhanced by IFNAR blockade (**Figure 5B**). This could be explained by the observation that a higher proportion of APCs were infected by LCMV13 following IFNAR blockade, along with higher viral load detected (**Figure 5C**). Consequently, interferon independent effects of viral PAMPs due to increased viral load could lead to the increase in MHC II expression on cDCs and inflammatory APCs, OX40L expression on inflammatory DC, and CD80 expression on inflammatory APCs after IFNAR blockade (**Figure 5A,B**). Thus, IFN-I coordinately induces several TNF family ligands on APCs during LCMV13 infection, with enhanced induction on inflammatory APCs.

By measuring the level of IFNAR on each APC subset pre-infection, as well as the level of pSTAT1 at day 1 post LCMV infection, Chang et al. showed that the overall ability to respond

to IFN-I did not fully explain the dichotomy in costimulatory molecule expression on different APC subsets (Chang, 2016). For example, despite having the highest MFI of IFNAR1 pre-infection and the highest MFI of phospho-STAT1 (pSTAT1) post-infection than other APCs (Chang, 2016), pDCs expressed only marginal surface levels of TNF family ligands (**Figure 2D**). Thus, we asked whether differences in TNF family versus B7 family induction on APCs could be a result of the induction of different IFN-induced genes in different APC subsets. Using the Qiagen qPCR array, we observed that pDCs, cDCs, and inflammatory APCs sorted from infected mice at day 1 p.i. showed distinct expression patterns of IFN-I induced genes (**Figure 6A,B**). Taken together, the above data demonstrated that while IFN-I is a general regulator of both B7 and TNF family molecule induction on APC, its effects are cell type specific, with IFN-I preferentially inducing B7 family ligands on cDCs, and higher surface expression of the four TNF family ligands on monocyte derived APCs.

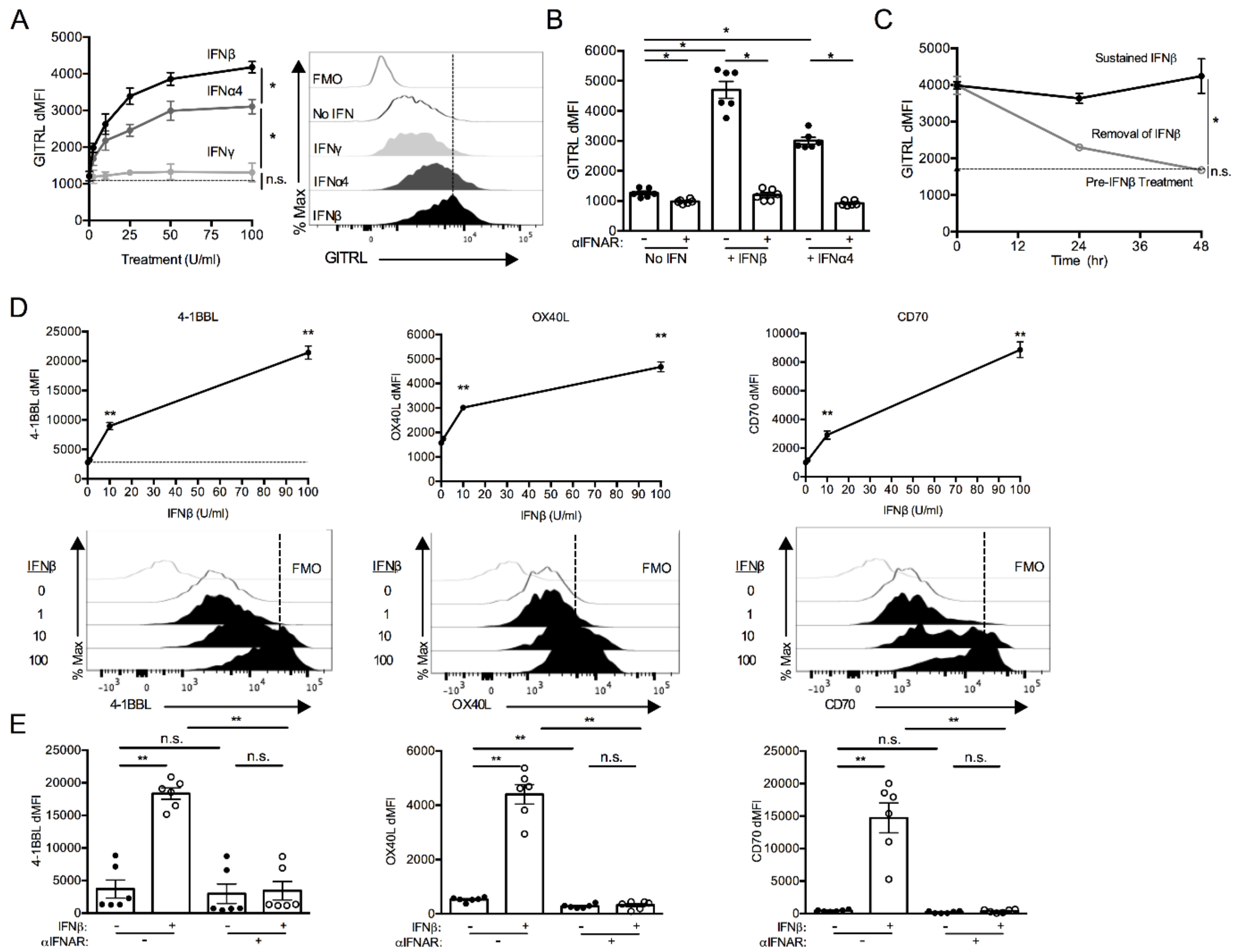


Figure 4. ***IFN-I induces GITRL protein and Tnfsf18-reporter activity in vitro.*** (A-E): Thioglycolate-elicited macrophages (TG MF) were harvested from mouse peritoneal cavity for *in vitro* culture. (A) TG MF were treated with IFNα4, β or γ at 0 - 100 U/ml for 24 hrs and analyzed for GITRL expression by flow cytometry with summary data and representative histograms shown. (B) TG MF were pre-treated with 10μg/ml of IFNAR blocking antibody or isotype control 1 hr prior to culturing with 0 or 100 U/ml of IFNα4 or IFNβ for 24hrs, followed by flow cytometry analysis of GITRL expression. (C) TG MF pre-treated *in vitro* with 25U/ml of IFNβ for 24 hrs were placed in fresh media with either 0 or 25U/ml of IFNβ. GITRL expression was analyzed at 0, 24 and 48 hrs following change of media. (D) TG MF were treated with IFNβ at 0, 1, 10 or 100 U/ml for 24 hrs and analyzed for expression of 4-1BBL, OX40L and CD70 by flow cytometry. (E) TG MF were pre-treated with 10μg/ml of IFNAR blocking antibody or isotype control 1 hr prior to culturing with 0 or 100 U/ml IFNβ. Expression of TNF family ligands as indicated was analyzed after 24 hrs by flow cytometry. Data in (A-E) represent mean ± SEM of 3-6 independent cultures harvested from separate mice. Two-tailed, non-parametric paired t-test was used for statistical analyses (A-E).

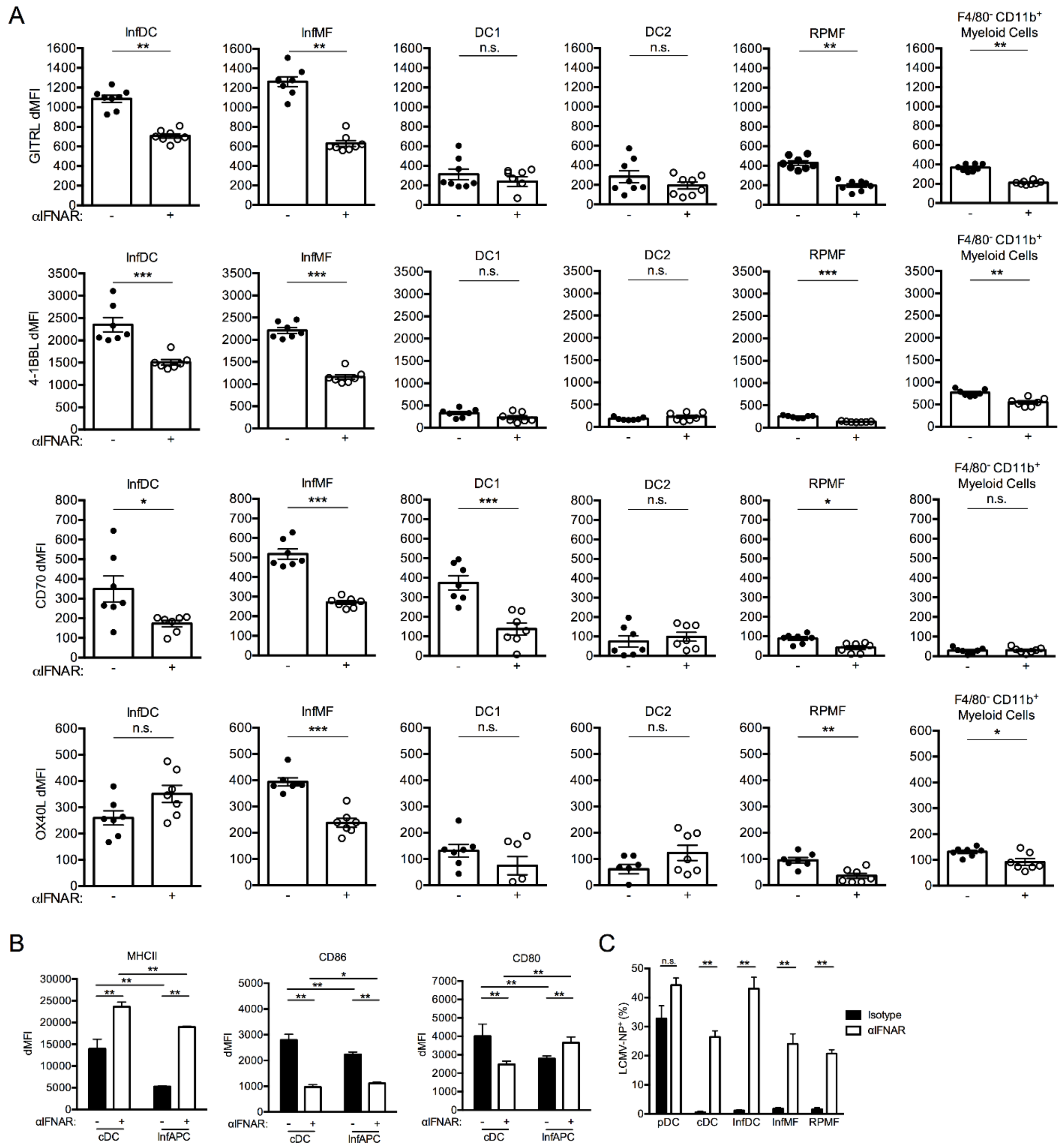


Figure 5. ***IFN-I regulates TNF family induction during LCMV clone 13 infection in vivo.*** Mice were injected i.p. with IFNAR blocking or isotype antibodies at D-1 and 0 prior to LCMV13 infection (500µg each injection). Splenic APC subsets was analyzed by flow cytometry at D2 p.i. using gating strategy defined in Figure 1: (A) GITRL, 4-1BBL, CD70 and OX40L surface expression was analyzed. (B) MHC class II, CD80 and CD86 expression was analyzed. (C) Percent infection of APC subsets by LCMV13 was measured at D2 p.i. using intracellular flow cytometry staining for LCMV-NP. Bars show mean ±

SEM of 6 to 8 mice pooled from 2 independent experiments. Two-tailed, non-parametric p unpaired t-test were performed for statistical analyses.

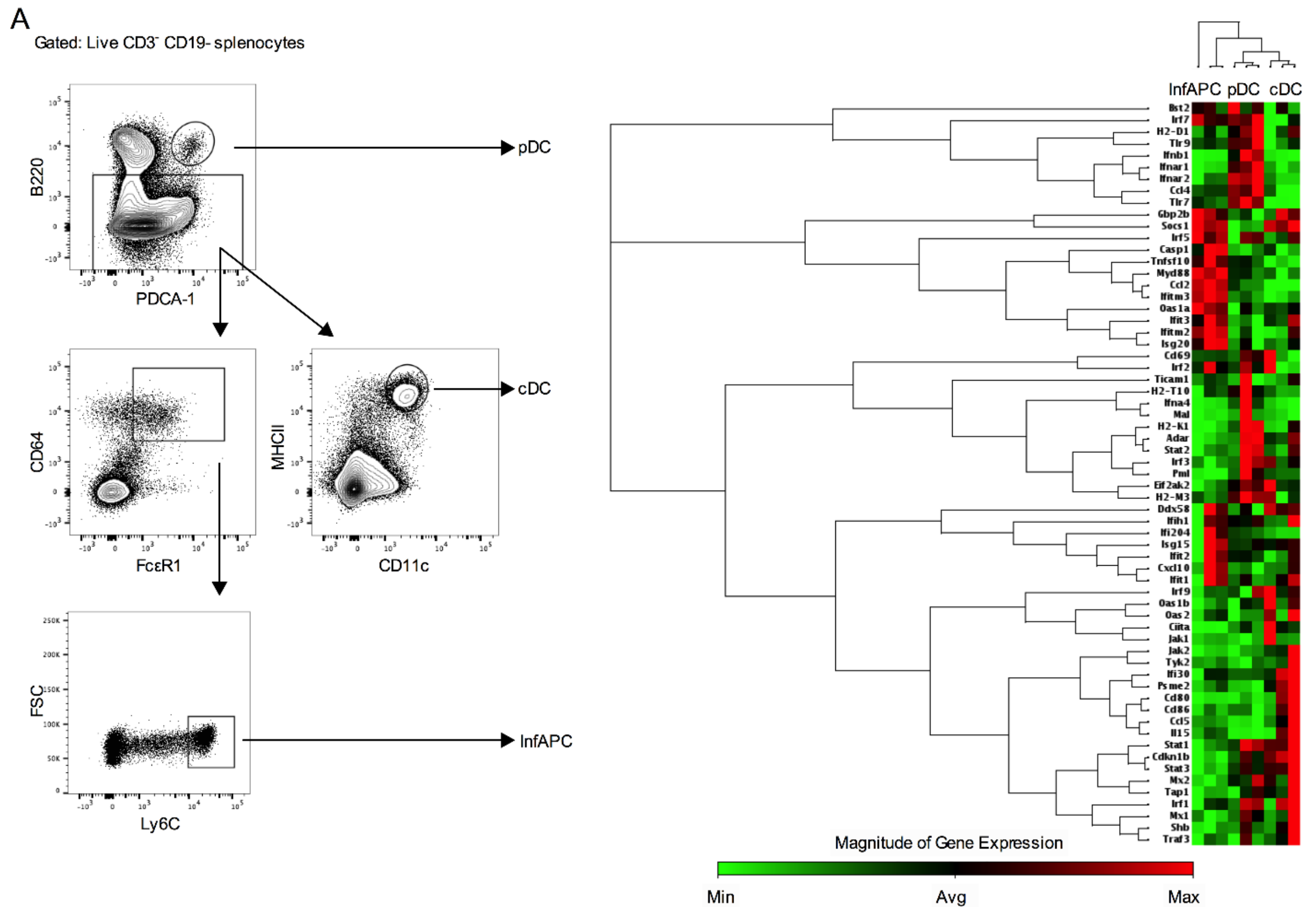


Figure 6. ***Type I IFN induces differential gene expression in APC subsets.*** (A, B) pDCs, cDCs and InfAPCs were sorted from splenocytes at D1 post-LCMV13 infection. RNA was isolated from individual APC subsets and analyzed for differential gene expression using the Qiagen Mouse Type I Interferon Response RT^2 Profiler PCR Array. (A) Sorting strategy. (B) ClusterGram illustrating differential expression of 63 IFN-I responsive genes from 3 independent sorting experiments.

3.1.3. Part I Conclusion

Overall, the results in part I of this thesis showed that during chronic infection with LCMV clone 13 as well as during acute respiratory infection with influenza A virus, TNF family ligands are preferentially induced on monocyte derived inflammatory APC. These findings provide evidence that the costimulatory signal provided by TNF family ligands is provided on a different cell type than the CD28-B7 family signal. Of note, in the LCMV clone 13 infection model, GITRL expression was limited to the first few days of infection and therefore, the first part of the thesis focused largely on the first few days after infection. In the next part of my thesis, I wanted to extend the analysis to investigate the expression of other TNF family members in response to different interferons over the course of LCMV infection.

Part II – Expression and regulation of TNF family ligands in later stages of infection and different tissues

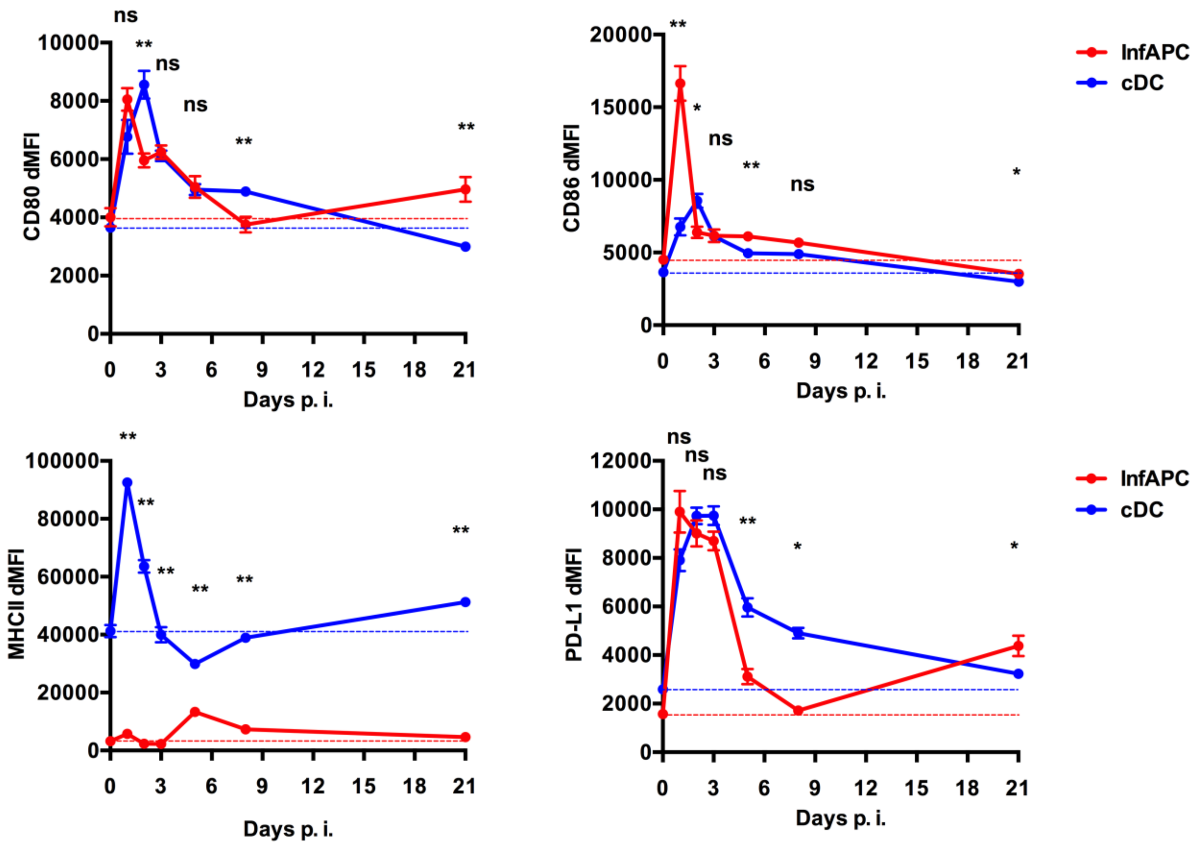
Part II of the thesis represents unpublished data not previously reported. All experiment in figures 9-15 were done by Kuan-Chung Wang with help from Kuan-Lun Chu with processing of mediastinal lymph node and lung tissues in Figures 9 and 10.

3.2.1. Expression kinetics of B7 and TNF family ligands on APC during LCMV13 infection

The work in part I of the thesis which showed dichotomous expression on TNF versus CD28 family members on APC, had largely focused on day 2 post-infection, the time of peak GITRL expression. Here, using the same 11-parameter flow cytometry panel with slight modifications, we investigated the expression of B7 and TNF family ligands across the entire 21-day period post LCMV13 infection. In contrast to the results observed at day 2 post infection (**Figure 2**), B7 family ligand expression was comparable between inflammatory APCs and cDCs across most of the 21-day period, except for the first 3 days, where there were significant differences at day 2 and 3 (**Figure 7A**). Noticeably, inflammatory APCs expressed higher levels of B7 family ligands day 1 p.i. and lower levels on day 2 p.i. than cDCs. Similarly, PD-L1 was higher on inflammatory APCs day 1 p.i. but cDCs took over and maintained higher levels on day 2 p.i. and onwards. MHC II expression stayed consistently higher on cDCs throughout the course of the experiment (**Figure 7A**).

Previous work had established that GITRL protein expression peaks at day 1.5 - 2 post LCMV clone 13 infection before declining below baseline between day 5 and 8 (Clouthier, Zhou and Watts, 2014; Chang, 2016). Here we investigated the kinetics of the other 3 TNF family ligands. Similar to what we had observed with GITRL expression, the expression of CD70, OX40L, and 4-1BBL was highest on inflammatory APCs and much lower on cDCs and pDCs, throughout the course of the infection. CD70 demonstrated a similar pattern to GITRL where it also peaked at day 2 p.i. before declining to at or below baseline levels by day 8 p.i. (**Figure 7B**) (Chang, 2016). In contrast, OX40L and 4-1BBL maintained higher levels of expression throughout the 21 days (**Figure 7B**).

A



B

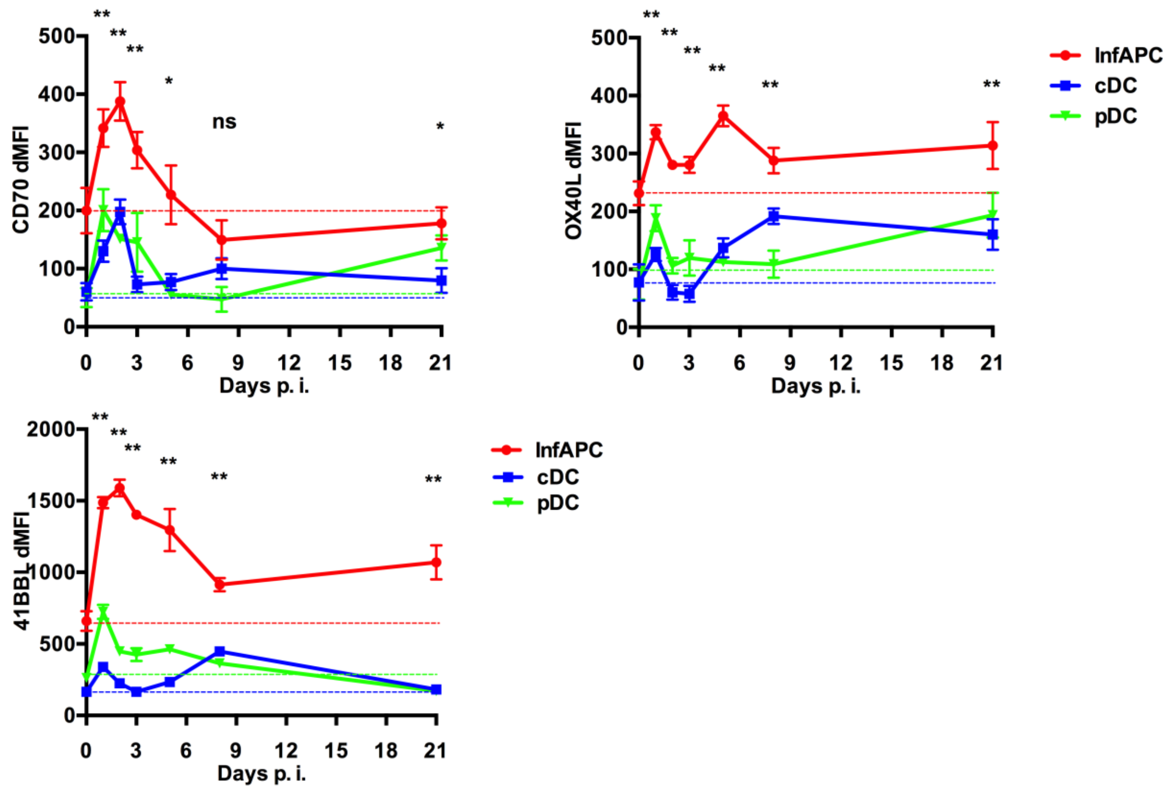
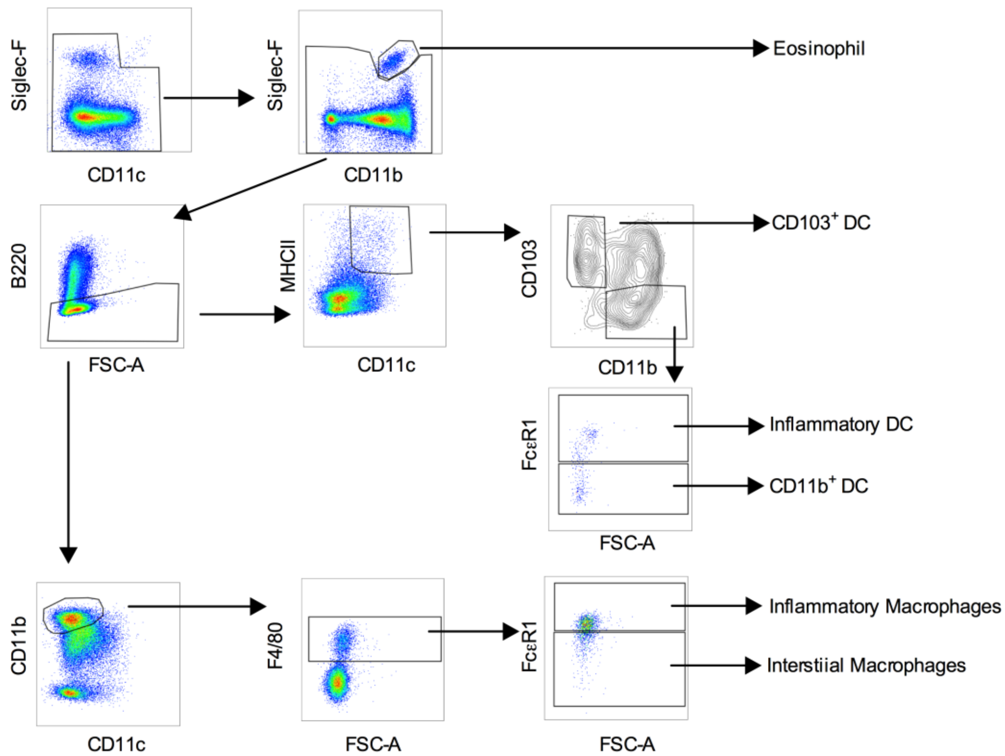


Figure 7. ***B7 and TNF family ligands in vivo expression kinetics*.** Splenocytes were analyzed by flow cytometry on D0 to D21 post-LCMV13 infection for (A) CD80, CD86, MHC II, and PD-L1 expression on cDCs and InfAPC. (B) And CD70, OX40L, and 4-1BBL on cDCs, InfAPC, and pDCs. Using the gating strategy from Figure 1 for cDCn and InfAPC and Figure 2C for pDCs. Results show the mean ± SEM of 6 to 9 mice pooled from at least 2 independent experiments.

3.2.2. B7-TNF dichotomy on different APC subsets in the lung and mLN during LCMV13 infection

To determine whether the dichotomy of expression between B7 and TNF family ligands on cDCs and inflammatory APCs is a specific feature to the spleen or a general feature across different organs and tissues, we investigated TNF family ligand expression in the lung after LCMV infection. We devised 12-parameter flow cytometry panels to characterize different APC subsets (**Figure 8**). Previous work in the lab had shown that $B220^+$ $PDCA1^+$ pDCs showed only minimal GITRL expression on pDC in both the lung and mLN at day 3 p.i with influenza virus. Thus, pDCs were not included in the panels (Chu *et al.*, 2018, submitted). Furthermore, GITRL expression kinetic in the lung during influenza infection, was shown to peak between day 3 and 5 p.i. (Chu *et al.*, 2018, submitted). Therefore, for our investigation of LCMV13 infection in the lung and mLN we chose to investigate day 3 p.i.. At day 3 post-LCMV infection in the lung, inflammatory APCs were again the main expressers of GITRL in both the lung and mLN over other migratory and tissue-resident APCs (**Figure 9A**). Medullary macrophages in the mLN also demonstrated high MFI of GITRL, however, the cell number was very low at day 3 p.i. and signals were weak (**Figure 9B**).

To ask if B7 and TNF family ligands showed dichotomous expression in the lung, we stained for B7 and TNF family ligands together with MHC II and Ly6C in the lung during LCMV13 infection. Similar to what was seen in the spleen at day 2 p.i., the dichotomy of expression of B7 and TNF family ligand on $CD80^{hi}$ versus $GITRL^{hi}$ subsets was observed in the lung and the mLN at day 3p.i., where the $GITRL^{hi}$ subset expressed higher levels of 4-1BBL and Ly6C and $CD80^{hi}$ subset expressed higher CD86 and MHC II, with the slight difference of $GITRL^{hi}$ subset also having slightly higher CD86 expression in the lung (**Figure 10**). Of note, by day 8 p.i., the dichotomy of expression was no longer observed in the lung nor the mLN. 4-1BBL expression was still moderately higher on $GITRL^{hi}$ subset in the lung but reversed in the mLN and Ly6C was higher on $CD80^{hi}$ subsets. The differential expression of MHC II and CD86 was also not observed significantly in both the lung and the mLN at day 8 p.i. (**Figure 10**). Therefore, the dichotomy of expression of B7 and TNF family ligand on cDCs versus inflammatory APCs appears to be limited to the early stages of LCMV infection.


A
Gated: Live CD3⁻ CD19⁻ Lung Single Cell Suspension at D3 p.i.
Siglec-F
CD11c
Siglec-F
CD11b
Eosinophil
B220
FSC-A
MHCII
CD11c
CD103
CD11b
CD103+ DC
FcεR1
FSC-A
Inflammatory DC
CD11b+ DC
CD11b
CD11c
F4/80
FSC-A
FcεR1
FSC-A
Inflammatory Macrophages
Interstiial Macrophages

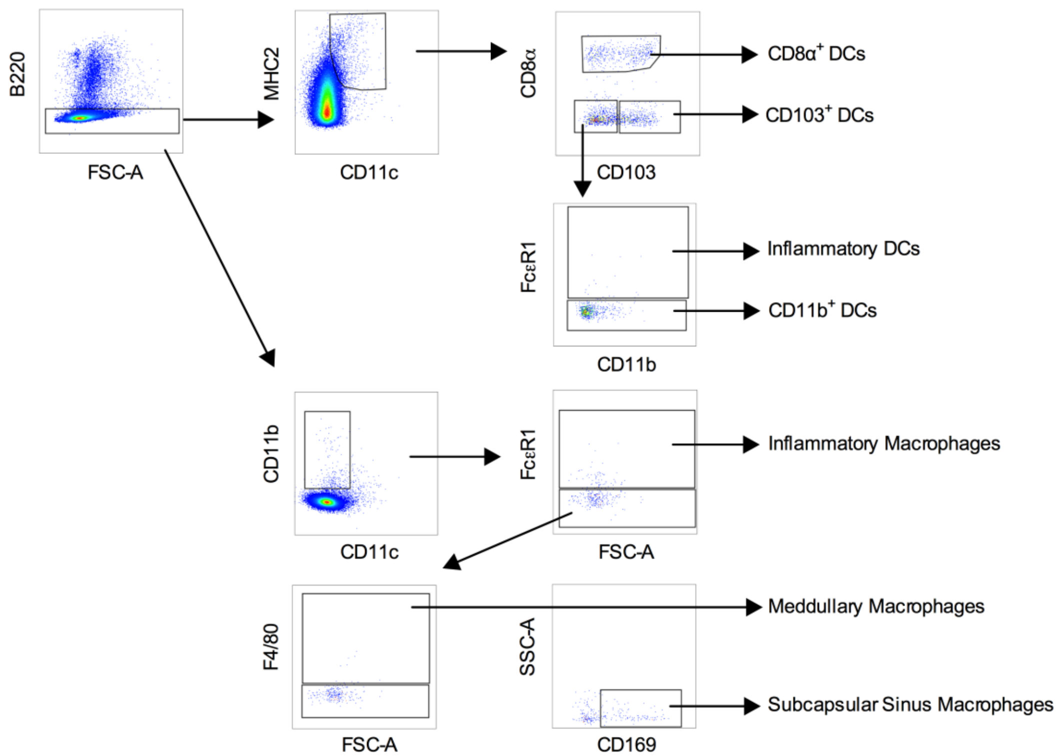

B
Gated: Live CD3⁻ CD19⁻ mLN Single Cell Suspension at D3 p.i.
B220
FSC-A
MHC2
CD11c
CD8α
CD103
CD8α+ DCs
CD103+ DCs
FcεR1
CD11b
Inflammatory DCs
CD11b+ DCs
CD11b
CD11c
FcεR1
FSC-A
Inflammatory Macrophages
F4/80
FSC-A
SSC-A
CD169
Meddullary Macrophages
Subcapsular Sinus Macrophages

Figure 8 ***Lung and mLN APC subsets during LCMV13 infection can be delineated by multiparameter flow cytometry*****.** (A) Gating strategy for Eosinophil, CD103+ DC, InfDC, CD11b+ DC, InfMF, and interstitial macrophages in the lung. Representative flow cytometry plots are shown pre-gated on Live $CD3^-$ $CD19^-$ lung single cell suspension from D3 p.i. (B) Gating strategy for CD8a+ DC, CD103+ DC, InfDC, CD11b+ DC, infMF, medullary macrophages, and subcapsular sinus macrophages in the mLN. Representative flow cytometry plots are shown pre-gated on Live $CD3^-$ $CD19^-$ mLN single cell suspension from D3 p.i.

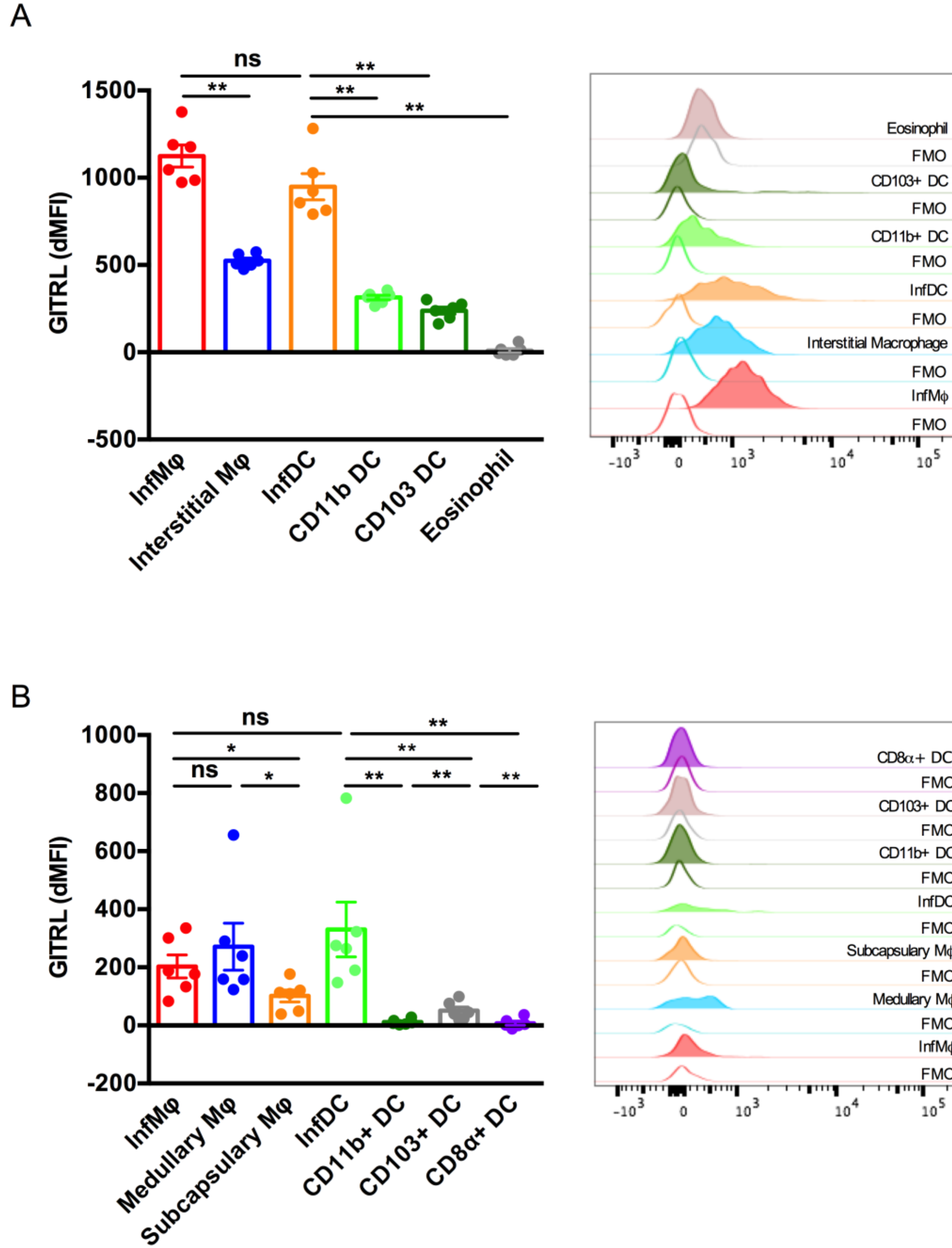
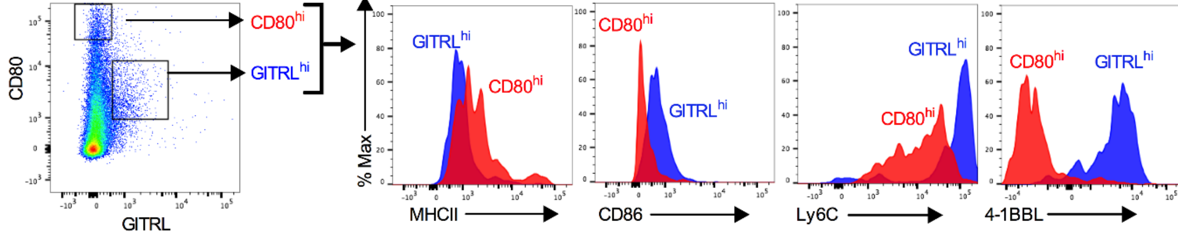
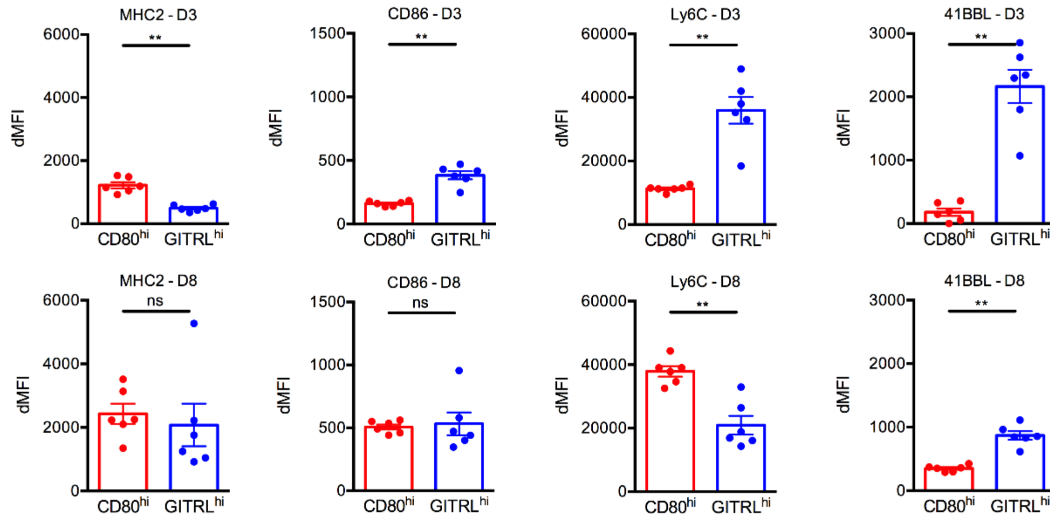


Figure 9 ***Inflammatory APC show highest expression of GITRL.*** (A-B): Lung and mLN APC subsets from D3 p.i. with LCMV13 were analyzaed by flow cytometry using the gating strategies described in Figure 20 for GITRL expression. Right: representative histograms. Left: summary plots. Data represent mean ± SEM with 6 mice pooled from 2 independent experiments. Two-tailed, non-parametric unpaired t-test was used for statistical analysis for all experiments. dMFI refers to the MFI for the specific antibody stain minus the FMO control.

A

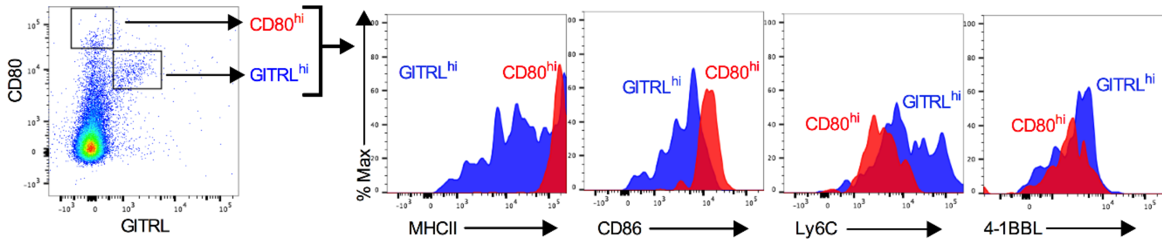
Gated: Live CD3⁻ CD19⁻ B220⁻ Lung Single Cell Suspension





B

Gated: Live CD3⁻ CD19⁻ B220⁻ mLN Single Cell Suspension



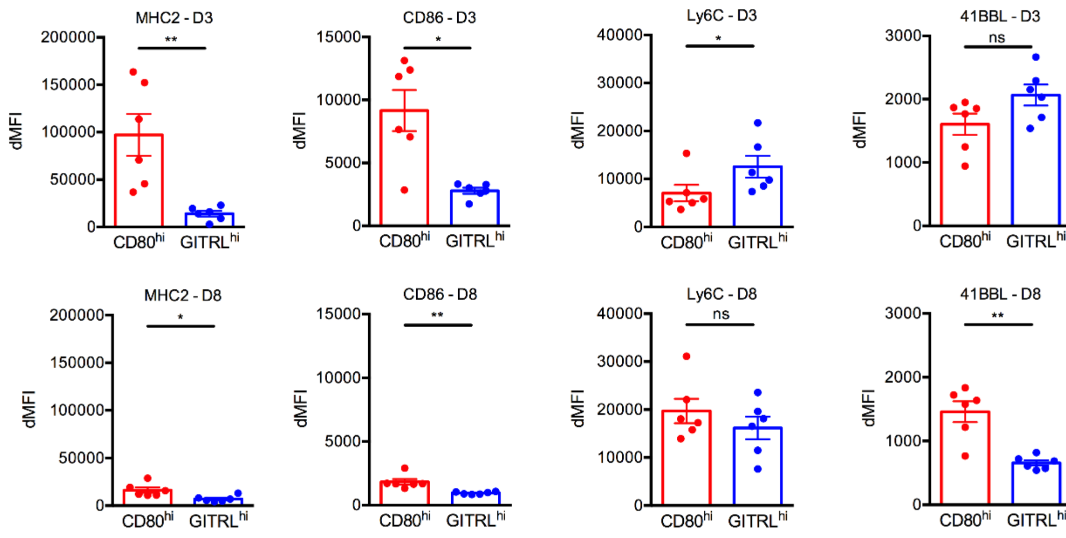


Figure 10 ***Dichotomous expression of CD28 and TNFR family ligands in lung and mLN after LCMV 13 viral infection***. (A) Lung and (B) mLN APC subsets from D3 and D8 p.i. with LCMV13 were analyzed by flow cytometry where $GITRL^{hi}$ and $CD80^{hi}$ subsets from $CD3^{-}$ $CD19^{-}$ $B220^{-}$ single cell suspension was analyzed for MHCII, CD86, Ly6C, and 4-1BBL. Data are displayed as mean ± SEM, from 6 mice pooled from 2 independent experiments. Representative histograms are shown at the top and summary data shown in the middle and at the bottom. dMFI refers to the MFI for the specific antibody stain minus the FMO control. Two-tailed, non-parametric paired t-test was used for statistical analysis for all experiments.

3.2.3. IFNγ regulation of TNF family ligands

The findings that 4-1BBL and OX40L maintained expression well into the later stages of the infection, whereas CD70 and GITRL showed more transient expression, suggest differential regulation. GITRL is regulated by type I interferon early during infection, however, this induction is lost after a few days likely due to a combination of reduced IFN production, receptor downregulation and interference of LCMV with the IFN response in infected cells (Chang, 2016). Thus, the continued expression of OX40L and 4-1BBL suggested that they may be maintained by signals other than IFN-I. As mentioned in the introduction, IFNγ is critical in viral control and its dynamic interaction with IFNβ is also important for LCMV13 persistence. Therefore, we first investigated IFNγ for its ability to induce 4-1BBL, CD70, and OX40L on thioglycolate-elicited peritoneal macrophages *ex vivo*. Previous work had shown minimal induction of GITRL by IFNγ (Chang, 2016). 4-1BBL was induced by IFNγ in a dose-dependent manner, and this was blocked by antibodies to the IFNγ receptor, IFNGR (**Figure 11A,B**). CD70 and OX40L also showed weak, but IFNGR dependent induction by IFNγ,

As 4-1BBL was most highly induced by IFNγ, we focused on 4-1BBL for *in vivo* experiments. To determine whether IFNγ was required for LCMV-mediated induction of the 4-1BBL *in vivo*, mice were treated with isotype control or anti-IFNGR blocking antibody at days 1, 2, and 3 p.i. and 4-1BBL protein level was measured at day 5 p.i. (**Figure 11C**). Looking at inflammatory APCs and cDCs, there was a reduction in the MFI of 4-1BBL on inflammatory APCs as compared to cDCs (**Figure 11C**), but this did not quite reach statistical significance. Thus, IFNγ may be involved in maintenance of expression of 4-1BBL in the later stages of LCMV13 infection.

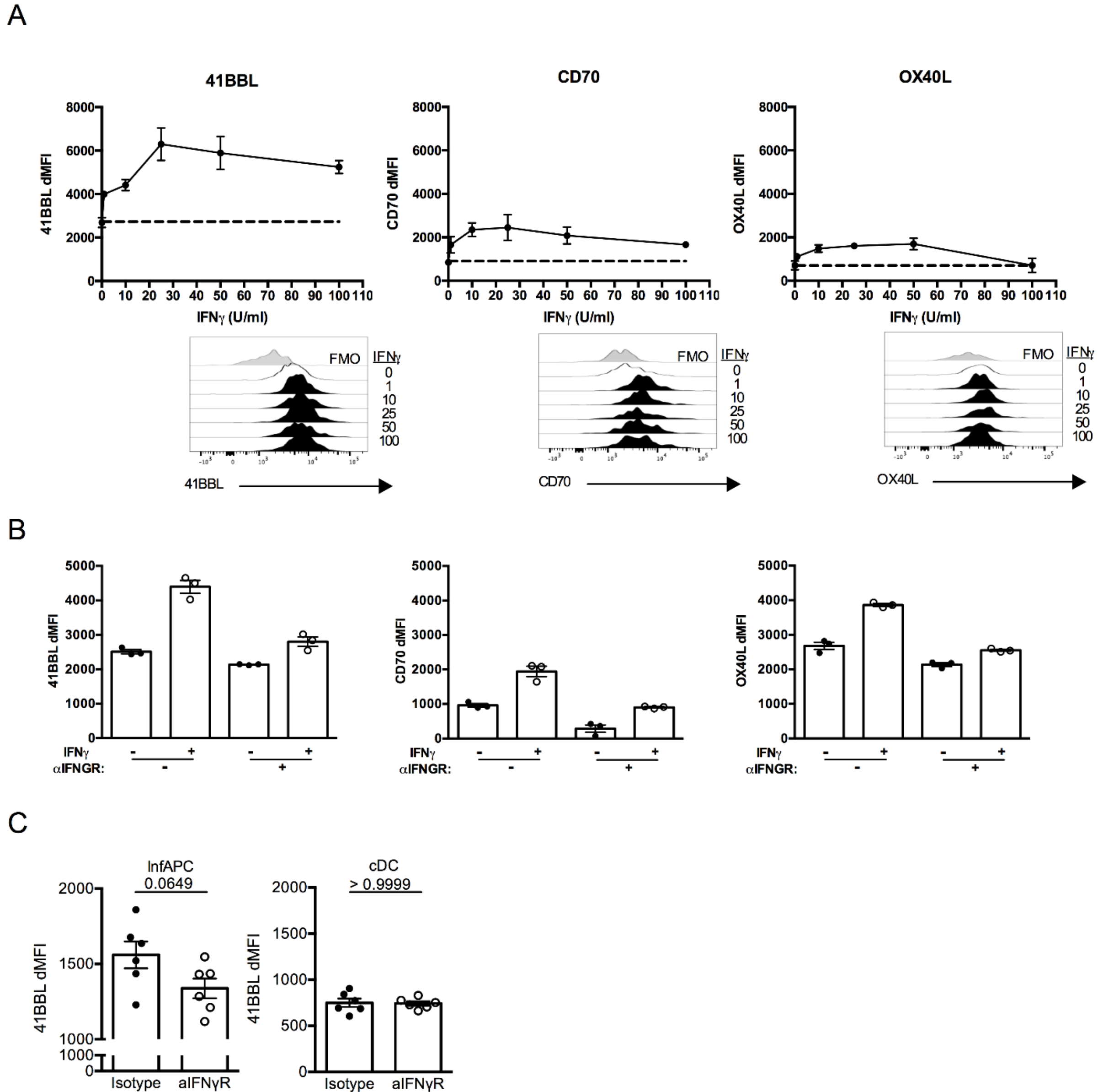


Figure 11. ***IFNγ induces TNF family ligand protein expression in vitro and in vivo*****.** (A-B): TG MF were harvested from mouse peritoneal cavity for *in vitro* culture. (A) TG MF were treated with IFNγ at 0 – 100U/ml for 24 hrs and analyzed for 4-1BBL, CD70, and OX40L expression by flow cytometry with summary data and representative histograms shown. Results represent mean ± SEM of 6 independent cultures harvested from separate mice and 2 independent experiments. (B) TG MF were pre-treated with 25ug/mL of IFNGR blocking antibody or isotype control 3 hr prior to culturing with 0 or 25 U/mL of IFNγ followed by flow cytometry analysis of the three ligands' expression. Results represent mean ± SEM of 1 experiment of 3 independent cultures harvested from separate mice but similar results were observed in another experiment of 3 independent cultures from separate mice. (C) Mice were injected i.p. with IFNGR blocking or isotype antibodies at D1, 2, and 3 p.i. (500μg each injection). Splenic APC subsets was analyzed by flow cytometry at D5 p.i. using gating strategy defined in Figure 1 for 4-1BBL surface expression. Bars show mean ± SEM of 6 mice pooled from 2 independent experiments. Two-tailed, non-parametric paired t-test was used for statistical analyses (A). Two-tailed, non-parametric p unpaired t-test were performed for statistical analyses (C).

3.2.4 LPS induction of TNF family ligands

Lipopolysaccharide (LPS) has been reported in the literature to induce 4-1BBL expression on peritoneal macrophages (Futagawa *et al.*, 2002). As LPS induces type I interferon in macrophages (Toshchakov *et al.*, 2002; Barton and Medzhitov, 2003; Bogdan, Mattner and Schleicher, 2004), we asked if LPS could induce TNF family ligands on TG MF and whether this was type I interferon dependent. LPS induced dose-dependent upregulation of 4-1BBL, CD70, and OX40L but not GITRL on TG MF *ex vivo* (**Figure 12A**). Interestingly, the induction by LPS was completely dependent on IFNAR for CD70 and OX40L. In contrast, LPS induction of 4-1BBL was only partially IFNAR dependent (**Figure 12B**). In sum, while GITRL expression is largely mediated by Type I IFN and restricted to the early stages of LCMV infection, 4-1BBL can be induced by both Type I and type II interferon and shows sustained expression throughout the first 3 weeks of LCMV clone 13 infection.

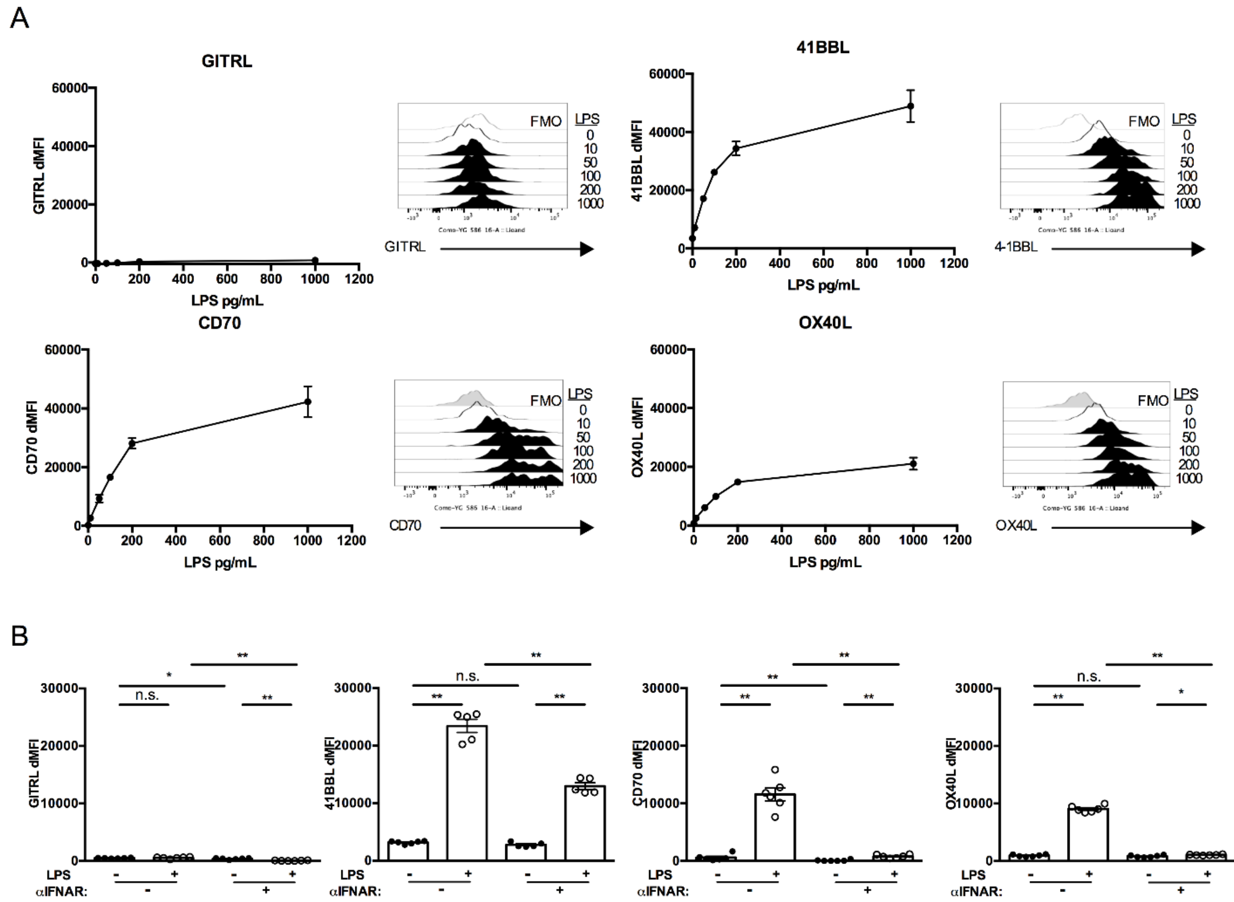


Figure 12 ***LPS induces TNF family ligand protein expression in vitro*****.** (A-B): TG MF were harvested from mouse peritoneal cavity for *in vitro* culture. (A) TG MF were treated with LPS at 0 – 1000pg/ml for 24 hrs and analyzed for GITRL, 4-1BBL, CD70, and OX40L expression by flow cytometry with summary data and representative histograms shown. (B) TG MF were pre-treated with 10ug/mL of IFNAR blocking antibody or isotype control 1 hr prior to culturing with 0 or 1000 pg/mL of LPS followed by flow cytometry analysis of the ligands' expression. Results represent mean ± SEM of 6 independent cultures harvested from separate mice and 2 independent experiments. Two-tailed, non-parametric paired t-test was used for statistical analyses (A-B).

Chapter 4
Discussion and Summary

Discussion

APCs are crucial to the immune response against viral infections. Their diverse and dynamic nature allows them to participate in multiple tissue and cell type-specific roles including antigen presentation, costimulation, immunomodulation, etc. (Gordon and Taylor, 2005; Auffray, Sieweke and Geissmann, 2009; Davies *et al.*, 2013; Merad *et al.*, 2013; Segura and Amigorena, 2013; Guilliams *et al.*, 2014). In this thesis, we revaluated the heterogeneity of different APC subsets with respect to TNF family ligand expression and demonstrated that costimulatory molecules are differentially distributed amongst APC subsets during LCMV13 infection. We discovered a dichotomy of expression between the B7 and TNF family ligands on cDCs versus inflammatory APCs at day 2 p.i. Using the combination of CD64 and FcεR1, we resolved the inflammatory APCs from both the cDCs and resident Mϕ (Langlet *et al.*, 2012; Plantinga *et al.*, 2013; Segura and Amigorena, 2013). They are monocyte derived as evident by the expression of CCR2, a chemokine receptor required for monocyte to egress from the bone marrow (Boring *et al.*, 1997; Auffray, Sieweke and Geissmann, 2009), and the lack of expression of zbtb46 (Satpathy *et al.*, 2012; Murphy *et al.*, 2016). Importantly, inflammatory APCs, including $MerTK^+$ inflammatory macrophages and $MerTK^-$ inflammatory DCs, expressed the highest MFI of TNF family ligands, GITRL, 4-1BBL, OX40L, and CD70 than any other APC subsets. Whereas $zbtb46^+$ cDCs only expressed low levels of the TNF family ligands but were higher in surface expression of MHCII, CD80, and CD86 than inflammatory APCs at day 2 p.i. of LCMV13. This dichotomy in B7 and TNF family ligands was further emphasized by the segregation of the $GITRL^{hi}$ from the $CD80^{hi}$ populations in LCMV13 infected mice. Data from spleen, lung, and mLN early during LCMV infection shows that this dichotomy is found in both lymphatic and non-lymphatic tissues. These findings suggest that in the face of a systemic infection such as LCMV13, monocyte-derived inflammatory APCs are poised to infiltrate various tissues from the blood stream to potentiate system-wide costimulation. Of note, a similar dichotomy was observed during influenza infection in the lung and mLN demonstrating that this dichotomous expression of B7 and TFN family ligands might be a general feature of both chronic and acute viral infections.

Analysis of the kinetics of surface costimulatory ligand expression on inflammatory APCs versus cDCs, demonstrated that the inflammatory APCs are the main expresser of TNF

family ligands throughout the first 21 days of LCMV13 infection. In contrast, the dichotomy of expression between the B7 and TNF family ligands on cDCs and inflammatory APCs appears limited to a rather specific time point, at day 2 post-LCMV clone 13 infection, which also coincides with the timing of a post-priming TNF-dependent costimulatory check point (Chang, 2016). At day 1 p.i., during the acute phase of the infection, high levels of inflammatory cytokines and viral load likely contributed to the high expression of both B7 and TNF family ligands on inflammatory APCs. It should be noted that these changes in expression levels of costimulatory molecules measured on the APC are taking place at a time when the populations of APC are also highly dynamic, with dramatic changes. After the dip in B7 ligand expression observed at day 2 p.i., the plasticity of the inflammatory APCs was evident from the comparable expression of the B7 family ligands to cDCs day 3 p.i and onwards. Nevertheless, MHC II expression remained higher on cDCs throughout the course of the infection. Overall, cDC are the highest expressors of MHC II throughout the response but have low levels of TNF family ligands, whereas inflammatory APC, despite lower levels of MHC II, have the highest levels of TNF family ligands throughout the response. These data are consistent with Inflammatory APC providing signals for post-priming accumulation of T cells (Chang, 2016).

Recent studies on immune exhaustion have led to the observation that a monocyte-derived APCs subset plays a crucial part in immune exhaustion and viral persistence as iregAPCs (Wilson *et al.*, 2012; Cunningham *et al.*, 2016). However, the inflammatory APCs that we focused on in this thesis were a distinct population of monocyte-derived APC subset that did not express inhibitory profile consistent with this population.

Comparing the expression kinetics of the 4 TNF family ligands, conserved versus unique features were identified. In agreement with the findings from Clouthier et al. (Clouthier, Zhou and Watts, 2014), GITRL expression was limited to day 1 to day 3 p.i., confirming its role as the limiting factor for GITR costimulation *in vivo*. Furthermore, GITRL and CD70 both demonstrated restricted early induction followed by rapid downregulation to at or below baseline levels across all APC subsets regardless of their maximal expression in both lymphoid and non-lymphoid tissues. The conserved rapid induction across different APC subsets was also noted for 4-1BBL and OX40L expression, however, following rapid induction, the 2 ligands were maintained through the later stages of the infection. The rapid induction of TNF family ligands

early in infection with similar kinetics of induction across subsets, suggested that a common regulatory factor was responsible. Indeed, coordinate the expression of all 4 TNF family ligands is IFNAR dependent early on. However, as IFN-I signaling becomes more limited later in the infection (Wilson *et al.*, 2013; Snell and Brooks, 2015; Cunningham *et al.*, 2016; Snell, McGaha and Brooks, 2017), it is likely that other factors maintain the expression of 4-1BBL and OX40L throughout the course of the infection.

Regulation of TNF family ligands remains largely unexplored during persistent viral infection *in vivo*. The kinetics of expression of mRNA for IFN-I precedes that of *Tnfsf18*, and *Tnfsf18* mRNA showed a similar kinetics of induction as other ISGs, leading us to explore the regulatory relationship between IFN-I and the TNF family ligands (Chang, 2016). The rapid induction of 4 TNF ligands observed in this study, correlate well with IFN-I levels during LCMV13 infection as shown by others (Zuniga *et al.*, 2008; Lee *et al.*, 2009; Y.Wang, Swiecki, *et al.*, 2012; Teijaro *et al.*, 2013). IFN-I, especially IFNβ, are potent regulators of both TNF family and B7 family costimulatory ligands during viral infection. Both subsets of cDCs and inflammatory APCs shared similar sensitivity to IFNβ signaling as reflected by the reduction in induction of B7 and TNF family ligands respectively via IFNAR blockade. However, sensitivity to IFN-I signaling by different APC does not explain the differential expression of TNF family ligands on different APC subsets shown here. For instance, pDC, which only express minimal levels of TNF family ligands, had the highest levels of IFNAR preinfection and the highest pSTAT1 signaling at day 1 p.i. compared to both inflammatory APCs and cDCs (Chang, 2016). Thus, a preferential response to IFNAR signaling alone does not explain differential costimulatory molecule expression by APC subsets and we believe that the downstream effects of IFNAR in inducing B7 versus TNF family ligands are cell type specific. This is plausible, as the 3 APC subtypes represent different lineages of APCs and induction of gene transcription by interferon signaling is regulated at many levels, including differential expression of STAT proteins in different cell types, cooperation with other cell specific transcription factors as well epigenetic regulation of IFN response genes (Ivashkiv and Donlin, 2014). Consistently, sorting of APC populations revealed a different pattern of *Isg* expression in pDCs, cDCs and inflammatory APCs.

With the findings that blockade of IFNAR only partially impaired the induction of the TNF family ligands and 4-1BBL and OX40L showed maintained expression throughout the course of LCMV infection, we believe that other factors contribute to the regulation of these ligands in the later stages of LCMV13 infection. Naturally, we turned our attention to IFNγ, as IFNγ plays important roles during viral infection, both augmenting cell mediated immunity, but also contributing to viral persistence, depending on the timing of IFNγ production, the nature of the infection, and the cytokine milieu (Murphy and Reiner, 2002; Dong, 2006; Weaver *et al.*, 2006; Schoenborn and Wilson, 2007; Cunningham *et al.*, 2016). GITRL expression is restricted to only the first few days of LCMV infection and IFNγ is a very poor inducer of GITRL (Chang et al. 20160 and this thesis). Here we showed that IFNγ only weakly induced OX40L and CD70, with slightly greater induction of 4-1BBL. The induction of 4-1BBL, was partially blocked by anti-IFNGR blocking antibody on inflammatory APCs at day 5 p.i. of LCMV13 infection (did not reach statistical significance with p-value of 0.0649). In agreement with the literature, we also observed LPS induced the upregulation of costimulatory molecule 4-1BBL (Futagawa *et al.*, 2002), CD70, and OX40L on TG MF. In addition, we observed this induction to be partially independent of IFNAR for 4-1BBL and completely dependent of IFNAR for CD70 and OX40L. It is likely that during persistent viral infection such as LCMV13, multiple factors participate in the regulation of the TNF family ligands at different stages of the infection.

The immunological impact of the difference in expression kinetics between the transiently expressed GITRL and CD70 versus maintained 4-1BBL and OX40L still needs to be further investigated. Persistent CD27 signaling has been reported to contribute to the pathological cytokine production and disruption of splenic microarchitecture during LCMV13 infection (Matter *et al.*, 2006; Penaloza-MacMaster *et al.*, 2011), thus it is reasonable that CD70 expression is only transient early on during infection to prevent pathology. On the other hand, disruption of OX40 signaling has been shown to result in higher viral burden later on in the infection (> day 50 p.i.) (Boettler *et al.*, 2012), thus providing insight to why OX40L expression should be maintained longer during LCMV13 infection. However, the effect of the maintenance of 4-1BBL expression needs to be further investigated as the signaling adaptor of 4-1BB, TRAF1, is downregulated during the chronic phase of LCMV13 infection to desensitize T cells to 4-1BB costimulation (C.Wang *et al.*, 2012).

In sum, this thesis provides exploration of various aspects of TNF superfamily ligand expression and regulation during viral infections. A dichotomy of expression between B7 and TNF family ligands was observed on cDCs versus tissue-infiltrating monocyte-derived inflammatory APCs in the spleen, lung, and mLN during LCMV13 or Influenza infection. These ligands are regulated temporally by an array of different molecules including IFN-I, IFNγ, and LPS. Such variety of regulatory mechanism is a representation of the complexity of the interaction between host immune response and viral persistence. Both unique and conserved features amongst the TNF family ligands signify the important role that they play in providing the post-priming survival signals for T cells.

Chapter 5

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